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Laboratory of Microbiology

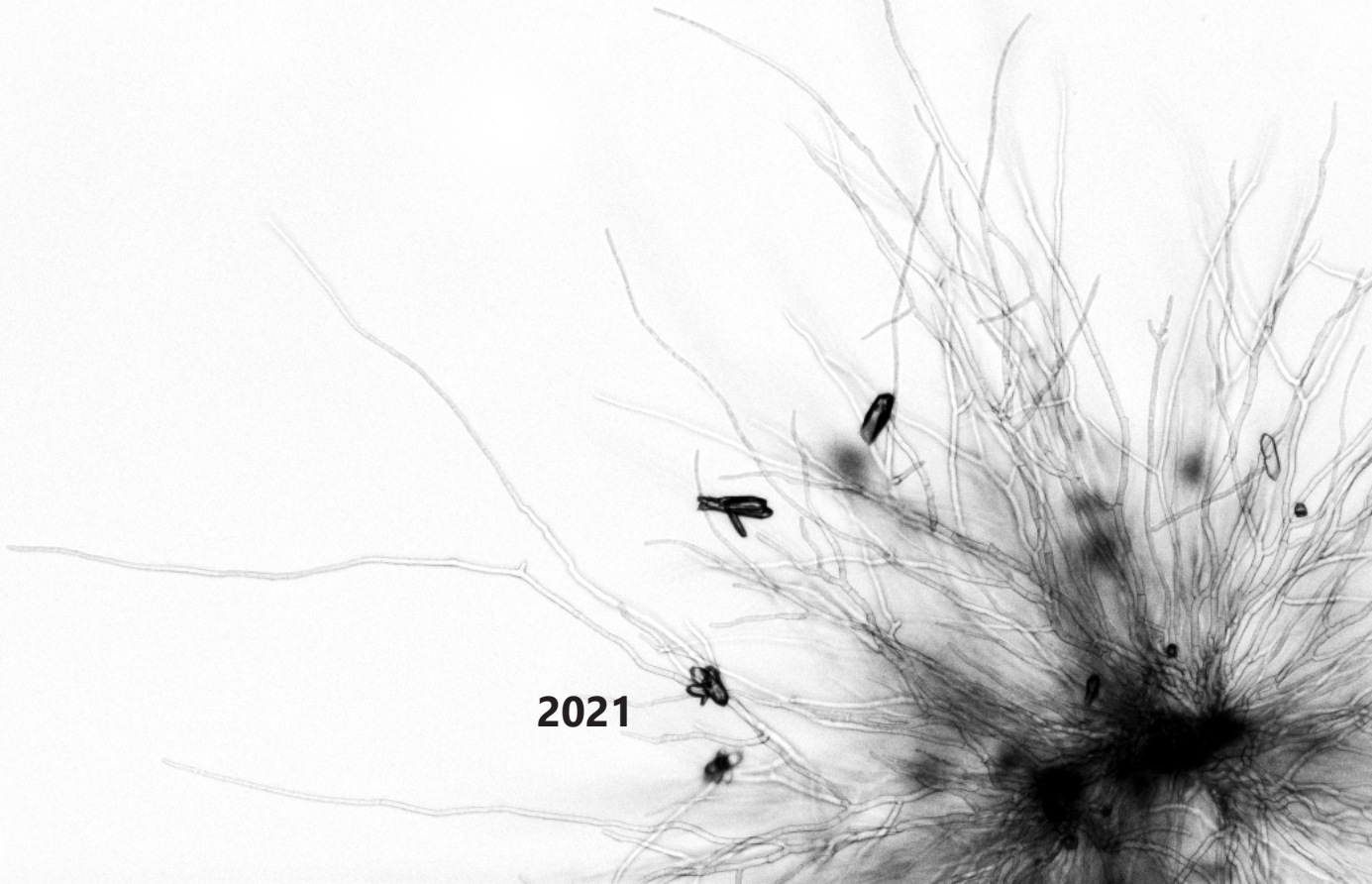
Bacterial oxalotrophy as an alternative biocontrol approach for the fight against pulmonary aspergillosis

A dissertation submitted to

University of Neuchâtel

by

Fabio Palmieri



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A dissertation submitted to the

University of Neuchâtel, Switzerland

for the degree of

Docteur ès sciences

by

Fabio Palmieri

Accepted on the recommendation of the Jury:

Prof. Pilar Junier, *thesis director, University of Neuchâtel*

Dr. Saskia Bindschedler, *internal expert, University of Neuchâtel*

Prof. Daniel Croll, *internal expert, University of Neuchâtel*

Dr. Eric Bernasconi, *external expert, Lausanne University Hospital (CHUV)*

Dr. Alix Coste, *external expert, Lausanne University Hospital (CHUV)*

Prof. Salomé Leibundgut-Landmann, *external expert, University of Zürich*

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University of Neuchâtel

UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Monsieur Fabio PALMIERI

Titre:

“Bacterial oxalotrophy as an alternative biocontrol approach for the fight against pulmonary aspergillosis”

sur le rapport des membres du jury composé comme suit:

- Prof. Pilar Junier, directrice de thèse, Université de Neuchâtel, Suisse
- Dr Saskia Bindschedler, Université de Neuchâtel, Suisse
- Prof. Daniel Croll, Université de Neuchâtel, Suisse
- Dr Eric Bernasconi, CHUV, Lausanne, Suisse
- Dre Alix Coste, CHUV, Lausanne, Suisse
- Prof. Salomé Leibundgut-Landmann, Université de Zürich, Suisse

Neuchâtel, le 16 juin 2021

Le Doyen, Prof. A. Bangerter

You will fail. That's inevitable. It's what you do with it.

- J. K. Rowling

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Summary

Although fungi are estimated to kill more than 1.5 million people every year worldwide, the issue of fungal pathogenesis is largely neglected. Moreover, the rise of emergence of multi-resistant fungal pathogens worldwide is a major threat for human health. This is notably the case of the opportunistic fungal pathogens of the genus *Aspergillus*. The prevalence of *Aspergillus*-related infections, also known as aspergillosis, has dramatically increased in the last few years. *Aspergillus* species, such as *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. terreus* or *A. niger*, are known to cause a vast spectrum of respiratory diseases, ranging from mild allergies to life-threatening invasive infections. Interestingly, the formation of calcium oxalate crystals has been previously reported in the latter cases. Oxalic acid is known to play a key role in the pathogenesis of plant fungal pathogens, such as for instance *Sclerotinia sclerotiorum*. However, a link between the production of oxalic acid and pathogenicity has not been made yet in the case of *Aspergillus*.

Oxalic acid is commonly produced by soil fungi, along with other low molecular weight organic acids. In soils, oxalic acid generally occurs in the form of calcium oxalate crystals. Despite its chemical stability and low solubility, calcium oxalate is rarely found in the geological records, something that has been suggested to be the results of its metabolization by oxalotrophic bacteria. Oxalogenic fungi are known to interact with oxalotrophic bacteria in soils within the oxalate-carbonate pathway, where fungi, along with plants, are the source of oxalate, and oxalotrophic bacteria are its sink. Oxalotrophy is concomitant with a pH increase, which eventually leads to the precipitation of calcium carbonate, if the pH increases above a value of 8.4.

The aim of the present thesis was to translate the metabolic interaction between oxalogenic fungi and oxalotrophic bacteria occurring in soils to human health. Specifically, we developed and assessed a novel biocontrol strategy for the treatment of pulmonary aspergillosis based on the manipulation of the environment through bacterial oxalotrophy, a process we named *environmental interference*. For this, the influence of the composition of the culture medium on the production of low molecular weight organic acids was tested for selected fungal strains, as well as their interaction with non-oxalotrophic and oxalotrophic bacterial strains. The fungal strain *Aspergillus niger* was selected because of its systematic production of oxalic acid in all culture media tested, and because of its medical relevance. The first demonstration of the principle of environmental interference *in-vitro* was made by showing the biocontrol exerted by the oxalotrophic bacterial species *Cupriavidus oxalaticus* on the growth of *A. niger*. The use of soil bacteria was shown to be problematic, as they induce important cellular damage. Therefore, the genome of *C. oxalaticus* was analyzed in order to better understand the oxalotrophy

metabolism in this model bacterial species. This highlighted the presence of an operon containing all the genes that are required for the degradation of oxalate. These data will be used for the design of an entirely enzymatic degradation pathway of oxalate that will be more suitable as a potential therapeutic option. Finally, the fungal:fungal:bacterial interaction between *A. niger*, *Candida albicans* and *C. oxalaticus* was investigated. This interaction is relevant in the case of pulmonary co-infection in immunocompromised patients and patients suffering from cystic fibrosis. The interaction of both opportunistic fungal pathogens and the oxalotrophic bacterium depended on the inoculation mode (simultaneous versus sequential). To conclude, while the presented results on the biocontrol concept of environmental interference are promising, a preclinical *in-vivo* demonstration of this concept in a murine infection model is crucial for the development of a potential clinical application. Indeed, a more comprehensive approach by integrating the immune system is necessary in order to better comprehend the interplay between the host, the pathogen and the lung microbiota in disease development.

Keywords

Opportunistic fungal pathogens | antifungal resistance; *Aspergillus* spp. | *Aspergillus niger* | oxalic acid | calcium oxalate | oxalotrophic bacteria | *Cupriavidus oxalaticus* | bacterial:fungal interaction | environmental interference | *Candida albicans* | fungal:fungal:bacterial interaction | microbial ecology

Résumé

On estime que les champignons tuent plus de 1,5 million de personnes chaque année dans le monde. Malgré cela, la question de la pathogenèse fongique est largement négligée. De plus, l'augmentation de l'émergence de pathogènes fongiques multi-résistants dans le monde constitue une menace majeure pour la santé humaine. C'est notamment le cas des moisissures du genre *Aspergillus*, dont la prévalence des infections, également connues sous le nom d'aspergilloses, a augmenté de façon spectaculaire au cours des dernières années. Les espèces d'*Aspergillus*, telles que *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. terreus* ou *A. niger*, sont à l'origine d'un vaste éventail de maladies respiratoires, allant des allergies légères aux infections invasives potentiellement mortelles. Dans ces derniers cas, il est intéressant de noter que la formation de cristaux d'oxalate de calcium a été précédemment observée. L'acide oxalique est connu pour jouer un rôle clé dans la pathogenèse des champignons phytopathogènes, comme par exemple *Sclerotinia sclerotiorum*. Cependant, un lien entre production d'acide oxalique et pathogénicité n'a pas encore été établi dans le cas d'*Aspergillus*.

L'acide oxalique, ainsi que d'autres acides organiques de faible poids moléculaire, est couramment produit par les champignons telluriques. Dans les sols, l'acide oxalique se présente généralement sous la forme de cristaux d'oxalate de calcium. Malgré sa stabilité chimique et sa faible solubilité, l'oxalate de calcium est rarement retrouvé dans les archives géologiques, ce qui peut être expliqué par l'activité de bactéries oxalotrophes. Dans le sol, l'interaction entre les champignons oxalogènes et les bactéries oxalotrophes au sein de la voie oxalate-carbonate est connue. L'oxalotrophie s'accompagne d'une augmentation locale de pH conduisant à la précipitation de carbonate de calcium si le pH augmente au-dessus d'une valeur de 8.4.

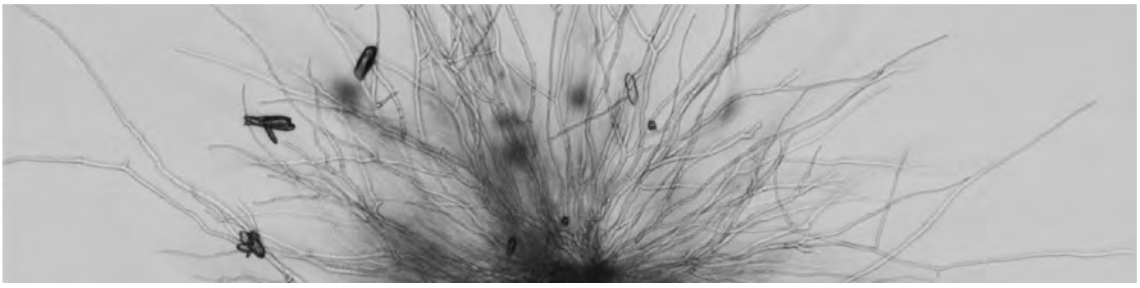
L'objectif de cette thèse était d'appliquer à la santé humaine les principes écologiques régissant l'interaction métabolique entre les champignons oxalogènes et les bactéries oxalotrophes dans les sols. Plus spécifiquement, nous avons développé et évalué une nouvelle stratégie de biocontrôle pour le traitement de l'aspergillose pulmonaire basée sur la manipulation de l'environnement par l'oxalotrophie bactérienne, un processus que nous avons nommé *interférence environnementale*. Pour cela, l'influence de la composition du milieu de culture sur la production d'acides organiques de faible poids moléculaire par certaines souches fongiques, ainsi que sur leur interaction avec des souches bactériennes oxalotrophes ou non-oxalotrophes, a été testée. La souche fongique *Aspergillus niger* a été sélectionnée en raison de sa production systématique d'acide oxalique dans tous les milieux de culture testés, et de son intérêt médical. La première démonstration *in-vitro* du principe d'interférence environnementale a été faite en montrant le contrôle exercé par l'espèce bactérienne oxalotrophe *Cupriavidus oxalaticus* sur

la croissance de *A. niger*. L'utilisation d'une bactérie tellurique s'est avérée problématique, car elle induit des dommages cellulaires importants. Par conséquent, le génome de *C. oxalaticus* a été analysé afin de mieux comprendre le métabolisme de l'oxalotrophie chez cette bactérie modèle. Ceci a permis de mettre en évidence la présence d'un opéron contenant les gènes impliqués dans la dégradation de l'oxalate. Ces données seront utilisées pour la conception d'une voie de dégradation de l'oxalate entièrement enzymatique qui sera plus adaptée pour une potentielle option thérapeutique. Enfin, l'interaction champignon:champignon:bactérie entre *A. niger*, *Candida albicans* et *C. oxalaticus* a été étudiée. Cette interaction est pertinente dans le cas de co-infection pulmonaire chez les patients immunodéprimés et les patients souffrant de mucoviscidose. L'interaction des deux pathogènes fongiques opportunistes et de la bactérie oxalotrophe dépendait du mode d'inoculation (simultané ou séquentiel). En conclusion, bien que les résultats présentés sur le concept de biocontrôle par interférence environnementale soient prometteurs, une démonstration préclinique *in-vivo* de ce concept dans un modèle d'infection murin est cruciale pour le développement d'une potentielle application clinique. En effet, une approche plus complète intégrant le système immunitaire est nécessaire afin de mieux comprendre l'interaction entre l'hôte, le pathogène et le microbiote pulmonaire dans le développement de la maladie.

Mots clés

Champignons pathogènes opportunistes | résistance antifongique | *Aspergillus* spp. | *Aspergillus niger* | acide oxalique | oxalate de calcium | bactéries oxalotrophes | *Cupriavidus oxalaticus* | interaction bactérie:champignon | interférence environnementale | *Candida albicans* | interaction champignon:champignon:bactérie | écologie microbienne.

CHAPTER 1



Introduction

1. Introduction

1.1. Antimicrobial resistance – a problem for and of society

Infectious diseases have been part of human history since the settlement of hunter-gatherer societies into villages, and the domestication and cultivation of animals and crops, respectively (1, 2). The most notorious historical infectious diseases are probably the *Plague of the Justinian* and the *Black Death*, which were caused by the bacterial species *Yersinia pestis* and which killed millions of people (3, 4). Another bacterial disease: tuberculosis (caused by *Mycobacterium tuberculosis*), has been pointed out as the deadliest in the world in the last decades (5, 6), with close to 1.5 millions of deaths in 2019 alone (7). The discovery of antibiotics at the beginning of the 20th century was a true revolution for the treatment of bacterial infections and for medicine in general (8, 9). The discovery of penicillin, the very first antibiotic accidentally discovered by Alexander Fleming in 1928 (10), marked the beginning of the Golden Age of the antibiotic era, which reached a peak in the mid-1950s (8, 11). During this period, new classes of antibiotics, such as sulfonamides, tetracyclines, or aminoglycosides, were discovered almost on a yearly basis through the isolation of soil antibiotic-producing microorganisms, mostly Actinobacteria (8). Penicillin resistance was first identified in 1940 (8). The elucidation of the chemical structure of penicillin by Dorothy Hodgkin in 1945 permitted to develop semi-synthetic penicillin-derived antimicrobial compounds to circumvent penicillin resistance (8).

However, in 1945, around fifteen years after his serendipitous discovery of penicillin, Fleming already predicted the obsolescence of antibiotics:

"*The time may come when penicillin can be bought by anyone in the shops. Then, there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant*."

Nowadays, the world is currently facing one of the most challenging public health emergencies, the so-called antibiotics crisis. This crisis refers to the increasing prevalence of pathogens resistant to most of the available antibiotic compounds in medical use (12, 13). After the initial use of antibiotics in medicine, their use became a common practice in agriculture for livestock production (14). Their overuse, inappropriate prescription, and extensive use in human medicine and agriculture lead to the emergence of resistance to these antimicrobial drugs (12-14).

Although resistance has been traditionally viewed as a problem in clinical settings, the role of non-clinical environments in the dissemination and emergence of resistance is becoming increasingly obvious. One of the most important discoveries made by scientists during the initial phases of the antibiotic

era was the realization that resistance is not restricted to pathogens but can be also widely spread in apparently harmless environmental microorganisms (15, 16). This raises the question as to the role of the environmental reservoirs of antimicrobial resistance in the development of clinical resistance (16).

While the risk of untreatable bacterial infections has been widely acknowledged, an equally concerning problem involves an increasing rate of resistance among opportunistic fungal pathogens. The reasons for this increase are multiple. Indeed, the extensive use of the same classes of antifungal compounds in both agriculture and human health has led to the emergence and spread of cross-resistances in human opportunistic fungal pathogens with multiple hosts (17). This is particularly well highlighted in the case of resistance to azoles in *Aspergillus fumigatus*. Azoles are frontline antifungal compounds used in crop protection and in human and animal health (17). These compounds have different effects depending on the fungal species. Azoles have a fungistatic effect in yeasts such as *Candida albicans*, but act as fungicides against filamentous fungi such as *A. fumigatus*. Its fungicidal effect against *A. fumigatus* has been shown to be linked to defects in the cell wall remodeling induced by azoles, which cause cell wall integrity loss and death (18). However, despite the fungicidal effect of triazoles in filamentous fungi, their application in agriculture at sub-inhibitory concentrations has led to the emergence and rapid spread of resistance among natural populations of *A. fumigatus* in soil (17). This resistance to azoles in turn greatly compromise the success of treatment in human patients suffering from *A. fumigatus* infections (19). Antifungal resistance to azoles is also a worrisome issue in the well-known commensal yeast *C. albicans*. Given their fungistatic effect, *Candida* spp. is known to rapidly develop resistance to azoles (20). In addition to azoles, *Candida* spp. can also develop resistance to echinocandins, which are highly active against this genus and yeasts in general (21), or even acquire multidrug resistance to azoles and echinocandins as in the case of *C. glabrata* (22).

The problem of antimicrobial resistance in fungal pathogens described above (common use of the same antimicrobial compounds in different context) presents the perfect example for the advocacy of a One-Health approach to disease management (23). According to this initiative, attaining better health for people, animals and the environment requires the concerted effort of multiple disciplines working at different scales (24). Indeed, in the case of antimicrobial resistance, humans, animals and the environment are interconnected. The fact that the same family of antimicrobial compounds can be used in human and animal health might promote the transfer of antimicrobial resistance determinants between animal and human pathogens via an environmental reservoir (23). This is again nicely illustrated by the emergence of azole resistance in *Aspergillus* spp., and more specifically in the case of *A. fumigatus*, for which the emergence of resistance to azoles has been hypothesized to be selected in the environment due to the extensive use of triazoles in agriculture (25).

Azole fungicides such as propiconazole, difenoconazole, or tebuconazole have a very similar structure than those used in clinical practice, and their use is correlated with the increased emergence of clinical azole-resistant *A. fumigatus* strains (26). The emergence of this resistance led to the hypothesis that the extensive use of azole fungicides in agriculture selected for azole resistant *A. fumigatus* in the environment (27). This is supported by several studies that reported the presence of azole-resistant *A. fumigatus* strains in patient who have never been treated with azoles before (25, 28), which accounts for two third of the patients suffering from azole-resistant aspergillosis. Resistance has been attributed to specific mutations in the tandem repeat (RT) of the promoter region of the *cyp51A* gene, which is involved in the biosynthesis of ergosterol in fungi (29), in combination with single or multiple point mutations (TR34/L98H; TR53; TR46/Y121F/T289A) (25). Clinical azole-resistant *A. fumigatus* strains have also shown cross-resistance to azoles commonly used in agriculture (25). Thus, considering an integrated disease management approach through the One Health initiative that bring together scientists, medical doctors, veterinarians, and plant pathologists, is badly needed to reduce our reliance on chemical control alone to stop the spread of resistance among opportunistic pathogens (17).

1.2. Fungal diseases

Fungal diseases are estimated to kill more than 1.5 million people per year, with the number of affected people approaching the billion (30). In spite of this, the issue of fungal pathogenesis has been largely neglected (17, 31). Over the past two decades, the frequency of invasive fungal diseases has considerably increased (30). This has also been pointed out in the case of healthcare-associated invasive fungal infection (32, 33), for which a call to action was recently issued in the journal Cell Host & Microbe (34). Indeed, in order to address the issue of nosocomial fungal infections, several parameters such as fungal pathogenesis and antifungal immunity, the mycobiota and colonization resistance, and the associated risk factors in order to reduce the number of infections, need to be taken into account (34).

This increase in frequency is correlated with an increasing number of at-risk vulnerable patients, which include individuals undergoing major surgery or being immunosuppressed due to transplants, AIDS, cancer, corticosteroid therapies or auto-immune diseases (30, 35). Tuberculosis, chronic obstructive pulmonary disease (COPD) and the increased incidence of cancers are also known risk factors for fungal diseases all across the world (30).

The most prevalent human fungal pathogens are airborne opportunists such as *Aspergillus* spp., *Cryptococcus* spp., *Pneumocystis* spp., and human-associated commensals like *Candida albicans* (35, 36), which are responsible for more than 90% of all reported fungal disease-related deaths (37). The latest

estimates of annual burden of fungal diseases amounts to approximately 3 million cases of chronic pulmonary aspergillosis, over 200'000 cases of cryptococcal meningitis, 700'000 cases of invasive candidiasis, 500'000 cases of *Pneumocystis jirovecii* pneumonia, 250'000 cases of invasive aspergillosis, and over 10 million cases of fungal asthma (30, 37). Besides these well-known opportunistic pathogens, new and emerging fungal pathogens are appearing, and these include other *Candida* spp., such as *Candida auris* (38), rare *Aspergillus* spp. species (39), opportunistic yeast-like fungi such as *Trichosporon* spp., members of the Mucoromycota (*Rhizopus* spp. and *Mucor* spp.), hyaline molds such as *Fusarium* spp., *Acremonium* spp. or *Trichoderma* spp (35, 40), or the dimorphic *Emergomyces* spp. fungi (41).

If left untreated, invasive fungal infections (IFIs) can be fatal. This is notably the case of transplanted patients for which IFIs are major post-organ transplantation complications (42). For instance, *Aspergillus* spp. have been pointed out as the most common opportunistic fungal pathogen causing an IFI in lung transplant recipients, with an incidence of 40.5 cases per 1000 patients annually, despite the use of prophylactic antifungal treatments (42). Invasive aspergillosis most commonly occurs within 1 year after transplantation, with the majority of the cases reported within the first 6 months (42, 43). The most commonly isolated *Aspergillus* spp. are *A. fumigatus* and *A. flavus* (42).

IFIs occurring during an antifungal treatment, as in the case of a prophylactic treatment, are called breakthrough invasive fungal infections (44). These can occur even by fungi that are outside the spectrum of activity of an antifungal compound (44), as reported by Lerolle *et al.* (45) who reported pulmonary aspergillosis, disseminated fusariosis, pulmonary mucormycosis, and candidaemia in neutropenic patients treated with posaconazole.

Fungal pathogens have also been reported to occur as a mixed fungal infection. This is notably the case of mixed pulmonary infections by *Aspergillus* spp. and *Candida* spp. in immunocompromised patients (46, 47), an *Aspergillus* spp., *Candida* spp., and *Mucor* spp. mixed fungal infection in a severe hand injury (48), and a *Candida tropicalis* and *Cryptococcus laurentii* mixed fungal facial infection in a non-Hodgkin's lymphoma patient (49).

Invasive aspergillosis has also been found to be associated with viral infections, such as influenza (50, 51), and more recently COVID-19. Invasive aspergillosis was recently portraited by the Open Forum of Infectious Diseases as an under recognized super infection in the case of SARS-CoV-2 positive patients (52, 53). This is supported by various case studies that are emerging from affected countries. A recent editorial by Prof. J.-P. Gangneux of the Journal de Mycologie Médicale (54) warns about the need to readiness in the case of co-infections given that COVID-19 patients in intensive care units (ICUs) shared

many risks factors as those populations in the grip of the fungal infection epidemy that preceded the current global pandemic.

1.3. Pulmonary aspergillosis

Fungi of the genus *Aspergillus* are widespread in the environment and are commonly isolated from both outdoors and indoors environments (55, 56). They are found in large quantities in soil and decaying biomass, especially in compost piles, where these saprophytic fungi participate to the degradation of organic matter (25, 57, 58). They produce large numbers of small conidia that are dispersed through air (58, 59). It is estimate that a human inhales between 100 to 1000 conidia per day, which can, due to their small size (2 to 5 µm in diameter), reach the alveoli in the lungs (58). Moreover, *Aspergillus* is the most frequently isolated filamentous fungus in humans and animals (55, 60). A remarkable plasticity in their ecology and stress-response is believed to be at the basis of the success of *Aspergillus* spp. as opportunistic pathogens. The wide environmental distribution of this genus can be explained by its competitiveness and adaptability (61). Indeed, *Aspergillus* spp. can use multiple organic substrates and adapt to a broad range of environmental conditions (55).

Aspergillus spp. are associated with a variety of clinical manifestations ranging from allergic disease to life-threatening systemic infections. Infections are principally caused by *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus*, with *A. fumigatus* being responsible for 90% of the reported cases (55). Lung infections due to *Aspergillus* spp. are caused by inhalation of airborne conidia (57). Concentration of conidia in the air can range from 1 to 100 per m^3, but it can reach up to 10^8 per m^3 in some environments (60). Inhaled conidia are usually eliminated either by mucocilliary clearance or by macrophages (innate immune system) in immunocompetent individuals. However, depending on the virulence of the fungal strain, as well as the immunological status and the pulmonary structure and function of the host, *Aspergillus* can lead to a variety of pathologies (55). In view of the variability in pathogenicity within *Aspergillus*, there is an urgent need to understand the conditions that make the respiratory tract permissive to conidial germination in susceptible individuals, and in particular, to determine whether the composition of the airway microbiota plays a role in this regard.

Pulmonary aspergillosis is classified into three different groups with distinct clinical manifestations depending on the immune status of the host (56, 60, 62) (Fig. 1). These clinical manifestations include hypersensitivity responses (asthma or allergic bronchopulmonary aspergillosis – ABPA), colonization (i.e. presence of the fungus without any clinical symptoms or radiological or laboratory indications of active fungal infection), or infection (chronic or invasive aspergillosis).

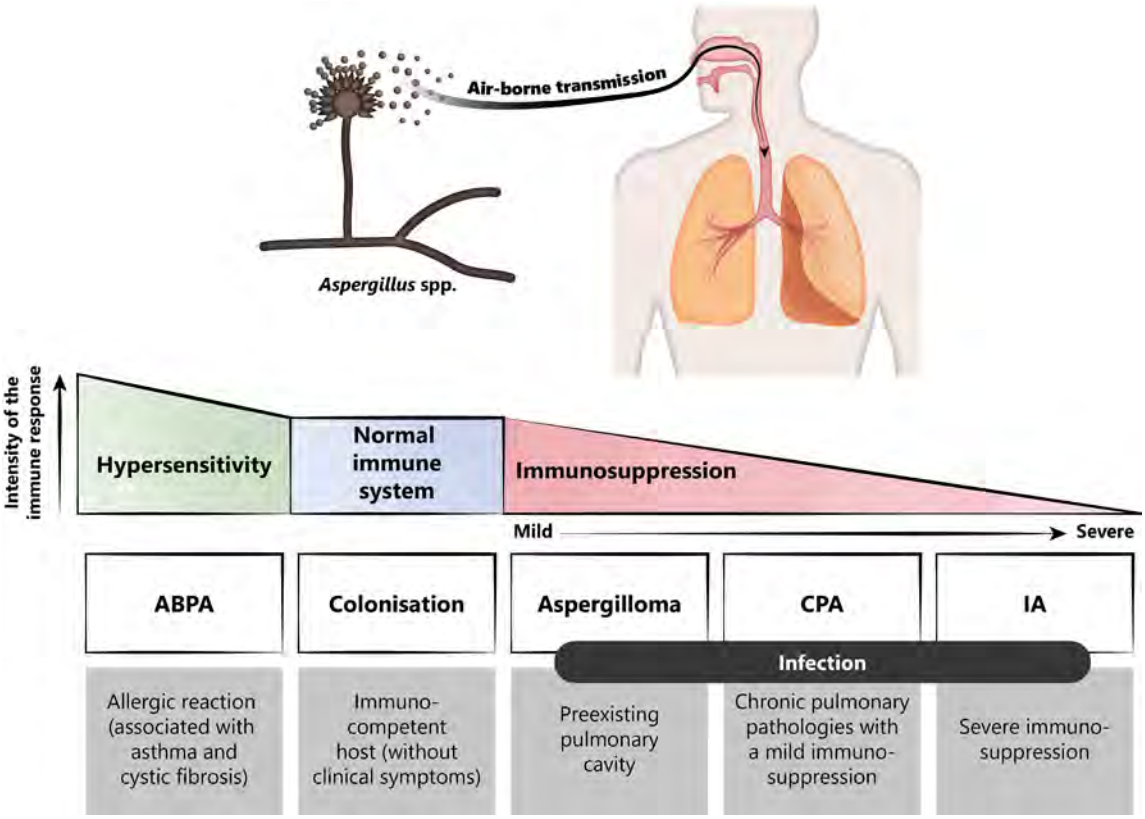


Fig. 1. Summary diagram of the disease spectrum of pulmonary aspergillosis. Upon inhalation of Aspergillus spp. conidia and unproper elimination of those by mucocilliary clearance or macrophages, conidia germination and growth can lead to a variety of diseases depending on the immune status of the host, ranging from allergic reaction (hypersensitivity) to life-threatening invasive infection (severe immunosuppression). ABPA = allergic bronchopulmonary aspergillosis; CPA = chronic pulmonary aspergillosis; IA = invasive aspergillosis.

On one side, *Aspergillus* spp. can cause hypersensitivity responses. In the atopic patient, the most severe form of aspergillosis is the allergic bronchopulmonary aspergillosis (ABPA). This disease is almost always related to *A. fumigatus* and develops following sensitization to *A. fumigatus* allergens in patients with cystic fibrosis (CF) or chronic asthma. ABPA affects close to 5 million patients worldwide (30).

In immunocompetent hosts, *Aspergillus* spp. can simply colonize the lungs without any damage and clinical symptomatic manifestations (63). Moreover, *Aspergillus* spp. can also lead to a chronic, non-invasive form of infection called chronic pulmonary aspergillosis (CPA). One form of CPA is aspergilloma, which is usually characterized by the colonization and proliferation of the fungus inside a preexisting cavity, leading to the development of a fungus ball (64). Aspergilloma typically occur in immunocompromised patients previously suffering from lung pathologies such as tuberculosis, lung abscess, cysts or tumors (64). Chronic cavitary pulmonary aspergillosis, also called chronic necrotizing aspergillosis or complex aspergilloma, is an inflammatory form of the infection characterized by the production of serum IgG antibodies directed to *Aspergillus*, elevated acute-phase inflammation markers, and the absence of pulmonary or vascular invasion. CPA usually occur in patients with an immune status ranging from

normal to mild immunosuppression. The global burden of CPA, including aspergilloma, is estimated to more than 3 million people affected worldwide (30).

On the other side of the spectrum, invasive pulmonary aspergillosis (IPA) is the most severe and lethal form of *Aspergillus* infection occurring in immunosuppressed patients. The incidence of IPA is approximately 250'000 cases annually and mortality rate ranges from 30 to 80% (30). IPA is characterized by the invasion of the lung tissue by *Aspergillus* hyphae, which can be followed by angioinvasion and dissemination in other organs in patients with prolonged neutropenia. Other at-risk patients include individuals with hematopoietic stem cell transplantation, solid-organ transplantation, prolonged corticosteroid therapy, AIDS or COPD (56, 60, 62).

Currently, the available treatments for the whole aspergillosis disease spectrum are very limited. The primary treatment in the case of CPA or IPA is the use of azoles such as voriconazole or itraconazole. Surgical resection is performed on patients suffering from aspergilloma and presenting associated complications such as severe hemoptysis. Corticosteroids are the mainstay for the treatment of ABPA, with or without the administration of antifungal drugs (65).

1.4. Innate immune defense against *Aspergillus* spp.

Despite constant exposure to *A. fumig*atus conidia, most humans do not develop any illness. This suggests an efficient clearance of the conidia by the innate immunity in immunocompetent individuals before the adaptive immunity is activated (59). Following inhalation, resting conidia begin to swell and become metabolically active. If the innate immune system does not effectively eliminate conidia, they germinate and form invasive hyphae that can penetrate the lung tissues (66). The innate immune response is stage-specific: depending on the developmental stage of the pathogen, different components of the host's defenses are activated in order to clear it (66).

Upon inhalation, conidia arriving in the respiratory tract are subjected to a turbulent airflow due to branching pattern of the respiratory tract, resulting in the deposition of most inhaled particles against the airway fluid (59). The trapped conidia are then removed by the ciliary action of the respiratory epithelium, which is the first line of defense in the lung (59, 67). Moreover, respiratory epithelial cells, i.e., bronchial and alveolar epithelial cells, have been shown to participate actively to the innate immune response against *Aspergillus* spp. by phagocytosing and killing conidia *in-vitro* (59, 68, 69). However, due to their small size, some of the inhaled conidia can reach the respiratory alveoli. After 4 to 5 h, resting conidia become swollen, and if not cleared, germinate and form hyphae within 12 to 15 h after arrival into the lungs (59). The maturation of conidia triggers a morphological change involving the exposure

of the inner cell wall following the loss of the thin hydrophobic RodA protein layer, thus exposing the immunogenic component of the cell wall (59, 66). These cell wall pathogen-associated molecular patterns (PAMPs) comprises polysaccharides such as β-glucan, mannan, chitin, and galactomannan, all of which are recognized by different pattern recognition receptors (PRRs) (58, 70).

*A. fumig*atus conidia and hyphae are recognized by the host via soluble and cell-associated microbial PRRs (59). The soluble PRRs belong to the humoral part of the innate immune system and include mainly pentraxins, complement proteins, and pulmonary collectins (59, 66). The presence of conidia can rapidly induce pentraxins production, and more specifically pentraxin-3 (PTX3). PTX3 is secreted by various cells, including neutrophils, dendritic cells, mononuclear phagocytes, and pulmonary epithelial cells (59, 66). It binds to galactomannan on *A. fumig*atus conidia and facilitates recognition by phagocytes such as alveolar macrophages (66). The complement system is an essential mechanism of the innate immunity that leads to the direct or indirect death of the pathogen. Three different pathways of complement activation exist, the classical, the lectin, and the alternative pathways, all of which converge on a common pathway in which C3 convertase cleaves the complement protein C3 in C3a and C3b (71, 72). These cleavage molecules of C3 can either opsonize pathogens to facilitate phagocytosis, act as chemoattractant for proinflammatory cells such as neutrophils and eosinophiles or produce a membrane pore-forming complex leading to the lysis of the pathogen (66). Pulmonary collectins include lung surfactant proteins A and D and serve as opsonins. They have been shown to bind to *A. fumig*atus conidial carbohydrate structures in a calcium-dependent manner. Surfactant proteins A and D have also been shown to promote the agglutination of conidia and their binding to neutrophils and alveolar macrophages, and improve the phagocytosis and killing of conidia by neutrophils (59).

Cell-associated PRRs include Toll-like receptors (TLRs) and C-type Lectin receptors (CLRs) such as Dectins or Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (59). TLR recognition of pathogen triggers a signaling cascade leading to the activation of transcriptional factors such as NF-κB, which controls the expression of pro- and anti-inflammatory cytokines and chemokines (66). The universal adaptor molecule MyD88 has been shown to play a significant role in the signaling of TLRs, which induce the production of various inflammatory cytokines and reactive oxygen species (59, 66). TLR2 and TLR4 have been implicated in the recognition of *A. fumig*atus conidia and hyphae (66). However, available data concerning their roles in *A. fumig*atus-associated immunity are conflicting. Indeed, the *A. fumigatus*-associated PAMPs for TLR2 and TLR4 remain indeterminate. TLR9 has also been shown to play a role in the innate immunity against *A. fumigatus* by recognizing fungal unmethylated CpG DNA (59, 66). Dectin-1 is a primary receptor for recognizing fungal β-glucan and is essential for the mediation of the proinflammatory response (66). Dectin-1 is widely expressed on macrophages,

dendritic cells, and neutrophils (59). Dectin-1 signaling activates the NF-κB and induces the expression of cytokines and chemokines including TNF-α, IL-6, IL-1α, IL-1β, G-CSF, GM-CSF, MIP-1α, and MIP-1β, all of which participate in the recruitment of the cellular effectors of the innate immune system (66, 67). Moreover, Dectin-1 also induces the expression of the anti-inflammatory cytokine IL-10, indicating its immunomodulatory role in modulating the inflammatory response (66). Dectin-2 has been recently shown to be implicated in the innate immune response against *A. fumig*atus. Dectin-2 is expressed in macrophages and dendritic cells and recognizes α-mannan in the fungal cell wall's outer layer. Dectin-2 is highly expressed by alveolar macrophages in response to *A. fumig*atus and was also shown to mediate an NF-κB-dependent proinflammatory response against swollen conidia (66). Finally, DC-SIGN is expressed at the surface of dendritic cells and some macrophages, and binds to *Aspergillus* conidia via the recognition of fungal galactomannan (59).

Alveolar macrophages (AMs) are the major leukocytes found in the lung. They constitute the first line of defense against inhaled *A. fumigatus* conidia that have reached the alveoli (59). Their role is to phagocytose and kill conidia either via oxidative mechanisms through the generation of ROS, or by non-oxidative mechanisms through phagosomal acidification (66). Corticosteroids have been shown to impair the capacity of AMs to kill conidia (59). Neutrophils are also crucial in the defense against *A. fumigatus*. Initially thought to kill hyphae exclusively, they have also been essential in killing germinating conidia. Neutrophils bind and phagocytose swollen conidia to trigger respiratory burst and degranulation. While the size of the hyphae prevents phagocytosis, direct contact with neutrophils can induce oxidative and non-oxidative mechanisms to damage the hyphae (59). Human peripheral blood monocytes and Natural Killer (NK) cells have also been shown to have a role in the innate immune defense against *A. fumigatus* (59). Dendritic cells (DCs), as to them, have well-documented roles in the defense against *A. fumigatus*. Immature DCs (iDCs) have been shown to phagocytose opsonized and non-opsonized conidia and hyphae, both of which are recognized through PRRs such as Dectin-1, among others. TNF-α, IL-6, IL-12, IL-1α, and IL-1β are the central proinflammatory cytokines produced by iDCs upon recognition of *A. fumigatus* conidia and hyphae (66).

1.5. Importance of oxalate in aspergillosis

Although many aspects of the ecology of *Aspergillus* spp. have been investigated in relationship to their pathogenicity, one aspect that has been largely ignored is its ability to lower the pH of its environment, necessary for their capacity to colonize or cause infection, via the secretion of oxalic acid or other low molecular weight organic acids. Oxalic acid is a known pathogenicity factor of plant fungal pathogens, such as *Sclerotinia sclerotiorum* or *Botrytis cinerea* (73-75). This acid is secreted in the host tissues and

accumulates in the form of oxalate, leading to a pH decrease that stimulates the production and activity of fungal enzymes . Moreover, as oxalate is a strong chelator of divalent metallic cations, it can sequester calcium ions, with multiple possible structural and physiological consequences for the host (76). In the case of plant pathogens, the formation of calcium oxalate crystals in the middle lamella weakens the cell wall structure and facilitates infection. Moreover, oxalate can inhibit plant defenses and induce programmed cell death, which is also beneficial for necrotrophic pathogens (61, 73, 76).

Given the importance of oxalic acid production in plant fungal pathogens, this compound might also play a role during human infection by opportunistic fungal pathogens. In fact, several studies have reported the presence of calcium oxalate crystals for pulmonary aspergillosis (77-86), and the detection of calcium oxalate crystals has been proposed as an easy tool for differential diagnosis (83). In most of the reported cases, oxalate deposition is associated to *A. niger* infection, but some reports also include infection caused by *A. flavus* or *A. fumigatus* (82). Oxalic acid and oxalate crystals are thought to cause damage to host tissues (including pulmonary blood vessels), as well as tissue injury via iron-dependent generation of free radicals (84, 87). A recent case report indicates the presence of calcium oxalate crystals around and within the blood vessel walls of a man with Burkitt's lymphoma who developed pneumonia after profound neutropenia following immunosuppressing treatment, suggesting a potential mechanical role of oxalate crystals in the angioinvasion by *Aspergillus* (85). Aside from mechanical damage to the host tissues, the formation of calcium oxalate could also have a dramatic effect on cell physiology. Indeed, calcium is a secondary messenger in many cell types, including those of the immune system (88). During immune stimulation, Ca^{2+} entry from extracellular medium or cellular compartments increases intracellular Ca^{2+} concentration (89), and thus Ca^{2+} chelation has been shown to inhibit the immune response *in vitro* (90, 91). All this suggests a role of oxalic acid also in the inhibition of the immune response. Despite these converging indications, a link between oxalic acid production and pathogenicity for *Aspergillus* spp. in animals has only been recently demonstrated (cf. Chapter 4).

1.6. *Candida albicans* pathogenesis and the role of oxalate and other low-molecular weight organic acids

Candida albicans is a polymorphic commensal yeast and is widespread in the human mycobiome. It colonizes different niches of the human body – the skin, mucosal surfaces of the mouth, reproductive and gastrointestinal tracts, or even the lungs – without causing any harm (92-94). Moreover, *C. albicans* is the most common human opportunistic fungal pathogen affecting immunocompromised people (95). One key component of the virulence of *C. albicans* is its ability to switch from yeast to hyphal growth forms, its two main morphotypes, during infection (95-98). This growth shift is induced by several factors

including incubation at 37°C, high CO_2 concentration, neutral or alkaline pH, growth in embedded conditions, carbon and nitrogen starvation, or direct contact with peptidoglycan (95, 98, 99). Moreover, oxalate has also been reported to trigger the shift from the yeast to the hyphal growth forms (100). Furthermore, when the environmental pH is not optimal for its growth, *C. albicans* has been shown to self-induce the yeast-to-hyphae growth shift by excreting ammonium ions in order to alter the pH of its environment (101).

Besides its ability to alkalinize its environment by the secretion of ammonia, *Candida* spp. has also been shown to acidify it (102). *Candida* spp. are well known to produce low-molecular weight organic acids (LMWOA) such as acetate, formate, pyruvate or propionate, under different culture conditions (103-106). *Candida* spp. were also shown to produce citric acid (106-108). The production of LMWOA has been suggested to have a key role in the pathogenesis of oral *Candida* infections (104). Indeed, acidification through acetate production, for example, by *C. albicans* has been shown to allow the production of aspartyl proteases (99, 102, 103, 109), which are major virulence factors (110). Interestingly, *Candida* spp. is also listed as an oxalate-producing fungal genus in the Human Metabolome Database (HMDB0002329). However, this has been never confirmed experimentally. To the best of our knowledge, the only studies linking the observation of calcium oxalate crystals to *Candida* spp. infections are the one from Takeuchi *et al.* (111), which reports the presence of *Candida*-like yeast cells in a bladder stone, and the one from Muntz (79), which describes the presence of *C. albicans* in a case of pulmonary aspergillosis in alpaca associated with the presence of calcium oxalate crystals. Moreover, no genes related to oxalic acid production were found in any of the *Candida* species (i.e. *C. albicans*, *C. auris*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. guilliermondii*, *C. lusitaniae*, *C. orthopsilosis*, and *C. parapsilosis*) present in the *Candida* Genome Database (CGD).

1.7. Microbiomes & ecological medicine – translating ecological concepts into human medicine

Each individual, as well as each specific tissue, harbors a complex community of microorganisms that constitute the so-called human microbiome. Although initially associated with disease, it is nowadays increasingly clear that these microbial communities are of great beneficial importance for human health (112). Indeed, the human microbiome has been shown to contribute to many aspects of human physiology, ranging from metabolic activity to the homeostasis of the immune system (113).

Traditionally, medicine has used a reductionist approach, ignoring completely complex interactions. In contrast, biology often deals with complex systems, such as complex communities, or entire ecosystems

(112, 114). Recently, there has been a shift in the paradigm concerning the principles directing the relationship of the human body and the microbial communities that can colonize it. This shift is the result of the realization that humans can be considered as an ecosystem, following the same ecological rules determining habitat colonization in other non-human ecosystems. Nowadays, it is becoming increasingly clear that differences in the taxonomic composition of the microbiome between individuals are important to determine the susceptibility to diseases as complex as cancer, as well as the success of medical treatments (115). Because of this implication in susceptibility to disease and medical care, there is an increasing interest into better understanding the factors that shape our microbiome, with the purpose, one day, of being able to modify those factors and contribute to a healthier microbiome composition.

This paradigm shift also led to an update of the famous Koch's postulates, which aim to determine the causative agent of infectious diseases. In this updated vision, a more integrated and ecological definition considers the role of the microbiome and its dysbiosis in health and disease (116, 117). In its original postulates, Koch states that the causative microorganism occurs in every individual having the disease symptoms, but not in healthy ones; and that after isolation and culturing from diseased individuals, the pathogen can cause disease after exposure of a new and healthy individual (118). This last assumption can be challenged in the case where a particular microorganism, or even a consortium, is present and can protect the host from the infection by the pathogen, a process called colonization resistance (116). For instance, Buffie *et al.* (119) found that the bacterium *Clostridium scindens* had a colonization resistance effect against the pathogen *Clostridium difficile* in a mouse model. In the same way, Lawley *et al.* (120) showed that the administration of feces from healthy-donor mice have a protective effect and helped pathogen clearance in *C. difficile*-infected mice. Moreover, a very recent study showed that the addition of *Lactobacillus murinus* in a respiratory dysbiosis murine model after Influenza A virus infection offered protection against pneumococcal colonization (121). A similar concept is widely used in soil ecology, which is that of disease-suppressive and conducive soils (122). This concept links the composition of the soil microbiota with the natural protection against plant fungal infections. Mendes *et al.* (123) showed that a specific assemblage of rhizospheric bacteria lead to disease-suppression and confer protection against the root fungal pathogen *Rhizoctonia solani* in sugar beet seedlings. In the same way, a specific microbiota composition could lead to more pathogen-suppressive communities, or conversely, dysbiosis of the microbiota could lead to pathogen-permissive communities. Translating soil ecological concepts into human medicine could be of great benefit as this will bring the necessary knowledge to understand the complex interspecies interaction dynamics within the human microbiome and between itself and the host (112, 114).

1.8. The role of the lung microbiota in pulmonary fungal infections and the gut-lung axis

Contrary to the gut, whose microbiota have been extensively studied, the lungs were considered sterile for a long time (124). However, the lung is now known to harbor a diverse microbiota composed of bacteria, fungi and viruses (125, 126). In the healthy lung, this microbiota is dominated by the genera *Prevotella*, *Streptococcus,* and *Veillonella* (127, 128). Its composition differs in the case of a disease attacking the lung (129-131) and after transplantation (132). Although most studies are descriptive, recent data provide clear evidence for the role of the airway microbiota (133), for instance, in the modulation of the host immune response or mucus production (134).

It can be expected that the lung microbiota plays a role in the development of fungal diseases, such as *Aspergillus* spp. infections, as there is increasing evidence that the lung microbiota plays a key role in immune homeostasis, chronic inflammatory diseases, and other physiological processes (135). Indeed, the *bacterial* microbiome is likely to have an influence on the composition of the *fungal* microbiome through bacterial-fungal interactions, thus making the lung environment more permissive or restrictive to fungal growth (135).

Moreover, respiratory diseases have not only been associated with lung microbiota dysbiosis, but also with the gut microbiota (136). This crosstalk between the gut and the lung compartment is called the gut-lung axis (137). The intestine has been shown to have an influence on the immune responses of distant organs, including lungs, through the systemic dissemination of metabolites such as short chain fatty acids (SCFAs), or through the direct seeding of bacteria from the gut to the lungs. These bacteria may then have a stimulatory effect of the local immune cells (135-137). However, mechanisms through which the lung could influence the gut are poorly known (137).

Currently, there are many knowledge gaps regarding the link between the pulmonary microbiota and *Aspergillus* spp. infections. For instance, alteration of the air-blood barrier, particularly access to the extracellular matrix, is a known risk factor for fungal infection (138), especially in the case of *Aspergillus* (139). Therefore, investigating the role of the lung microbiota on the strengthening of the air-blood barrier is of clinical importance. The integration of principles from ecological theory will be key in order to understand the interactions between the bacterial and fungal members of the gut and the pulmonary microbiota and their human host in order to identify manipulable elements from within the airway microbiota, or their metabolites, for the development of therapeutic tools to control *Aspergillus* (128, 135).

1.9. Bacterial-Fungal Interactions (BFI) in human diseases

Bacteria and fungi are known to co-occur and interact in a wide variety of ecosystems, ranging from soils and plant hosts up to all niches of the human body (140, 141). The interaction between the *bacterial* and *fungal* microbiota can affect both host health and disease (141). Indeed, BFI can be beneficial, in the case of eubiosis and high microbial diversity, to detrimental, in the case of dysbiosis and damage, for the host (141). Bacteria and fungi can interact through different modes, ranging from physical interaction, consumption or secretion of metabolites, changes in the environment, biofilm formation, or competition for nutrients or space (141, 142).

Several reviews have documented diverse BFI and their role in host health and disease, mainly *Candida*- and *Aspergillus*-bacteria interactions (98, 141-149). A recent review from d'Enfert *et al.* (98) summarized the current knowledge on the interaction of *C. albicans* with bacterial species present in the human microbiota. Most of the studied interactions between *C. albicans* and bacteria are antagonistic, as these could be exploited as therapeutic approaches based on biocontrol. However, around 30% of all *Candida* bloodstream infections are estimated to be polymicrobial, suggesting these types of interactions between *Candida* and bacteria might be synergistic (98, 150). Indeed, *C. albicans* is known to establish synergistic, as well as antagonistic, interactions in various locations of the human body, such as the vagina, the oral cavity or the gastrointestinal tract (98). These synergistic interactions include notably interactions between *C. albicans* and *Staphylococcus aureus* (151) or Streptococci (148, 152, 153), which for instance promote biofilm formation, and thus enhance virulence. On the contrary, *Enterococcus faecalis* is known to reduce biofilm formation by inhibiting the yeast-to-hyphal growth shift in *C. albicans* via the production of the bacteriocin inhibitor *EntV* (154). In addition, *Streptococcus mutans* has been shown to inhibit hyphal formation via the formation of the fatty acid Streptococcus Diffusible Signal Factor (SDSF) (155). *C. albicans* is also known to interact with *Pseudomonas aeruginosa*. An early report from Hogan *et al.* (156) showed that *P. aeruginosa* kills *C. albicans* in its hyphal form by forming a dense biofilm on it, most probably through the use of type IV pili and production of specific virulence factors such as phospholipase C and phenazines. *C. albicans* and *P. aeruginosa* were also found to mutually enhance their virulence (98, 157). Indeed, *C. albicans* was shown to produce ethanol, which promotes the production of phenazines such as pyocyanin. These phenazines in turn increase ethanol production by *C. albicans* and inhibit hyphal growth and biofilm formation by the latter (98, 157). Lactobacilli are known to inhibit *C. albicans* filamentation through the production of lactic acid, which maintains an acidic pH in the vagina (158, 159). Moreover, *Lactobacillus* spp. were shown to protect against *C. albicans* infection by inducing the production of IL-22 through the production of indole-3-aldehyde which binds to AhR, a receptor that modulate disease resistance (98, 160, 161). *Salmonella enterica* serovar Typhimurium was

also shown to kill *C. albicans* hyphae via the injection of SopB effectors through the SipB translocase (98, 162).

Concerning *Aspergillus* spp., the study of its interactions with bacteria is less extended. Indeed, to our knowledge, all the data available on *Aspergillus*-bacteria interactions almost exclusively concern *P. aeruginosa* (147, 149). Indeed, both *A. fumigatus* and *P. aeruginosa* are the most common fungus and bacterium isolated from patients suffering from cystic fibrosis (163), in which they often co-occur as polymicrobial infections (164). *A. fumigatus* has been shown to interact both positively and negatively with *P. aeruginosa* through the secretion of molecules by the latter (147). *P. aeruginosa* has been shown to stimulate fungal growth and increase iron uptake by *A. fumigatus* through the production of pyochelin and phenazines (concentration <100µM) (165). The volatile dimethylsulfide produced by *P. aeruginosa* was also shown to stimulate *A. fumigatus* growth (166). Dirhamnolipids, another type of molecule secreted by *P. aeruginosa*, were shown to induce the formation of a thick cell wall, the production of melanin and galactosaminogalactan (GAG) in the extracellular matrix, and also provide resistance to caspofungin in *A. fumigatus* (167). In terms of negative interactions, the production of homoserine lactones by *P. aeruginosa* is well known to inhibit *A. fumigatus* growth (168). Moreover, pyoverdine and pyochelin have also been shown to have a negative impact on *A. fumigatus* growth resulting from iron starvation (147). Pyochelin, together with phenazine 1-HP, was shown to kill *A. fumigatus* through the induction of reactive oxygen and nitrogen species production (169). Finally, dirhamnolipids were also found to inhibit the synthesis of β1,3 glucan (167). Outside *P. aeruginosa*, *Klebsiella pneumoniae* was found to inhibit spore germination and growth in *Aspergillus* spp. (170). Another study showed that *A. fumigatus* inhibits biofilm formation in *Acinetobacter baumannii* via the production of gliotoxin (171). Additionally, *Staphylococcus aureus* and *Streptococcus pneumoniae* were shown to inhibit *A. fumigatus* development (149). Finally, *Stenotrophomonas maltophilia* was shown to hinder hyphal formation, reduce biofilm formation, and block conidia production in *A. fumigatus* (149).

Many research gaps still exist concerning the interactions between *Aspergillus* spp. and the lung microbiota, and their contribution to health and disease. More systematic studies are thus needed in order to decipher the impact of these interactions in the onset of pulmonary aspergillosis.

Beside *Aspergillus* and *Candida* spp., Frases *et al.* (172) showed that *Klebsiella aerogenes* promoted *Cryptococcus neoformans* melanization. *Saccharomyces cerevisiae* was found to improve *Acinetobacter* spp. growth and pathogenicity *in-vitro* and in a *Caenorhabditis elegans in-vivo* infection model (173). Additionally, *Mucorales* molds, such as *Rhizopus* spp. and *Mucor* spp., are known to infect the lungs of immunocompromised people, and they thus must inevitably interact with the bacterial microbiota (141).

Mucorales fungi are well known to harbor endosymbiotic bacteria, such as the famous *Burkholderia rhizoxinica* of *Rhizopus microsporus,* which produces the plant mycotoxin rhizoxin (174). Unlike *Mucorales* pathogens infecting plants, the presence of *Burkholderia* endosymbionts was not found to increase pathogenicity in *Rhizopus* spp. and *Mucor* spp. animal pathogens in mice and fly infection models (175). However, a recent study from Itabangi *et al.* (176) showed that the *R. microsporus* endobacterium *Ralstonia pickettii* was required for virulence in zebrafish and mice, and that it also permitted its host to escape phagocytosis by the soil amoeba *Dictyostelium discoideum*.

1.10. Outline of the thesis

The present thesis studies a specific BFI in the case of human health: the biocontrol potential of oxalotrophic bacteria on oxalic acid-producing *Aspergillus niger* for the fight against pulmonary aspergillosis.

The rationale for using *A. niger*, which accounts only for 5% of the reported cases of pulmonary aspergillosis (55, 177), as compared to *A. fumigatus*, which is the major opportunistic fungal pathogen in humans (60), is biosafety reasons. The former is regarded as a safe relative of the latter, facilitating the work (178). Moreover, *A. niger* has been extensively studied for its ability to produce oxalic acid in various culture conditions (179, 180), something we confirmed in Chapter 3. Additionally, besides being abundant in the environment, the *Aspergillus* genus has also been shown to be part of the normal upper and lower respiratory tract mycobiota (60, 181).

The commensal yeast *Candida albicans* was also used in this thesis. The rationale for selecting this second opportunistic fungal pathogen model is because the genus *Candida* has been shown to co-occur in mixed fungal lung infections with *Aspergillus* spp. in immunocompromised (46) and cystic fibrosis patients (182). Moreover, oxalate has been shown to have a role in the yeast-to-hyphal growth shift in *C. albicans* (100). Finally, as in the case of the *Aspergillus* genus, *Candida* spp. have been shown to commonly colonize the lower respiratory tract (94).

Chapter 2 presents a review of the literature available on oxalic acid as a mediator of BFI. More specifically, the production and consumption of oxalic acid by fungi and bacteria, their interactions and contributions to the general cycling of this molecule in the soils, plants and human ecosystems, as well as the research gaps in the field are presented. This chapter has been published in Advances in Applied Microbiology (Vol. 106, DOI: 10.1016/bs.aambs.2018.10.001).

In Chapter 3, the impact of the environmental (trophic) conditions on the interaction between fungi and bacteria are tested. Moreover, the implications of these results from a biocontrol point of view are discussed.

Chapter 4 presents the first demonstration of biocontrol activity of oxalotrophic bacteria over *A. niger* in *in-vitro* conditions in Petri dishes and 3D-lung cell tissues. This chapter has been published as a preprint on BioRxiv (DOI: 10.1101/2020.08.20.259929) and it is currently under revision in PLOS One.

Chapter 5 presents the full genome of *Cupriavidus oxalaticus* Ox1 and the mechanisms involved in oxalotrophic growth in this model bacterium, as well as the genomic markers for oxalotrophy for the future identification of oxalotrophic bacteria in the lung microbiota.

In Chapter 6, we explored for the first time Fungal-Fungal-Bacterial interactions by investigating the interaction between *Candida albicans, Aspergillus niger* and *Cupriavidus oxalaticus*. Indeed, fungal co-infections are of clinical importance, as they occur frequently in immunocompromised, and cystic fibrosis patients.

Finally, Chapter 7 presents a general discussion of the present dissertation, a summary of the findings of each chapter, as well as future perspective of research, and a conclusion.

1.11. Research objectives and hypotheses

The general aim of this thesis was to study the interaction between *Aspergillus niger* and the oxalotrophic bacterium *Cupriavidus oxalaticus* and to develop a biocontrol model based on the environmental interference of oxalate-producing *A. niger* by oxalatrophic bacteria under laboratory conditions. More specifically, the research objectives of this study were:

- To present a review of the literature available on oxalic acid production and consumption by fungi and bacteria, their interactions and contributions to the general cycling of this molecule in the soils, plants and human ecosystems (Chapter 2).
- To identify a fungal model relevant to human health based on its ability to produce low molecular weight organic acids (LMWOA) on different culture media, and investigate the impact of trophic conditions on the interaction between bacteria and fungi (Chapter 3).
- To investigate the biocontrol potential of the oxalotrophic bacterium *C. oxalaticus* to control the growth of *A. niger in vitro* in Petri dishes and in 3D-lung cell cultures in Transwell® inserts and Lung-on-a-chip systems (Chapter 4).
- To provide the full genome of the model oxalotrophic bacterium *C. oxalaticus* and to investigate the genomic determinants of oxalotrophy in this bacterial species (Chapter 5).
- To investigate the interaction between *A. niger* and *Candida albicans* as a frequent fungal co-infection, as well as their interaction with *C. oxalaticus* (Chapter 6).

Accordingly, the subsequent hypotheses are the following:

- Trophic conditions, and more specifically the C/N ratio, have an effect on LMWOA production by fungi, as well as on the interaction with bacteria (oxalotrophic and non-oxalotrophic) (Chapter 3).
- Oxalic acid production is needed for *Aspergillus* spp. to manipulate pH during lung cells infection and thus interfering with this process through bacterial oxalotrophy may limit its growth potential (Chapter 4).
- Oxalate produced by *A. niger* could trigger the shift from yeast to hyphal growth in *C. albicans*, and thus using oxalotrophic bacteria permits to revert back to yeast growth by inhibiting *A. niger* (Chapter 6).

References

1. Morens DM, Fauci AS. Emerging Pandemic Diseases: How We Got to COVID-19. Cell. 2020;182(5):1077-92.

2. Dobson AP, Carper ER. Infectious Diseases and Human Population History. BioScience. 1996;46(2):115-26.

3. Prentice MB, Rahalison L. Plague. The Lancet. 2007;369(9568):1196-207.

4. Wagner DM, Klunk J, Harbeck M, Devault A, Waglechner N, Sahl JW, et al. Yersinia pestis and the Plague of Justinian 541–543 AD: a genomic analysis. The Lancet Infectious Diseases. 2014;14(4):319-26.

5. Bourzac K. Infectious disease: Beating the big three. Nature. 2014;507(7490):S4-7.

6. Paulson T. Epidemiology: A mortal foe. Nature. 2013;502(7470):S2-3.

7. Global tuberculosis report 2020. 2020 15 October 2020.

8. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: past, present and future. Curr Opin Microbiol. 2019;51:72-80.

9. Smith PW, Watkins K, Hewlett A. Infection control through the ages. Am J Infect Control. 2012;40(1):35-42.

10. Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? Yale J Biol Med. 2017;90(1):135-45.

11. Gould K. Antibiotics: from prehistory to the present day. J Antimicrob Chemother. 2016;71(3):572-5.

12. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277-83.

13. Martens E, Demain AL. The antibiotic resistance crisis, with a focus on the United States. J Antibiot (Tokyo). 2017;70(5):520-6.

14. Chang Q, Wang W, Regev-Yochay G, Lipsitch M, Hanage WP. Antibiotics in agriculture and the risk to human health: how worried should we be? Evol Appl. 2015;8(3):240-7.

15. Benveniste R, Davies J. Mechanisms of antibiotic resistance in bacteria. Annu Rev Biochem. 1973;42:471-506.

16. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74(3):417-33.

17. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science. 2018;360(6390):739-42.

18. Geissel B, Loiko V, Klugherz I, Zhu Z, Wagener N, Kurzai O, et al. Azole-induced cell wall carbohydrate patches kill Aspergillus fumigatus. Nat Commun. 2018;9(1):3098.

19. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, et al. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis. 2008;46(3):327-60.

20. Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr., Calandra TF, Edwards JE, Jr., et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis. 2009;48(5):503-35.

21. Perlin DS. Current perspectives on echinocandin class drugs. Future Microbiol. 2011;6(4):441-57.

22. Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. The Lancet Infectious Diseases. 2017;17(12):e383-e92.

23. Robinson TP, Bu DP, Carrique-Mas J, Fèvre EM, Gilbert M, Grace D, et al. Antibiotic resistance is the quintessential One Health issue. 2016. p. 377-80.

24. Trinh P, Zaneveld JR, Safranek S, Rabinowitz PM. One Health Relationships Between Human, Animal, and Environmental Microbiomes: A Mini-Review. Front Public Health. 2018;6:235.

25. Chowdhary A, Meis JF. Emergence of azole resistant Aspergillus fumigatus and One Health: time to implement environmental stewardship. Environ Microbiol. 2018;20(4):1299-301.

26. Meis JF, Chowdhary A, Rhodes JL, Fisher MC, Verweij PE. Clinical implications of globally emerging azole resistance in Aspergillus fumigatus. Philos Trans R Soc Lond B Biol Sci. 2016;371(1709).

27. Verweij PE, Zhang J, Debets AJM, Meis JF, van de Veerdonk FL, Schoustra SE, et al. In-host adaptation and acquired triazole resistance in Aspergillus fumigatus : a dilemma for clinical management. The Lancet Infectious Diseases. 2016;16(11):e251-e60.

28. Arastehfar A, Carvalho A, van de Veerdonk FL, Jenks JD, Koehler P, Krause R, et al. COVID-19 Associated Pulmonary Aspergillosis (CAPA)-From Immunology to Treatment. J Fungi (Basel). 2020;6(2).

29. Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in Aspergillus fumigatus and other Aspergillus species. J Clin Microbiol. 2001;39(7):2431-8.

30. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases—estimate precision. 2017.

31. Rodrigues ML, Nosanchuk JD. Fungal diseases as neglected pathogens: A wake-up call to public health officials. PLoS Negl Trop Dis. 2020;14(2):e0007964.

32. Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med Mycol. 2007;45(4):321-46.

33. Suleyman G, Alangaden GJ. Nosocomial Fungal Infections: Epidemiology, Infection Control, and Prevention. 2016. p. 1023-52.

34. Lionakis MS, Hohl TM. Call to Action: How to Tackle Emerging Nosocomial Fungal Infections. Cell Host Microbe. 2020;27(6):859-62.

35. Pfaller MA, Diekema DJ. Rare and emerging opportunistic fungal pathogens: Concern for resistance beyond Candida albicans and Aspergillus fumigatus. 2004. p. 4419-31.

36. Brunke S, Mogavero S, Kasper L, Hube B. Virulence factors in fungal pathogens of man. 2016. p. 89-95.

37. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden killers: Human fungal infections. 2012. p. 165rv13-rv13.

38. Spivak ES, Hanson KE. Candida auris: an Emerging Fungal Pathogen. J Clin Microbiol. 2018;56(2).

39. Tsang CC, Tang JYM, Ye H, Xing F, Lo SKF, Xiao C, et al. Rare/cryptic Aspergillus species infections and importance of antifungal susceptibility testing. Mycoses. 2020.

40. Friedman DZP, Schwartz IS. Emerging fungal infections: New patients, new patterns, and new pathogens. 2019. p. 67-.

41. Schwartz IS, Govender NP, Sigler L, Jiang Y, Maphanga TG, Toplis B, et al. Emergomyces: The global rise of new dimorphic fungal pathogens. PLoS Pathog. 2019;15(9):e1007977.

42. Patel TS, Eschenauer GA, Stuckey LJ, Carver PL. Antifungal Prophylaxis in Lung Transplant Recipients. Transplantation. 2016;100(9):1815-26.

43. Herrera S, Davoudi S, Farooq A, Tikkanen J, Foroutan F, Kumar D, et al. Late Onset Invasive Pulmonary Aspergillosis in Lung Transplant Recipients in the Setting of a Targeted Prophylaxis/Preemptive Antifungal Therapy Strategy. Transplantation. 2020;104(12):2575-81.

44. Cornely OA, Hoenigl M, Lass-Flörl C, Chen SCA, Kontoyiannis DP, Morrissey CO, et al. Defining breakthrough invasive fungal infection–Position paper of the mycoses study group education and research consortium and the European Confederation of Medical Mycology. 2019. p. 716-29.

45. Lerolle N, Raffoux E, Socie G, Touratier S, Sauvageon H, Porcher R, et al. Breakthrough invasive fungal disease in patients receiving posaconazole primary prophylaxis: A 4-year study. Clinical Microbiology and Infection. 2014;20(11):O952-O9.

46. Jaya S, Vipparti H. Mixed fungal lung infection with Aspergillus fumigatus and Candida albicans in a immunocomprimised patient: case report. J Clin Diagn Res. 2014;8(4):DD08-10.

47. von Eiff M, Roos N, Fegeler W, von Eiff C, Zuhlsdorf M, Glaser J, et al. Pulmonary fungal infections in immunocompromised patients: incidence and risk factors. Mycoses. 1994;37(9-10):329-35.

48. Obradovic-Tomasev M, Popovic A, Vuckovic N, Jovanovic M. Mixed fungal infection (Aspergillus, mucor, and Candida) of severe hand injury. Case Rep Infect Dis. 2014;2014:954186.

49. Mostafa A, Gaber M, Abdelrahman H, ElAnwar N, Galal A. Case Report: Facial mixed fungal infection in a poorly responding non-Hodgkin's lymphoma patient with suspected immunodeficiency. F1000Research. 2019;8.

50. Schwartz IS, Friedman DZP, Zapernick L, Dingle TC, Lee N, Sligl W, et al. High Rates of Influenza-Associated Invasive Pulmonary Aspergillosis May Not Be Universal: A Retrospective Cohort Study from Alberta, Canada. Clin Infect Dis. 2020;71(7):1760-3.

51. Verweij PE, Brüggeman RJM, Wauters J, Rijnders BJA, Chiller T, Van De Veerdonk FL. Influenza coinfection: Be(a)ware of invasive aspergillosis. 2020. p. 349-50.

52. Thompson GR, Cornely OA, Pappas PG, Patterson TF, Hoenigl M, Jenks JD, et al. Invasive aspergillosis as an under-recognized superinfection in COVID-19. 2020. p. ofaa242-ofaa.

53. Verweij PE, Gangneux J-P, Bassetti M, Brüggemann RJM, Cornely OA, Koehler P, et al. Diagnosing COVID-19-associated pulmonary aspergillosis. The Lancet Microbe. 2020;1(2):e53-e5.

54. Gangneux JP, Bougnoux ME, Dannaoui E, Cornet M, Zahar JR. Invasive fungal diseases during COVID-19: We should be prepared. 2020. p. 1524-35.

55. Paulussen C, Hallsworth JE, Álvarez-Pérez S, Nierman WC, Hamill PG, Blain D, et al. Ecology of aspergillosis: insights into the pathogenic potency of Aspergillus fumigatus and some other Aspergillus species. Microbial Biotechnology. 2017;10(2):296-322.

56. Kousha M, Tadi R, Soubani AO. Pulmonary aspergillosis: A clinical review. 2011. p. 156-74.

57. Kwon-Chung KJ, Sugui JA. Aspergillus fumigatus—What Makes the Species a Ubiquitous Human Fungal Pathogen? PLoS Pathogens. 2013;9(12):e1003743-e.

58. Van De Veerdonk FL, Gresnigt MS, Romani L, Netea MG, Latgé JP. Aspergillus fumigatus morphology and dynamic host interactions. 2017. p. 661-74.

59. Park SJ, Mehrad B. Innate immunity to Aspergillus species. Clin Microbiol Rev. 2009;22(4):535-51.

60. Latge JP, Chamilos G. Aspergillus fumigatus and Aspergillosis in 2019. Clin Microbiol Rev. 2019;33(1).

61. de Oliveira Ceita G, Macêdo JNA, Santos TB, Alemanno L, da Silva Gesteira A, Micheli F, et al. Involvement of calcium oxalate degradation during programmed cell death in Theobroma cacao tissues triggered by the hemibiotrophic fungus Moniliophthora perniciosa. Plant Science. 2007;173(2):106-17.

62. Kosmidis C, Denning DW. The clinical spectrum of pulmonary aspergillosis. Thorax. 2015;70(3):270-7.

63. Gago S, Denning DW, Bowyer P. Pathophysiological aspects of Aspergillus colonization in disease. Med Mycol. 2019;57(Supplement_2):S219-S27.

64. Ofori A, Steinmetz AR, Akaasi J, Asafu Adjaye Frimpong GA, Norman BR, Obeng-Baah J, et al. Pulmonary aspergilloma: An evasive disease. Int J Mycobacteriol. 2016;5(2):235-9.

65. Dhooria S, Sehgal IS, Muthu V, Agarwal R. Treatment of allergic bronchopulmonary aspergillosis: from evidence to practice. Future Microbiology. 2020;15(5):365-76.

66. Margalit A, Kavanagh K. The innate immune response to Aspergillus fumigatus at the alveolar surface. FEMS Microbiol Rev. 2015;39(5):670-87.

67. Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. Nat Immunol. 2015;16(1):27-35.

68. Bertuzzi M, Hayes GE, Icheoku UJ, van Rhijn N, Denning DW, Osherov N, et al. Anti-Aspergillus Activities of the Respiratory Epithelium in Health and Disease. J Fungi (Basel). 2018;4(1).

69. Bigot J, Guillot L, Guitard J, Ruffin M, Corvol H, Balloy V, et al. Bronchial Epithelial Cells on the Front Line to Fight Lung Infection-Causing Aspergillus fumigatus. Front Immunol. 2020;11:1041.

70. Cunha C, Carvalho A, Esposito A, Bistoni F, Romani L. DAMP signaling in fungal infections and diseases. Front Immunol. 2012;3:286.

71. Speth C, Rambach G, Lass-Florl C, Dierich MP, Wurzner R. The role of complement in invasive fungal infections. Mycoses. 2004;47(3-4):93-103.

72. Holers VM. Complement and its receptors: new insights into human disease. Annu Rev Immunol. 2014;32:433-59.

73. van Kan JAL. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends in Plant Science. 2006;11(5):247-53.

74. Dutton MV, Evans CS. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology. 1996;42(9):881-95.

75. Moore D, Robson G, Trinci T. 21st Century Guidebook to Fungi. Cambridge: Cambridge University Press; 2011. 627- p.

76. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. In: Gadd GM, Sariaslani S, editors. 106: Academic Press; 2019. p. 49-77.

77. Kurrein F, Path FRC, Green GH, Rowles SL. Localized deposition of calcium oxalate around a pulmonary Aspergillus niger fungus ball. American Journal of Clinical Pathology. 1975;64(4):556-63.

78. Kauffman CA, Wilson KH, Schwartz DB. Necrotizing Pulmonary Aspergillosis with Oxalosis: Nekrotisierende pulmonale Aspergillose mit Oxalose. Mycoses. 1984;27(11):535-8.

79. Muntz FHA. Oxalate-producing pulmonary aspergillosis in an alpaca. Veterinary Pathology. 1999;36(6):631-2.

80. Kuwabara H, Shibayama Y. Pulmonary aspergilloma with prominent oxalate deposition. Indian Journal of Pathology and Microbiology. 2012;55(4):589-.

81. Oda M, Saraya T, Wakayama M, Shibuya K, Ogawa Y, Inui T, et al. Calcium oxalate crystal deposition in a patient with Aspergilloma due to Aspergillus niger. Journal of thoracic disease. 2013;5(4):E174-8.

82. Pabuççuoğlu U. Aspects of oxalosis associated with aspergillosis in pathology specimens. Pathology Research and Practice. 2005;201(5):363-8.

83. Maeno T, Sasaki M, Shibue Y, Mimura K, Oka H. Calcium oxalate in the sputum may aid in the diagnosis of pulmonary aspergillosis: A report of two cases. Medical Mycology Case Reports. 2015;8:32-6.

84. Payne CL, Dark MJ, Conway JA, Farina LL. A retrospective study of the prevalence of calcium oxalate crystals in veterinary Aspergillus cases. Journal of Veterinary Diagnostic Investigation. 2017;29(1):51-8.

85. Yi Y, Cho SY, Lee DG, Jung JI, Park YJ, Lee KY. Invasive Pulmonary Aspergillosis Due to Aspergillus awamori: Role of Calcium Oxalate Crystal Precipitation Mimicking Mucormycosis. 2020. p. 409-11.

86. Lee SH, Barnes WG, Schaetzel WP. Pulmonary aspergillosis and the importance of oxalate crystal recognition in cytology specimens. Arch Pathol Lab Med. 1986;110(12):1176-9.

87. Ghio AJ, Peterseim DS, Roggli VL, Piantadosi CA. Pulmonary oxalate deposition associated with Aspergillus niger infection. An oxidant hypothesis of toxicity. Am Rev Respir Dis. 1992;145(6):1499-502.

88. Vig M, Kinet JP. Calcium signaling in immune cells. 2009. p. 21-7.

89. Grinstein S, Klip A. Calcium homeostasis and the activation of calcium channels in cells of the immune system. 1989. p. 69-79.

90. Diamantstein T, Odenwald MV. Control of the immune response in vitro by calcium ions. I. The antagonistic action of calcium ions on cell proliferation and on cell differentiation. Immunology. 1974;27(4):531-53141.

91. Diamantstein T, Ulmer A. The control of immune response in vitro by Ca2+. II. The Ca2+ dependent period during mitogenic stimulation. Immunology. 1975;28(1):121-5.

92. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol. 2011;10(2):112-22.

93. Underhill DM, Iliev ID. The mycobiota: interactions between commensal fungi and the host immune system. Nat Rev Immunol. 2014;14(6):405-16.

94. Zuo YH, Wang WQ, Chen QJ, Liu B, Zhang FY, Jin XY, et al. Candida in Lower Respiratory Tract Increases the Frequency of Acute Exacerbation of Chronic Obstructive Pulmonary Disease: A Retrospective Case-Control Study. Front Cell Infect Microbiol. 2020;10:538005.

95. Kim J, Sudbery P. Candida albicans, a major human fungal pathogen. J Microbiol. 2011;49(2):171-7.

96. Noble SM, Gianetti BA, Witchley JN. Candida albicans cell-type switching and functional plasticity in the mammalian host. Nat Rev Microbiol. 2017;15(2):96-108.

97. Chen H, Zhou X, Ren B, Cheng L. The regulation of hyphae growth in Candida albicans. Virulence. 2020;11(1):337-48.

98. d'Enfert C, Kaune AK, Alaban LR, Chakraborty S, Cole N, Delavy M, et al. The impact of the Fungus-Host-Microbiota interplay upon Candida albicans infections: current knowledge and new perspectives. FEMS Microbiol Rev. 2020.

99. Mba IE, Nweze EI. Mechanism of Candida pathogenesis: revisiting the vital drivers. Eur J Clin Microbiol Infect Dis. 2020;39(10):1797-819.

100. Bonhomme J, Chauvel M, Goyard S, Roux P, Rossignol T, d'Enfert C. Contribution of the glycolytic flux and hypoxia adaptation to efficient biofilm formation by Candida albicans. Mol Microbiol. 2011;80(4):995-1013.

101. Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H, Lorenz MC. The fungal pathogen Candida albicans autoinduces hyphal morphogenesis by raising extracellular pH. mBio. 2011;2(3):e00055-11.

102. Vylkova S. Environmental pH modulation by pathogenic fungi as a strategy to conquer the host. PLoS Pathog. 2017;13(2):e1006149.

103. Samaranayake LP, Geddes DAM, Weetman DA, MacFarlane TW. Growth and acid production of Candida albicans in carbohydrate supplemented media. Microbios. 1983;37(148):105-15.

104. Samaranayake LP, Hughes A, Weetman DA, MacFarlane TW. Growth and acid production of Candida species in human saliva supplemented with glucose. J Oral Pathol. 1986;15(5):251-4.

105. Klinke T, Kneist S, de Soet JJ, Kuhlisch E, Mauersberger S, Forster A, et al. Acid production by oral strains of Candida albicans and lactobacilli. Caries Res. 2009;43(2):83-91.

106. Oliver JC, Laghi L, Parolin C, Foschi C, Marangoni A, Liberatore A, et al. Metabolic profiling of Candida clinical isolates of different species and infection sources. Sci Rep. 2020;10(1):16716.

107. West TP. Citric acid production by Candida species grown on a soy-based crude glycerol. Prep Biochem Biotechnol. 2013;43(6):601-11.

108. Odu NN, Akani NP, Uzah GA. Screening of Aspergillus and Candida Species with Utmost Potential to Synthesize Citric Acid. Journal of Advances in Microbiology. 2020:10-8.

109. Tsuboi R, Matsuda K, Ko IJ, Ogawa H. Correlation between culture medium pH, extracellular proteinase activity, and cell growth of Candida albicans in insoluble stratum corneum-supplemented media. Arch Dermatol Res. 1989;281(5):342-5.

110. Singh DK, Nemeth T, Papp A, Toth R, Lukacsi S, Heidingsfeld O, et al. Functional Characterization of Secreted Aspartyl Proteases in Candida parapsilosis. mSphere. 2019;4(4).

111. Takeuchi H, Konishi T, Tomoyoshi T. Detection by light microscopy of candida in thin sections of bladder stone. Urology. 1989;34(6):385-7.

112. Pepper JW, Rosenfeld S. The emerging medical ecology of the human gut microbiome. Trends Ecol Evol. 2012;27(7):381-4.

113. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811-8.

114. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJ, Relman DA. The application of ecological theory toward an understanding of the human microbiome. Science. 2012;336(6086):1255-62.

115. Califf K, Gonzalez A, Knight R, Caporaso J. The Human Microbiome: Getting Personal. Microbe Magazine.

116. Byrd AL, Segre JA. Infectious disease. Adapting Koch's postulates. Science. 2016;351(6270):224-6.

117. Vonaesch P, Anderson M, Sansonetti PJ. Pathogens, microbiome and the host: emergence of the ecological Koch's postulates. FEMS Microbiol Rev. 2018;42(3):273-92.

118. Kaufmann SHE, Schaible UE. 100th anniversary of Robert Koch's Nobel Prize for the discovery of the tubercle bacillus. 2005. p. 469-75.

119. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. Nature. 2015;517(7533):205-8.

120. Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, et al. Targeted Restoration of the Intestinal Microbiota with a Simple, Defined Bacteriotherapy Resolves Relapsing Clostridium difficile Disease in Mice. PLoS Pathogens. 2012;8(10):e1002995-e.

121. Yildiz S, Pereira Bonifacio Lopes JP, Berge M, Gonzalez-Ruiz V, Baud D, Kloehn J, et al. Respiratory tissue-associated commensal bacteria offer therapeutic potential against pneumococcal colonization. Elife. 2020;9.

122. Mazzola M. Mechanisms of natural soil suppressiveness to soilborne diseases. Antonie van Leeuwenhoek. 2002;81(1):557-64.

123. Mendes R, Kruijt M, De Bruijn I, Dekkers E, Van Der Voort M, Schneider JHM, et al. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science. 2011;332(6033):1097-100.

124. Chang D, Dela Cruz CS, Sharma L. Challenges in understanding lung microbiome: It is NOT like the gut microbiome. Respirology. 2020;25(3):244-5.

125. Enaud R, Prevel R, Ciarlo E, Beaufils F, Wieërs G, Guery B, et al. The Gut-Lung Axis in Health and Respiratory Diseases: A Place for Inter-Organ and Inter-Kingdom Crosstalks. 2020.

126. Mitchell AB, Oliver BGG, Glanville AR. Translational aspects of the human respiratory virome. 2016. p. 1458-64.

127. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, et al. Topographical continuity of bacterial populations in the healthy human respiratory tract. American Journal of Respiratory and Critical Care Medicine. 2011;184(8):957-63.

128. Foesel BU, Pfeiffer S, Raj ACD, Etschmann SK, Schloter M. Applying ecological theories in research: Lessons learned from microbial ecology and evolution? ERS Monograph. 2019;2019(9781849841023):50-66.

129. Cui L, Lucht L, Tipton L, Rogers MB, Fitch A, Kessinger C, et al. Topographic diversity of the respiratory tract mycobiome and alteration in HIV and lung disease. American Journal of Respiratory and Critical Care Medicine. 2015;191(8):932-42.

130. Dickson RP, Martinez FJ, Huffnagle GB. The role of the microbiome in exacerbations of chronic lung diseases. 2014. p. 691-702.

131. van Woerden HC, Gregory C, Brown R, Marchesi JR, Hoogendoorn B, Matthews IP. Differences in fungi present in induced sputum samples from asthma patients and non-atopic controls: A community based case control study. BMC Infectious Diseases. 2013;13(1):69-.

132. Charlson ES, Diamond JM, Bittinger K, Fitzgerald AS, Yadav A, Haas AR, et al. Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant. American Journal of Respiratory and Critical Care Medicine. 2012;186(6):536-45.

133. Budden KF, Shukla SD, Rehman SF, Bowerman KL, Keely S, Hugenholtz P, et al. Functional effects of the microbiota in chronic respiratory disease. 2019. p. 907-20.

134. Mathieu E, Escribano-Vazquez U, Descamps D, Cherbuy C, Langella P, Riffault S, et al. Paradigms of Lung Microbiota Functions in Health and Disease, Particularly, in Asthma. Frontiers in Physiology. 2018;9(1168).

135. Kolwijck E, van de Veerdonk FL. The potential impact of the pulmonary microbiome on immunopathogenesis of Aspergillus-related lung disease. European Journal of Immunology. 2014;44(11):3156-65.

136. Ubags NDJ, Marsland BJ. Mechanistic insight into the function of the microbiome in lung diseases. European Respiratory Journal. 2017;50(3).

137. Marsland BJ, Trompette A, Gollwitzer ES. The gut-lung axis in respiratory disease. Annals of the American Thoracic Society. 2015;12(November):S150-S6.

138. Kottom TJ, Köhler JR, Thomas CF, Fink GR, Limper AH. Lung Epithelial Cells and Extracellular Matrix Components Induce Expression of Pneumocystis carinii STE20, a Gene Complementing the Mating and Pseudohyphal Growth Defects of ste20 Mutant Yeast. Infection and Immunity. 2003;71(11):6463.

139. Kunst H, Wickremasinghe M, Wells A, Wilson R. Nontuberculous mycobacterial disease and Aspergillus-related lung disease in bronchiectasis. European Respiratory Journal. 2006;28(2):352.

140. Deveau A, Bonito G, Uehling J, Paoletti M, Becker M, Bindschedler S, et al. Bacterial-fungal interactions: Ecology, mechanisms and challenges. FEMS Microbiology Reviews. 2018;42(3):335-52.

141. Kruger W, Vielreicher S, Kapitan M, Jacobsen ID, Niemiec MJ. Fungal-Bacterial Interactions in Health and Disease. Pathogens. 2019;8(2).

142. Peleg AY, Hogan DA, Mylonakis E. Medically important bacterial-fungal interactions. Nat Rev Microbiol. 2010;8(5):340-9.

143. Wargo MJ, Hogan DA. Fungal--bacterial interactions: a mixed bag of mingling microbes. Curr Opin Microbiol. 2006;9(4):359-64.

144. Lopez-Medina E, Koh AY. The complexities of bacterial-fungal interactions in the mammalian gastrointestinal tract. Microb Cell. 2016;3(5):191-5.

145. Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME. Polymicrobial interactions: impact on pathogenesis and human disease. Clin Microbiol Rev. 2012;25(1):193-213.

146. Diaz PI, Strausbaugh LD, Dongari-Bagtzoglou A. Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. Front Cell Infect Microbiol. 2014;4:101.

147. Briard B, Mislin GLA, Latge JP, Beauvais A. Interactions between Aspergillus fumigatus and Pulmonary Bacteria: Current State of the Field, New Data, and Future Perspective. J Fungi (Basel). 2019;5(2).

148. Morales DK, Hogan DA. Candida albicans interactions with bacteria in the context of human health and disease. PLoS Pathog. 2010;6(4):e1000886.

149. Margalit A, Carolan JC, Kavanagh K. Bacterial Interactions with Aspergillus fumigatus in the Immunocompromised Lung. Microorganisms. 2021;9(2).

150. Klotz SA, Chasin BS, Powell B, Gaur NK, Lipke PN. Polymicrobial bloodstream infections involving Candida species: analysis of patients and review of the literature. Diagn Microbiol Infect Dis. 2007;59(4):401-6.

151. Carolus H, Van Dyck K, Van Dijck P. Candida albicans and Staphylococcus Species: A Threatening Twosome. Front Microbiol. 2019;10:2162.

152. Bamford CV, d'Mello A, Nobbs AH, Dutton LC, Vickerman MM, Jenkinson HF. Streptococcus gordonii modulates Candida albicans biofilm formation through intergeneric communication. Infect Immun. 2009;77(9):3696-704.

153. Metwalli KH, Khan SA, Krom BP, Jabra-Rizk MA. Streptococcus mutans, Candida albicans, and the human mouth: a sticky situation. PLoS Pathog. 2013;9(10):e1003616.

154. Graham CE, Cruz MR, Garsin DA, Lorenz MC. Enterococcus faecalis bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of Candida albicans. Proc Natl Acad Sci U S A. 2017;114(17):4507-12.

155. Vilchez R, Lemme A, Ballhausen B, Thiel V, Schulz S, Jansen R, et al. Streptococcus mutans inhibits Candida albicans hyphal formation by the fatty acid signaling molecule trans-2-decenoic acid (SDSF). Chembiochem. 2010;11(11):1552-62.

156. Hogan DA, Kolter R. Pseudomonas-Candida interactions: an ecological role for virulence factors. Science. 2002;296(5576):2229-32.

157. Chen AI, Dolben EF, Okegbe C, Harty CE, Golub Y, Thao S, et al. Candida albicans ethanol stimulates Pseudomonas aeruginosa WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. PLoS Pathog. 2014;10(10):e1004480.

158. Parolin C, Marangoni A, Laghi L, Foschi C, Nahui Palomino RA, Calonghi N, et al. Isolation of Vaginal Lactobacilli and Characterization of Anti-Candida Activity. PLoS One. 2015;10(6):e0131220.

159. Zangl I, Pap IJ, Aspock C, Schuller C. The role of Lactobacillus species in the control of Candida via biotrophic interactions. Microb Cell. 2019;7(1):1-14.

160. Cheng SC, van de Veerdonk F, Smeekens S, Joosten LA, van der Meer JW, Kullberg BJ, et al. Candida albicans dampens host defense by downregulating IL-17 production. J Immunol. 2010;185(4):2450-7.

161. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013;39(2):372-85.

162. Kim Y, Mylonakis E. Killing of Candida albicans filaments by Salmonella enterica serovar Typhimurium is mediated by sopB effectors, parts of a type III secretion system. Eukaryot Cell. 2011;10(6):782-90.

163. Sass G, Nazik H, Penner J, Shah H, Ansari SR, Clemons KV, et al. Aspergillus-Pseudomonas interaction, relevant to competition in airways. Med Mycol. 2019;57(Supplement_2):S228-S32.

164. Beswick E, Amich J, Gago S. Factoring in the Complexity of the Cystic Fibrosis Lung to Understand Aspergillus fumigatus and Pseudomonas aeruginosa Interactions. Pathogens. 2020;9(8).

165. Hunter RC, Klepac-Ceraj V, Lorenzi MM, Grotzinger H, Martin TR, Newman DK. Phenazine content in the cystic fibrosis respiratory tract negatively correlates with lung function and microbial complexity. Am J Respir Cell Mol Biol. 2012;47(6):738-45.

166. Briard B, Heddergott C, Latge JP. Volatile Compounds Emitted by Pseudomonas aeruginosa Stimulate Growth of the Fungal Pathogen Aspergillus fumigatus. mBio. 2016;7(2):e00219.

167. Briard B, Rasoldier V, Bomme P, ElAouad N, Guerreiro C, Chassagne P, et al. Dirhamnolipids secreted from Pseudomonas aeruginosa modify anjpegungal susceptibility of Aspergillus fumigatus by inhibiting beta1,3 glucan synthase activity. ISME J. 2017;11(7):1578-91.

168. Mowat E, Rajendran R, Williams C, McCulloch E, Jones B, Lang S, et al. Pseudomonas aeruginosa and their small diffusible extracellular molecules inhibit Aspergillus fumigatus biofilm formation. FEMS Microbiol Lett. 2010;313(2):96-102.

169. Briard B, Bomme P, Lechner BE, Mislin GL, Lair V, Prevost MC, et al. Pseudomonas aeruginosa manipulates redox and iron homeostasis of its microbiota partner Aspergillus fumigatus via phenazines. Sci Rep. 2015;5:8220.

170. Nogueira MF, Pereira L, Jenull S, Kuchler K, Lion T. Klebsiella pneumoniae prevents spore germination and hyphal development of Aspergillus species. Sci Rep. 2019;9(1):218.

171. Reece E, Doyle S, Greally P, Renwick J, McClean S. Aspergillus fumigatus Inhibits Pseudomonas aeruginosa in Co-culture: Implications of a Mutually Antagonistic Relationship on Virulence and Inflammation in the CF Airway. Front Microbiol. 2018;9:1205.

172. Frases S, Chaskes S, Dadachova E, Casadevall A. Induction by Klebsiella aerogenes of a melanin-like pigment in Cryptococcus neoformans. Appl Environ Microbiol. 2006;72(2):1542-50.

173. Smith MG, Des Etages SG, Snyder M. Microbial synergy via an ethanol-triggered pathway. Mol Cell Biol. 2004;24(9):3874-84.

174. Partida-Martinez LP, Hertweck C. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature. 2005;437(7060):884-8.

175. Ibrahim AS, Gebremariam T, Liu M, Chamilos G, Kontoyiannis D, Mink R, et al. Bacterial endosymbiosis is widely present among zygomycetes but does not contribute to the pathogenesis of mucormycosis. J Infect Dis. 2008;198(7):1083-90.

176. Itabangi H, Sephton-Clark PCS, Zhou X, Starling GP, Mahamoud Z, Insua I, et al. Environmental interactions with amoebae as drivers of bacterial-fungal endosymbiosis and pathogenicity. BioRXiv. 2020.

177. Person AK, Chudgar SM, Norton BL, Tong BC, Stout JE. Aspergillus niger: An unusual cause of invasive pulmonary aspergillosis. Journal of Medical Microbiology. 2010;59(7):834-8.

178. Schuster E, Dunn-Coleman N, Frisvad J, Van Dijck P. On the safety of Aspergillus niger - A review. 2002. p. 426-35.

179. Ruijter GJG, van de Vondervoort PJI, Visser J. Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology. 1999;145(9):2569-76.

180. Cameselle C, Bohlmann JT, Núñez MJ, Lema JM. Oxalic acid production by Aspergillus niger. Bioprocess Engineering. 1998;19(4):247-52.

181. Richardson M, Bowyer P, Sabino R. The human lung and Aspergillus: You are what you breathe in? Med Mycol. 2019;57(Supplement_2):S145-S54.

182. Kim SH, Clark ST, Surendra A, Copeland JK, Wang PW, Ammar R, et al. Global Analysis of the Fungal Microbiome in Cystic Fibrosis Patients Reveals Loss of Function of the Transcriptional Repressor Nrg1 as a Mechanism of Pathogen Adaptation. PLoS Pathog. 2015;11(11):e1005308.

CHAPTER 2

Oxalic acid, a molecule at the crossroads of Bacterial-Fungal Interactions

Foreword

This chapter presents a review summarizing the current literature on oxalic acid as a mediator of Bacterial-Fungal Interactions (BFI). The chapter was published as a book chapter in Advances in Applied Microbiology. More specifically, this review discusses the production and consumption of oxalic acid by fungi and bacteria, as well as their interactions and contributions to its general cycling in soils, and in the plants and human ecosystems. Finally, the research gaps in these fields are presented. My personal contribution as co-first author concerned mainly writing the parts on the biosynthetic pathways of oxalic acid in fungi and bacteria, the degradation of oxalic acid in fungi, the cell-to-cell interactions between fungi and bacteria, as well as BFI and oxalic acid cycling in the animal and human ecosystems. I contributed equally in the writing of the roles of oxalic acid in fungi, bacteria, plants and humans, and of the conclusion, as well as the general review of the paper.

CHAPTER TWO

Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions

Fabio Palmieri[a,†], Aislinn Estoppey[a,†], Geoffrey L. House[b], Andrea Lohberger[a], Saskia Bindschedler[a], Patrick S.G. Chain[b], Pilar Junier[a,*]

[a]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland
[b]Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, United States
*Corresponding author: e-mail address: pilar.junier@unine.ch

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Abstract

Oxalic acid is the most ubiquitous and common low molecular weight organic acid produced by living organisms. Oxalic acid is produced by fungi, bacteria, plants, and animals. The aim of this review is to give an overview of current knowledge about the microbial cycling of oxalic acid through ecosystems. Here we review the production

† These authors contributed equally to this work.

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and degradation of oxalic acid, as well as its implications in the metabolism for fungi, bacteria, plants, and animals. Indeed, fungi are well known producers of oxalic acid, while bacteria are considered oxalic acid consumers. However, this framework may need to be modified, because the ability of fungi to degrade oxalic acid and the ability of bacteria to produce it, have been poorly investigated. Finally, we will highlight the role of fungi and bacteria in oxalic acid cycling in soil, plant and animal ecosystems.

1. Low molecular weight organic acids (LMWOA) and oxalic acid (OA)

Low molecular weight organic acids (LMWOA) are compounds with a maximal, and arbitrary, molecular weight of approximately 300 g/mol, characterized by the presence of one or more carboxylic groups (Strobel, 2001). LMWOA can be found ubiquitously in all ecosystems. Examples of LMWOA include lactate, acetate, oxalate, glyoxylate, succinate, fumarate, malate, citrate, isocitrate or aconitate that are produced by most living organisms, including plants, animals, fungi, and bacteria; some LMWOA are also produced by archaea, although oxalic acid is not one of those (Yadav et al., 2015). These LMWOA are involved in many processes taking place in soil (Jones, 1998). Oxalic acid, one of the most prevalent LMWOA, seems to play a key role in the regulation of bacterial-fungal interactions (Deveau et al., 2018).

Either oxalic acid or oxalate, its conjugated base, can be found in various terrestrial, aquatic, and clinical environments (Chandra & Shethna, 1977; Dutton & Evans, 1996; Hervé, Junier, Bindschedler, Verrecchia, & Junier, 2016; Krieger et al., 2017; Sahin, 2003; Tamer & Aragno, 1980). Oxalic acid is a strong organic acid ($pK_{a_1} = 1.25$ and $pK_{a_2} = 4.27$) and therefore, in most environments, it is found as its conjugated base, oxalate (Strobel, 2001). Oxalate can form several salts depending on the metal availability and the corresponding salt solubility products (e.g., K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Cu^{2+}) (Gadd, 1999). As a result, metal-oxalate compounds are widespread in the environment, with the most common one in terrestrial ecosystems being calcium oxalate (CaOx). The two most common CaOx minerals occurring in soils are whewellite ($CaC_2O_4 \cdot H_2O$) and weddellite ($CaC_2O_4 \cdot 2H_2O$), the monohydrate and the dihydrate forms, respectively (Uren, 2018).

Because of its chemical versatility and widespread occurrence, it is increasingly evident that oxalic acid may play a major role in interactions and ecosystem functioning. Therefore, the aim of this review is to provide an overview of current knowledge about the roles of oxalic acid in different organisms, including the pathways of its production and degradation in both

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fungi and bacteria. Finally, we will summarize what is currently known about the contributions of bacteria and fungi in the cycling of oxalate, as well as the role of oxalate as a modulator of bacterial-fungal interactions, using different model ecosystems, from humans to soils.

1.1 Roles of oxalic acid

Several roles have been proposed for oxalic acid in different types of organisms, and these are summarized in Table 1.

Table 1 Roles of oxalic acid in fungi, bacteria, plants and animals.

Organism	Role	References
Fungi	Pathogenicity	Dutton and Evans (1996), Moore, Robson, and Trinci (2011), and van Kan, Shaw, and Grant-Downton (2014)
	Mineral weathering and nutrients acquisition	Arvieu, Leprince, and Plassard (2003), Dutton and Evans (1996), and Schmalenberger et al. (2015)
	Wood degradation	Mäkelä, Galkin, Hatakka, and Lundell (2002), Shimada, Ma, Akamatsu, and Hattori (1994), and Tsao (1963)
	Metal tolerance (Cu, Zn, Pb, Cd)	Fomina et al. (2005)
Bacteria	Metal tolerance (Al)	Hamel, Levasseur, and Appanna (1999)
	Pathogenicity	Nakata (2011) and Nakata and He (2010)
	Mineral weathering	Becerra-Castro et al. (2013), Cheng, Wang, He, and Sheng (2017), Frey et al. (2010), and Hess, Coker, Loutsch, & Russ (2007)
Plants	Calcium regulation	Çalişkan (2000), Nakata (2003), and Smith (2002)
	Ion homeostasis	Çalişkan (2000) and Smith (2002)
	Heavy metal detoxification	Nakata (2003)
	Plant defenses	Nakata (2003)
Animals	Stimulation of Na^+, Cl^- and H_2O uptake	Markovich and Aronson (2007) and Wang, Egbert, Abbiati, Aronson, and Giebisch (1996)
	Production of H_2O_2 to increase phagocytosis	Albrecht, Brandl, and Schonfels (1994)

1.1.1 Oxalic acid in fungi

A wide range of fungi produce oxalic acid, and different environmental factors such as carbon or nitrogen availability, mineral type, and pH influence its production (Arvieu et al., 2003; Dutton & Evans, 1996; Schmalenberger et al., 2015). As a matter of fact, calcium oxalate is the most common oxalate mineral associated to fungal hyphae, but other metal oxalates are regularly observed in function of metal availability in the environment (Gadd et al., 2014). Given the large diversity of physiologies and ecologies found among fungi, several functions have been proposed for the role of oxalic acid in fungal metabolism. Secretion of oxalic acid by fungi can increase their pathogenicity (Dutton & Evans, 1996; Moore et al., 2011; van Kan, 2006). When this secreted oxalic acid accumulates in the host tissues, it lowers the pH, which stimulates the production and activity of fungal enzymes (Dutton & Evans, 1996). Furthermore, as oxalate is a strong metal chelator, it can extract and sequester calcium ions, which can have different physiological and structural roles in the host. For example, in the case of plants, the formation of calcium oxalate in the middle lamellae within the plant cell wall weakens the structure and facilitates infection and degradation by the fungal pathogen (Dutton & Evans, 1996; Yang, Tewari, & Verma, 1993). Moreover, oxalate can inhibit host defenses and induce programmed cell death, which is also beneficial for necrotrophic pathogens (de Oliveira Ceita et al., 2007; van Kan, 2006). However, oxalic acid can also have a protective role against pathogen attack. For example, oxalic acid produced by mycorrhizal fungi can act as an antimicrobial chemical and influence the growth of pathogenic fungi in the rhizosphere (Duchesne, Ellis, & Peterson, 1989).

Secretion of oxalic acid in soil does not only protect plants from undesirable fungi. Changes in soil pH that are induced by oxalic acid secretion play a major role in mineral weathering and thus in determining nutrient availability (Dutton & Evans, 1996). In particular, ectomycorrhizal fungi have been shown to produce large amounts of oxalic acid in response to the mineral composition of the medium (Landeweert, Hoffland, Finlay, Kuyper, & Van Breemen, 2001; Schmalenberger et al., 2015). In addition to this, oxalate can chelate aluminum and iron, thus increasing the availability of phosphate. Likewise, oxalic acid facilitates weathering of clays and therefore increases the availability of trace nutrients (Cromack et al., 1979; Graustein, Cromack, & Sollins, 1977; Landeweert et al., 2001). Oxalic acid also influences calcium cycling, as the formation of calcium oxalate efficiently traps calcium in the soil matrix and prevents its leaching (Cromack et al., 1979).

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Oxalic acid also plays a major role in wood degradation (Mäkelä et al., 2002; Shimada et al., 1994; Tsao, 1963). Brown-rot fungi, which degrade only cellulose and hemicellulose, exploit the precipitation of calcium oxalate crystals in cell walls to aid the degradation of cell wall components (Dutton & Evans, 1996; Shimada et al., 1994). Moreover, cellulose degradation is more efficient at low pH and oxalic acid can act as a proton donor or provide reactive oxygen species, such as O_2^- and H_2O_2, required for hydrolytic and oxidative degradation. Oxalic acid also plays a key role in regulating the rate of enzymatic degradation of lignin in wood by manganese-dependent lignin peroxidases (Shimada et al., 1994) produced by white-rot fungi (Tsao, 1963). Oxalate chelates and stabilizes Mn(III), which otherwise converts to Mn(II). Mn(III) is a key co-factor of Mn-peroxidases, one class of enzyme acting on lignin degradation (Hakala et al., 2005; Watanabe, Hattori, Tengku, & Shimada, 2005). Finally, as wood has a low nitrogen content, wood-rotting fungi are thought to produce oxalic acid as a mean to dispose of the excess carbon in wood. This hypothesis is supported by the fact that oxalic acid production is stimulated by growth in media with a low nitrogen content (Kuan & Tien, 1993; Shimada et al., 1994). Interestingly, copper compounds are widely used in wood preservation and it appears that oxalic acid also plays a role in copper tolerance, by inducing the mineralization of copper oxalate from copper sulfate (Dutton & Evans, 1996). Other metals such as zinc, cadmium or lead are also detoxified by oxalic acid secretion and precipitation as metal-oxalate salts (Fomina et al., 2005).

1.1.2 Oxalic acid in bacteria

Up to now, only a few studies have reported oxalic acid production in bacteria. Hamel et al. (1999) showed that a strain of *Pseudomonas fluorescens* produces oxalic acid in response to aluminum stress. Other studies reported the use of oxalic acid as a pathogenicity factor in two bacterial species of the genus *Burkholderia* (Nakata, 2011; Nakata & He, 2010). Moreover, oxalic acid has been reported to participate in mineral weathering in several bacterial genera (Becerra-Castro et al., 2013; Cheng et al., 2017; Frey et al., 2010; Hess et al., 2007). However, given the limited number of studies on this topic, there is a clear gap of knowledge concerning the role of oxalic acid in bacterial metabolism.

1.1.3 Oxalic acid in plants

Most of plants are able to produce oxalic acid, and oxalate can be present in different forms in plant cells. Those forms include soluble oxalate salts

(potassium, sodium or magnesium oxalate) or insoluble oxalate salts (calcium oxalate). Oxalate generally accumulates within cells in vacuoles called crystal idioblasts, and it can represent between 3% and 80% of their dry weight (Franceschi & Horner, 1980; Franceschi & Nakata, 2005). Oxalic acid and oxalate are thought to play major roles in calcium regulation, ionic balance, heavy metal detoxification and in plant defense against herbivores (Çalişkan, 2000; Nakata, 2003; Smith, 2002). Details of each one of these roles are given below.

Calcium plays a major role in plant metabolism both through signal transduction and through biochemical regulation of cellular process (Smith, 2002). However, calcium is toxic to cells at a concentration of 10^{-6}–10^{-8} M and thus the formation of insoluble calcium oxalate complexes is a way to regulate its concentration in order to ensure the proper functioning of the cell. The size and quantity of calcium oxalate crystals varies as a function of the calcium concentration in the environment. In addition, calcium oxalate crystals can serve as a storage reservoir of calcium. When cellular calcium levels are too high more crystals will be formed, but if there is a deficiency in calcium, crystals will be dissolved to make calcium available for cellular function (Çalişkan, 2000; Nakata, 2003; Smith, 2002).

As plants do not have skeletal structure, ionic balance is essential to maintain the hydrostatic pressure required for a plant to stay upright. Therefore, oxalic acid could play a major role in ion homeostasis, as it can balance the charge of the positive ions and amino acids present in the cell (Çalişkan, 2000; Smith, 2002). Moreover, oxalic acid also plays a role in heavy metal detoxification (Nakata, 2003). Aluminum toxicity, for example, is a major problem for plants developing on acidic soils. Plants have evolved different mechanisms to prevent aluminum toxicity in these conditions. Both the exclusion of aluminum from plant roots and increased internal tolerance of aluminum are related to oxalate production. In either case, aluminum will be sequestered as non-toxic aluminum-oxalate salts, either in the rhizosphere or within the above-ground portion of the plant.

Oxalate may also provide herbivory protection in plants. Crystals may be produced pre-emptively as physical defenses or produced in leaves as a response to herbivory (Nakata, 2003).

1.1.4 Oxalic acid in humans

Although oxalate is a known metabolic by-product of cellular metabolism, only two potential functions in humans have been identified. First, oxalate has been shown to have a physiological function in the proximal tubule of

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the nephron in human kidneys, where the transport of oxalate by the membrane transporter SLC26A6 stimulates the uptake of chloride, water and sodium (Markovich & Aronson, 2007; Wang et al., 1996). Second, another study has suggested that an oxalate oxidase produces H_2O_2 from oxalate, and by this mechanism, increases the burst of phagocytes (Albrecht et al., 1994). No other physiological roles in humans have been described to-date.

1.1.5 Oxalic acid in other organisms

Calcium oxalate crystals associated to sporophores of Myxomycetes are also mentioned in the literature (Clark & Haskins, 2014). However, to the best of our knowledge, the role of oxalic acid and/or oxalate in Myxomycetes has not been investigated further.

2. Oxalogenesis

2.1 Biosynthesis of oxalic acid in fungi

Oxalic acid can be synthesized through three different pathways in Fungi (Fig. 1): (i) the cytoplasmic pathway; (ii) the tricarboxylic acid (TCA) pathway; and (iii) the glyoxylate (GLOX) pathway. In the case of the cytoplasmic pathway, it first involves the fixation of carbon dioxide onto a molecule of pyruvate by the pyruvate carboxylase (PYC, EC 6.4.1.1) to produce oxaloacetate, which is then cleaved into oxalate and acetate by the Mn^{2+}-dependent oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1) (Han et al., 2007). In the case of the TCA and GLOX pathways, in order to enter either cycle, pyruvate is first oxidized into acetyl-CoA by the pyruvate dehydrogenase multienzyme complex (PDC) (Mattevi et al., 1992). For the TCA pathway, which occurs in the mitochondria (Mäkelä, Hildén, & Lundell, 2010), oxalic acid is produced through the cleavage of oxaloacetate by the oxaloacetate hydrolase, as in the cytoplasmic pathway. In the case of the GLOX pathway, occurring in the glyoxysomes (Mäkelä et al., 2010), oxalic acid is synthesized through the hydrolysis of citrate into glyoxylate by the glyoxylate dehydrogenase (GLOXDH, EC 1.2.1.17) (Dutton & Evans, 1996; Plassard & Fransson, 2009). Once produced, oxalic acid is secreted in the environment through a dedicated transporter. Up to now, FpOAR, a membrane transporter found in *Fomitopsis palustris*, is the only one that has been characterized (Watanabe et al., 2010).

Oxalic acid production can be affected by numerous factors such as the source of carbon and nitrogen and environmental pH, as shown for

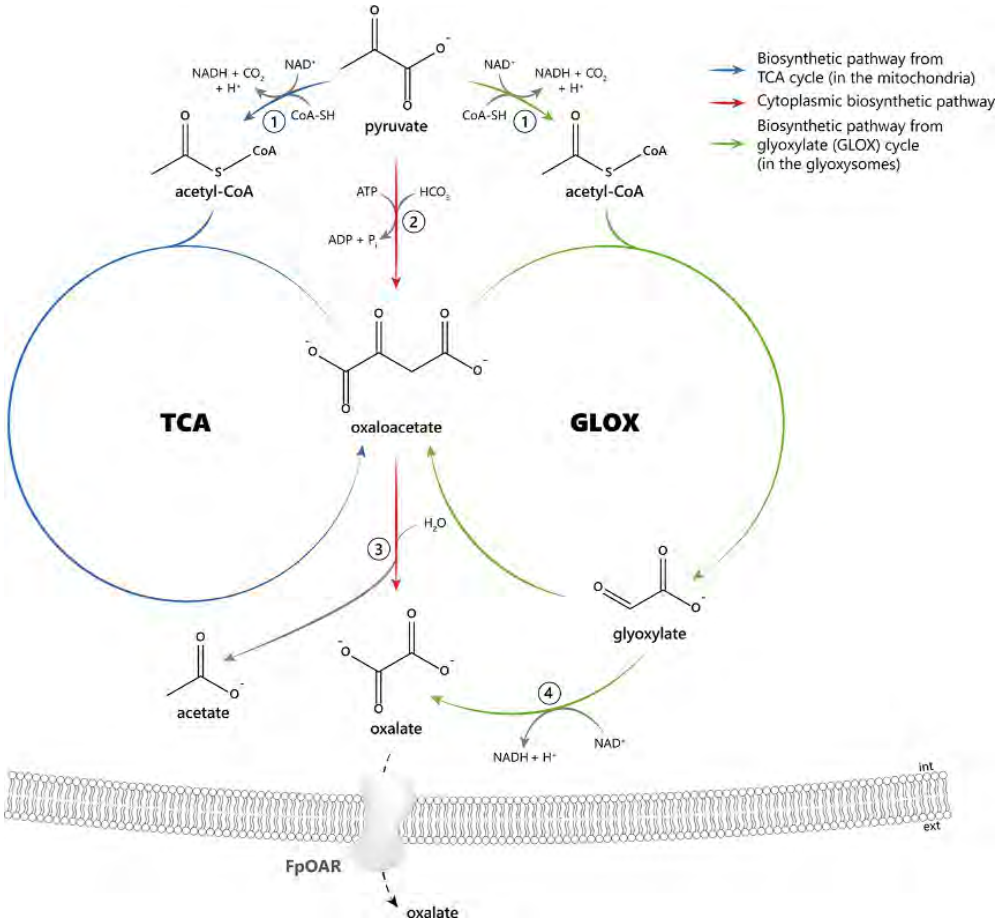

Fig. 1 Oxalogenesis pathways in fungi. Oxalic acid (OA) can be synthesized from a pyruvate precursor, which is either converted into acetyl-CoA by (1) the pyruvate dehydrogenase multienzyme complex that then enters the TCA or GLOX cycles, or is converted into oxaloacetate by (2) pyruvate carboxylase (PYC) (EC 6.4.1.1). The oxaloacetate (either produced from the TCA cycle or directly from pyruvate) can then be converted to oxalate by (3) oxaloacetate acetylhydrolase (OAH) (EC 3.7.1.1), whereas the glyoxylate from the GLOX cycle can be converted into oxalate by glyoxylate dehydrogenase (GLOXDH) (EC 1.2.1.17) (Dutton & Evans, 1996). Once produced, OA is secreted in the environment through an OA transporter. Up to now, FpOAR, an OA membrane transporter found in *Formitopsis palustris*, is the first one to be characterized (Watanabe et al., 2010).

Sclerotium rolfsii (Punja & Jenkins, 1984). Mineralogy has also been shown to influence oxalic acid production in the ectomycorrhizal fungus *Paxillus involutus* (Schmalenberger et al., 2015). Several studies have shown that the production of oxalic acid is stimulated by the presence of nitrate and inhibited by ammonium (Gharieb & Gadd, 1999; Kritzman, Chet, & Henis, 1977; Lapeyrie, Chilvers, & Bhem, 1987; Lapeyrie, Ranger, & Vairelles, 1991; Maxwell & Bateman, 1968). Optimal oxalic acid production occurs at pH 5–6 and is inhibited at a pH lower than 3, as shown in the case of the

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well-known producer *Aspergillus niger* (Kubicek, Schreferl-Kunar, Wöhrer, & Röhr, 1988; Ruijter, van de Vondervoort, & Visser, 1999). Ruijter et al. (1999) also demonstrated that Mn^{2+} is co-factor altering the activity of the OAH. Carbonate and bicarbonate ions are also shown to stimulate oxalic acid production (Arvieu et al., 2003; Lapeyrie, 1988; Lapeyrie et al., 1987). The addition of divalent cations, such as calcium or metals (e.g., copper) favors the production of oxalic acid (Fomina et al., 2005; Guggiari et al., 2011).

2.1.1 Assessing the prevalence of oxalate producers among fungal species

In order to screen for the diversity of fungal species capable of synthesizing oxalic acid, we searched for putative OAH amino acid sequences in annotated sequences of 1091 fungal isolates from the Joint Genome Institute (JGI). The search was performed using a Hidden Markov model trained with fungal sequences having the systematic name "oxaloacetate acetylhydrolase" in NCBI's protein database. The *oah* gene was selected for this analysis because oxalic acid is synthesized mainly from oxaloacetate through the OAH pathway (Han et al., 2007; Kubicek et al., 1988; Rio, de Oliveira, de Tomazella, Silva, & Pereira, 2008). We created a rooted cladogram of the identified OAH sequences and assigned trophic groups to the fungi represented by these OAH sequences by matching species (when possible) or genus against the FUNGuild database (Nguyen et al., 2016). The identified OAH amino acid sequences were found in fungi from all seven trophic groups that are recognized in the FUNGuild database (Nguyen et al., 2016) (Fig. 2). When multiple OAH amino acid sequences were identified in the same fungal isolate, these sequences were generally most closely related, consistent with their sharing of a common evolutionary origin. However, OAH sequences identified from different fungi generally assorted into clades based on taxonomy instead of trophic group, suggesting that the evolutionary history of these amino acid sequences may not have been constrained by different nutrient acquisition strategies (Fig. 2).

2.2 Biosynthesis of oxalic acid in bacteria

There are only a few reports of oxalic acid production in bacteria. *P. fluorescens* ATCC 13525 produced oxalic acid in a mineral medium containing citric acid as sole carbon source; the oxalic acid was thought to be produced from a glyoxylate intermediate in response to aluminum stress (Hamel et al., 1999). Two genes, *obcA* and *obcB*, are required for oxalic acid biosynthesis and have been described in *Burkholderia mallei*. The *obcA* and

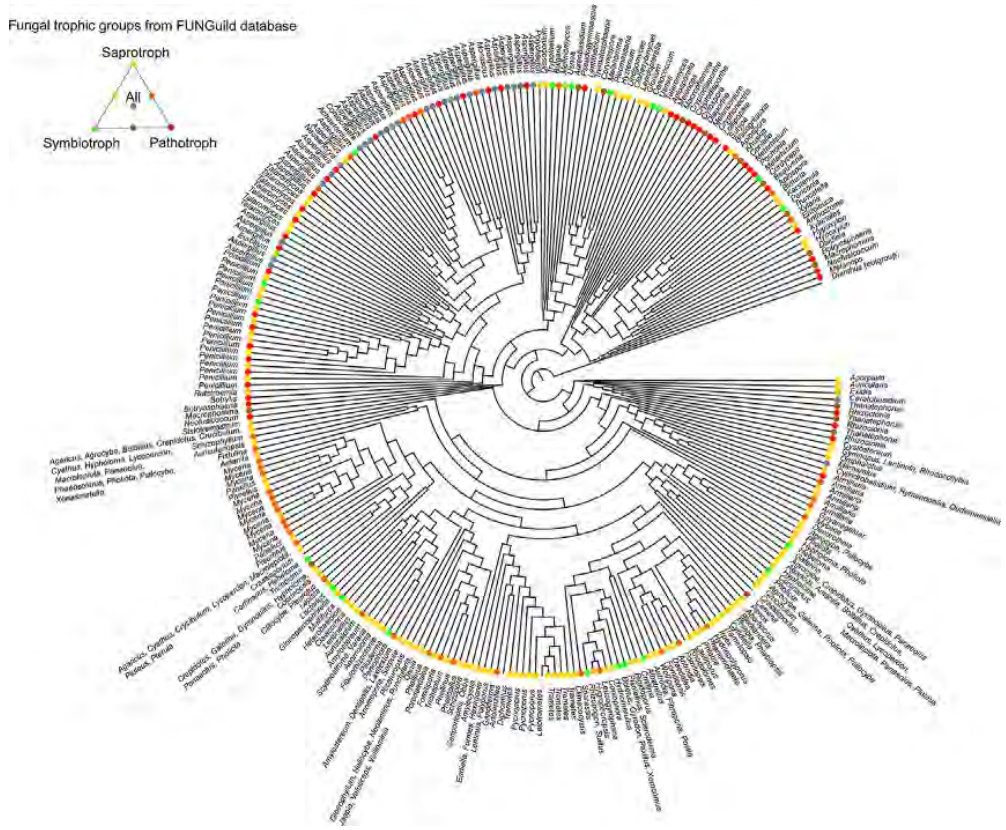

Fig. 2 Fungal *oah* gene screening. Cladogram of putative oxaloacetate hydrolase protein sequences (OAH) identified in fungal genera with diverse trophic strategies (different colors of points). The putative OAH amino acid sequences were identified using a Hidden Markov model using known fungal OAH sequences represented in the NCBI Protein database. This model was then used to screen all available fungal proteome datasets from JGI's Mycocosm portal (1091 fungal isolates) for putative OAH amino acid sequences. These putative OAH sequences typically group by genus instead of by trophic group, suggesting that they share common ancestry among closely related species instead of between fungi that share the same nutrient acquisition strategy.

obcB genes are located in the *obcAB* operon, which was also found in *Burkholderia glumae* (Nakata, 2011; Nakata & He, 2010). However, there is a significant gap of knowledge in the understanding of oxalogenesis in bacteria, as well as in the prevalence of this trait in bacteria.

3. Oxalotrophy

3.1 Oxalic acid degradation in bacteria

The first isolation of an oxalotrophic bacterium was reported more than a century ago (Bassalik, 1913). It has since become apparent that the taxonomic affiliation of oxalotrophic bacteria is heterogenous, with representatives

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found among three different bacterial phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Hervé et al., 2016). Some oxalotrophic bacteria, such as *Oxalobacter formigenes* depend on the presence of oxalic acid for their survival (Anantharam, Allison, & Maloney, 1989; Smith, 2002). Others can use different carbon sources, including oxalic acid, for their metabolism (Müller, Schubert, Röst, Aebersold, & Vorholt, 2016; Sahin, 2003).

As oxalic acid is the most highly oxidized organic compound (Chandra & Shethna, 1977; Jakoby & Bhat, 1958), it must undergo an activation step before it can be used either as carbon or energy source (Jakoby & Bhat, 1958; Quayle, Keecht, & Taylor, 1961). Moreover, it must be chemically reduced before it can be used in condensation reactions to form the C_3 and C_4 units, essential for the synthesis of cell constituents (Harder, 1973; Kornberg, 1959; Quayle et al., 1961). As shown in Fig. 3, the first step for the assimilation of oxalic acid into cells is the transfer of coenzyme-A (CoA) from acetyl-CoA or succinyl-CoA to oxalic acid to form oxalyl-CoA (Jakoby & Bhat, 1958; Jakoby, Homura, & Hayaishi, 1956; Quayle et al., 1961). Oxalyl-CoA is the key molecule of the metabolism of oxalic acid. It can be (a) decarboxylated to formate in order to produce energy or (b) reduced to glyoxylate in order to be incorporated in the cell constituents (Chandra & Shethna, 1977; Jakoby et al., 1956; Quayle et al., 1961).

For energy production, oxalyl-CoA is decarboxylated to formyl-CoA via an oxalyl-CoA decarboxylase (Fig. 3, reaction 1) (EC 4.1.1.8) and thiamine pyrophosphate (ThPP) is required for this reaction (Blackmore & Quayle, 1970; Jakoby & Bhat, 1958; Jakoby et al., 1956; Kornberg, 1959; Turroni et al., 2007). Coenzyme-A is then recycled from formyl-CoA to a new oxalic acid molecule by a formyl-CoA transferase (Fig. 3, reaction 2) (EC 2.8.3.16) (Baetz & Allison, 1990; Sidhu et al., 1997; Turroni et al., 2007). The formate produced can be either transported outside the cell (Anantharam et al., 1989) or be further oxidized to CO_2 by a NAD-linked formate dehydrogenase (Fig. 3, reaction 3) (EC 1.6.1.5) to produce NADH (Blackmore & Quayle, 1970; Dijkhuizen, Wiersma, & Harder, 1977). Some anaerobic bacteria have an oxalate^{2-}:formate^{1-} antiporter (OxlT) in their membranes (Anantharam et al., 1989; Ruan et al., 1992). The presence of this heterologous antiporter that is associated with the consumption of one H^+ for the decarboxylation of oxalic acid leads to both a continued polarization of the membrane and also to an indirect proton pump, with a net stoichiometry of one H^+ per turnover (Anantharam et al., 1989; Ruan et al., 1992). Therefore, oxalic acid decarboxylation supports ATP synthesis by an ATP synthase, with a ratio of one ATP produced for every three

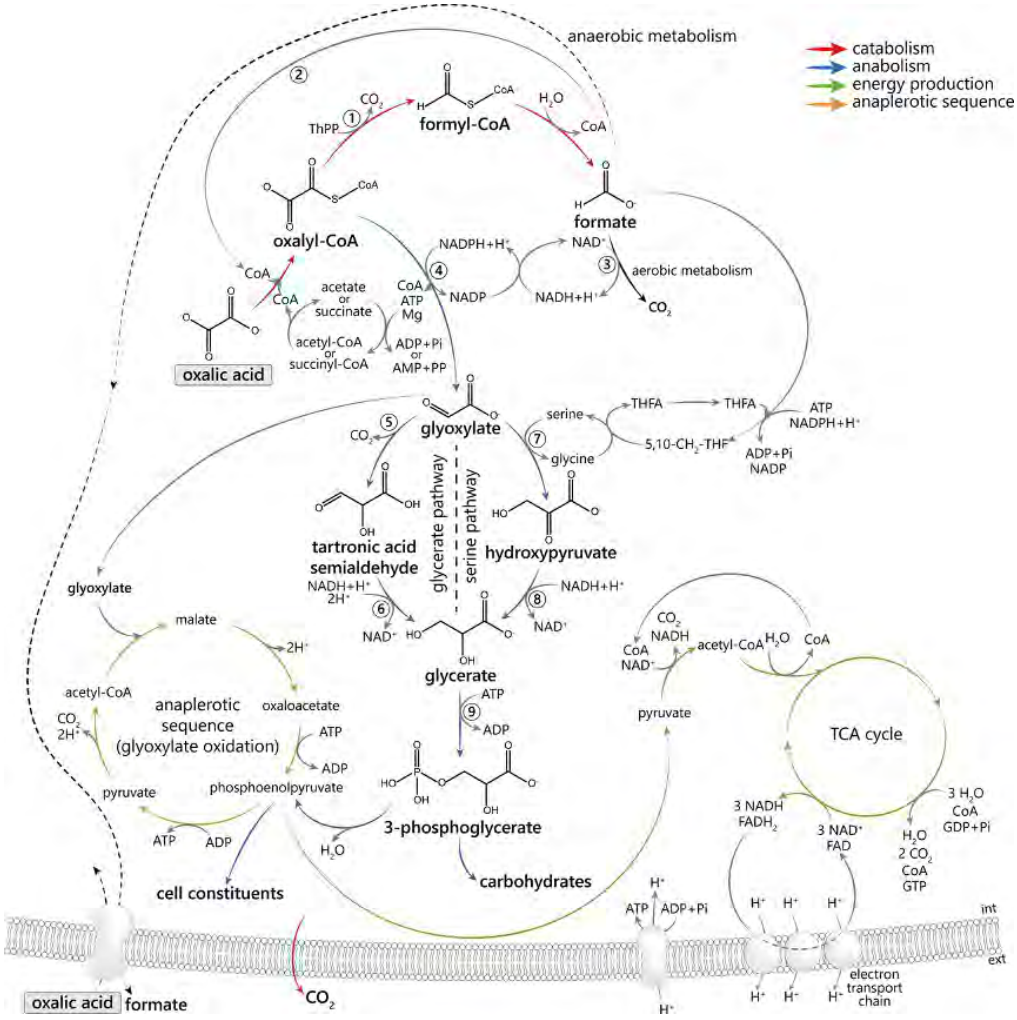

Fig. 3 Oxalotrophy in bacteria. In catabolic reactions (red arrows), oxalic acid (OA) is decarboxylated into formate (via an oxalyl-CoA intermediate) by (1) oxalyl-CoA decarboxylase (EC 4.1.1.8) and (2) formyl-CoA transferase (EC 2.8.3.16). In aerobic metabolism, the formate is then further oxidized in CO_2 by (3) NAD-linked formate dehydrogenase (EC 1.6.1.5); in anaerobic metabolism it is secreted via an oxalate:formate antiporter. For OA assimilation into cell constituents (blue arrows), oxalyl-CoA is converted to glyoxalate by (4) oxalyl-CoA reductase (EC 1.2.1.17) and is then converted into gylcerate either by the glycerate pathway: (5) glyoxylate carboligase (EC 4.1.1.47) followed by (6) tartronate semialdehyde reductase (EC 1.1.1.60), or by a variant of the serine pathway: (7) serine hydroxymethyl transferase (EC 2.1.2.1) followed by (8) hydroxypyruvate reductase (EC 1.1.1.29). Glycerate is finally phosphorylated into 3-phosphoglycerate by (9) glycerate 3-kinase (EC 2.7.1.31). The 3-phosphosglycerate can be used for carbohydrates synthesis or can act as a precursor either in the glyoxylate oxidation cycle (orange arrows) or in the tricarboxylic acid cycle (TCA) (green arrows) for the production of energy. For clarity, not all enzymatic reactions and co-factors required are depicted in this diagram of OA metabolism pathways in bacteria.

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decarboxylated oxalic acid molecules (Anantharam et al., 1989). For aerobic bacteria, it is unclear how oxalic acid enters the cell (Dijkhuizen et al., 1977). However, it has been recently shown that OxlT is present in the aerobic bacterium *Methylobacterium extorquens* during the colonization of leaves, and that formate is then oxidized to CO_2 in the periplasm (Müller et al., 2016).

To be assimilated into cell components, oxalyl-CoA is first reduced in glyoxylate by an oxalyl-CoA reductase (Fig. 3, reaction 4) (EC 1.2.1.17) and then to glycerate by two different pathways (Blackmore & Quayle, 1970; Quayle et al., 1961). The first one is the glycerate pathway, where the glyoxylate is first transformed in tartronic acid semialdehyde by a glyoxylate carboligase (Fig. 3, reaction 5) (EC 4.1.1.47) and then reduced to glycerate by a tartronate semialdehyde reductase (Fig. 3, reaction 6) (EC 1.1.1.60) (Kornberg, 1959; Quayle et al., 1961). The second pathway is a variant of the serine pathway (Blackmore & Quayle, 1970; Chandra & Shethna, 1977) that uses conversion of glyoxylate to glycine by a serine hydroxymethyl transferase (Fig. 3, reaction 7) (EC 2.1.2.1) to produce hydroxypyruvate, that is in turn reduced to glycerate by a hydroxypyruvate reductase (Fig. 3, reaction 8) (EC 1.1.1.29). Glycerate is then phosphorylated into 3-phosphogylcerate by a glycerate 3-kinase (Fig. 3, reaction 9) (EC2.7.1.31) and can be used to synthesize carbohydrates or be further transformed in phosphoenolpyruvate (Chandra & Shethna, 1977). Phosphoenolpyruvate can be incorporated into cell constituents, act as precursor of pyruvate for the tricarboxylic acid (TCA) cycle, or as an intermediate for the glyoxylate oxidation, which is an anaplerotic sequence that renews intermediates for the TCA cycle (Kornberg, 1965).

Thus, oxalic acid metabolism has a low energy yield, because: (1) ATP and reducing power are both required to assimilate it in the cell constituents, with five ATP and two NADH required per mole of 3-phosphoglycerate produced and (2) because the complete oxidation of 1 mol of oxalic acid only gives 1 mol of NADH (Dijkhuizen et al., 1977; Harder, 1973). Oxalyl-CoA plays a major role in oxalic acid metabolism, and because it represents a required intermediate for both energy production and carbon assimilation, this metabolic trade-off contributes to the low growth yield of oxalotrophic bacteria (Chandra & Shethna, 1977). Furthermore, in pink-pigmented organisms that use a variant of the serine pathway to assimilate oxalic acid into the cell constituents, formate is used as a precursor for 5,10-Methylenetetrahydrofolate (5,10-CH_2-THF) (Fig. 3), which means that some of the formate is not oxidized in CO_2 to produce NADH, thereby

adding to the low energy yield of this metabolism (Blackmore & Quayle, 1970; Schneider, Skovran, & Vorholt, 2012). Because of the constraints in using oxalic acid as a sole energy and carbon source, oxalotrophic bacteria can use different input molecules for carbon assimilation and energy production. In *M. extorquens*, the two key enzymes for the conversion of oxalic acid into formate: oxalyl-CoA decarboxylase (encoded by the *oxc* gene) and formyl-CoA transferase (encoded by the *frc* gene), as well as the oxalate: formate antiporter—OxlT (encoded by the *oxlT* gene) are up-regulated during phyllosphere colonization (Baetz & Allison, 1990; Müller et al., 2016; Ruan et al., 1992; Sidhu et al., 1997). In contrast, the key enzyme for oxalic acid assimilation, oxalyl-CoA reductase is down-regulated. Overall, this indicates that oxalic acid is used only for energy production (Müller et al., 2016).

3.1.1 Assessing the prevalence of oxalate degraders among bacterial species

In order to better assess the diversity of bacterial species putatively capable of metabolizing oxalic acid, we screened bacterial proteomes for OXC and FRC sequences, which enable the use of oxalic acid as a carbon source under aerobic and anaerobic conditions, respectively (Fig. 3). All bacterial amino acid sequences annotated as either OXC or FRC were downloaded from the UniProt database. A subset of sequences from each represented species was randomly selected and used to train two independent Hidden Markov models (HMMs). These HMMs were used to identify OXC and FRC sequences within all (8648) UniProt reference bacterial proteomes. Nearly all bacterial strains identified as having these amino acid sequences contained either only OXC or both OXC and FRC; only 159 bacterial strains (2.5%) contained just FRC sequences (Fig. 4). The *oxc* and *frc* genes can co-occur in an operon (Azcarate-Peril, Bruno-Bárcena, Hassan, & Klaenhammer, 2006), and this may in part explain their co-occurrence as found here. Additional screening of proteome data could determine the location of these two genes to identify their potential to be encoded by an operon.

3.2 Oxidation of oxalic acid in fungi

Oxalic acid degradation activities have been documented in several fungi (Dutton & Evans, 1996; Mäkelä et al., 2010) and two enzymes involved in this degradation have been described: (1) oxalate decarboxylase (ODC, syn. Oxalate carboxy-lyase, EC 4.1.1.2) and (2) oxalate oxidase (OXO, oxalate: oxygen oxidoreductase, EC 1.2.3.4) (Mäkelä et al., 2010). ODC oxidizes

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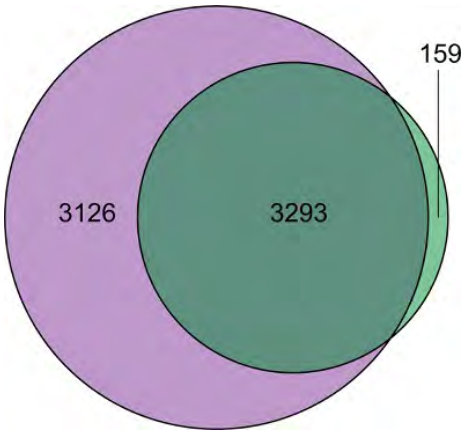

Fig. 4 Screening bacteria for OXC and FRC amino acid sequences. Counts of bacterial strains identified as only containing the oxalyl-CoA decarboxylase gene products (OXC) (purple), those only containing the formyl-CoA:oxalate CoA-transferase gene products (FRC) (light green), and those containing both gene products (dark green, overlap). Genes were identified using a Hidden Markov model using known bacterial OXC and FRC protein sequences from UniProt that had species-level assignment, and then querying all UniProt bacterial reference proteome accessions to independently identify those containing putative OXC sequences and those containing putative FRC sequences. The vast majority of proteomes contain only OXC sequences or both OXC and FRC sequences.

oxalic acid to formate and further to CO_2 and requires O_2 and Mn^{2+} as co-factors (Just et al., 2004). OXO, on the other hand, cleaves oxalic acid into two molecules of CO_2 and produces H_2O_2. OXO has been described in only two white-rot species so far, *Ceriporiopsis subvermispora* and *Abortiporus biennis* (Aguilar, Urzúa, Koenig, & Vicuña, 1999; Grąz, Jarosz-Wilkołazka, & Pawlikowska-Pawlęga, 2008). ODC seems to be the most prevalent oxalic acid degrading enzyme in the fungal world and has been shown to have a role in the maintenance of stable pH by controlling the levels of oxalic acid inside and outside fungal hyphae (Mäkelä et al., 2010; Micales, 1997). Formate dehydrogenase (FDH, EC 1.2.1.2 and 1.2.2.1) has been proposed to act sequentially with ODC to degrade formate—the end product of ODC—into CO_2 and NADH. This NADH produced through the combined activity of ODC and FDH could be used for ATP synthesis during fungal growth (Watanabe et al., 2005). Several fungal species have been shown to degrade oxalic acid in a calcium oxalate-amended mineral medium (Table 2, Guggiari et al., 2011; Simon, 2016). However, the mechanism of degradation is unknown and no enzymatic activity has been measured so far. Even if oxalic acid utilization as a carbon source has been described in many fungi, energy generation through oxalic acid consumption needs to be further investigated.

Table 2 Calcium oxalate (CaOx) mineral dissolution in CaOx-amended Schlegel mineral medium.

Species	CaOx dissolution on Schlegel-AB CaOx	References
Pleurotus tuber-regium	+	Guggiari et al. (2011)
Polyporus ciliatus	+	Guggiari et al. (2011)
Agaricus blazei	+	Guggiari et al. (2011)
Fusarium nygamai	+	Simon (2016)
Fusarium equiseti	+	Simon (2016)
Fusarium oxysporum	+	Simon (2016)
Fusarium chlamydosporium	+	Simon (2016)
Fusarium chlamydosporium	+	Simon (2016)

4. Cell-to-cell interactions

Oxalic acid has been shown to have a signaling role in the interaction between oxalogenic fungi and oxalotrophic bacteria. For instance, mycophagous bacteria of the genus *Collimonas* use oxalic acid to localize their fungal host and to move toward it by quorum sensing-dependent chemotaxis (Rudnick, Veen, & Boer, 2015). An earlier study by Mela et al. (2011) confronting *C. fungivorans* Ter331 with *A. niger* demonstrated that several genes encoding for oxalate:formate antiporter, formyl-CoA transferase and oxalyl-CoA decarboxylase were up-regulated in *C. fungivorans* Ter331, indicating a possible use of oxalic acid as carbon and/or energy source. However, *C. fungivorans* Ter331 does not seem to grow on oxalic acid (Mela et al., 2011; Rudnick et al., 2015). Four soil *Paraburkholderia terrae* strains have also been shown to be attracted by oxalic acid produced by two soil fungi, *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302. One of these strains, *P. terrae* BS001, is able to use oxalic acid as carbon source (Haq, Zwahlen, Yang, & van Elsas, 2018). These findings suggest that oxalic acid can be used as a signaling molecule for bacteria to locate fungi in soil in order for both partners to interact. A similar chemotactic behavior toward selected plant exudates such as oxalate has been demonstrated for rhizospheric bacteria (Alexandre, Greer, & Zhulin, 2000).

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5. Bacterial-fungal interactions (BFI) in the framework of oxalic acid cycling: Parallels in soils, plants and animals

5.1 BFI and oxalic acid cycling in soils: The oxalate-carbonate pathway (OCP)

LMWOA are involved in many processes occurring in soil, particularly in the rhizosphere (Jones, 1998). By exuding LMWOA, plants and fungi create micro-niches for the development of bacteria around their roots or hyphae, respectively (Badri & Vivanco, 2009; Braissant, Verrecchia, & Aragno, 2002; De Boer, Folman, Summerbell, & Boddy, 2005). Indeed, LMWOA such as acetate, glyoxylate, malate or oxalate can be used as sole carbon and energy source by bacteria (Braissant et al., 2002). Despite the release of oxalic acid into the soil by both fungi and plants, either through exudates or through decomposition, oxalic acid appears not to accumulate in soils. While several soil properties, such as pH and sorption sites influence the presence of oxalic acid in soils, bacterial metabolism is the main factor influencing the persistence of this compound in soils (Martin et al., 2012; Uren, 2018).

Oxalotrophic bacteria readily metabolize oxalic acid and they play a major role in the oxalate-carbonate pathway (OCP) (Fig. 5), which is a biogeochemical process that provides two important ecosystem functions: (1) a potential sink for atmospheric CO_2 (Aragno & Verrecchia, 2012; Cailleau, Mota, Bindschedler, Junier, & Verrecchia, 2014; Verrecchia, Braissant, & Cailleau, 2006) and (2) an increase in soil nutrient content (Pons et al., 2018). Through photosynthesis, plants fix CO_2 and produce the precursors of oxalic acid (Franceschi & Horner, 1980). Decay of plant biomass and fungal excretion leads to the release of oxalate crystals in soils and to their availability as part of the soil carbon pool (Aragno & Verrecchia, 2012; Verrecchia et al., 2006). Oxalotrophic bacteria oxidize calcium oxalate to CO_2, which is then excreted in the local microenvironment and turns into one of the four carbonate species (CO_2*, H_2CO_3, HCO_3^-; CO_3^{2-}) in function of the alkalinity of the soil solution (Aragno & Verrecchia, 2012). The microbial metabolism of oxalic acid exchanges a strong acid—oxalic acid ($pK_{a_1} = 1.25$ and $pK_{a_2} = 4.27$)—for a weak one—carbonic acid ($pK_{a_1} = 6.35$ and $pK_{a_2} = 10.33$)—which may lead to a strong local increase of pH. Such an influence on the soil pH prevents cation leaching from soil as a result of low pH (Pons et al., 2018) and

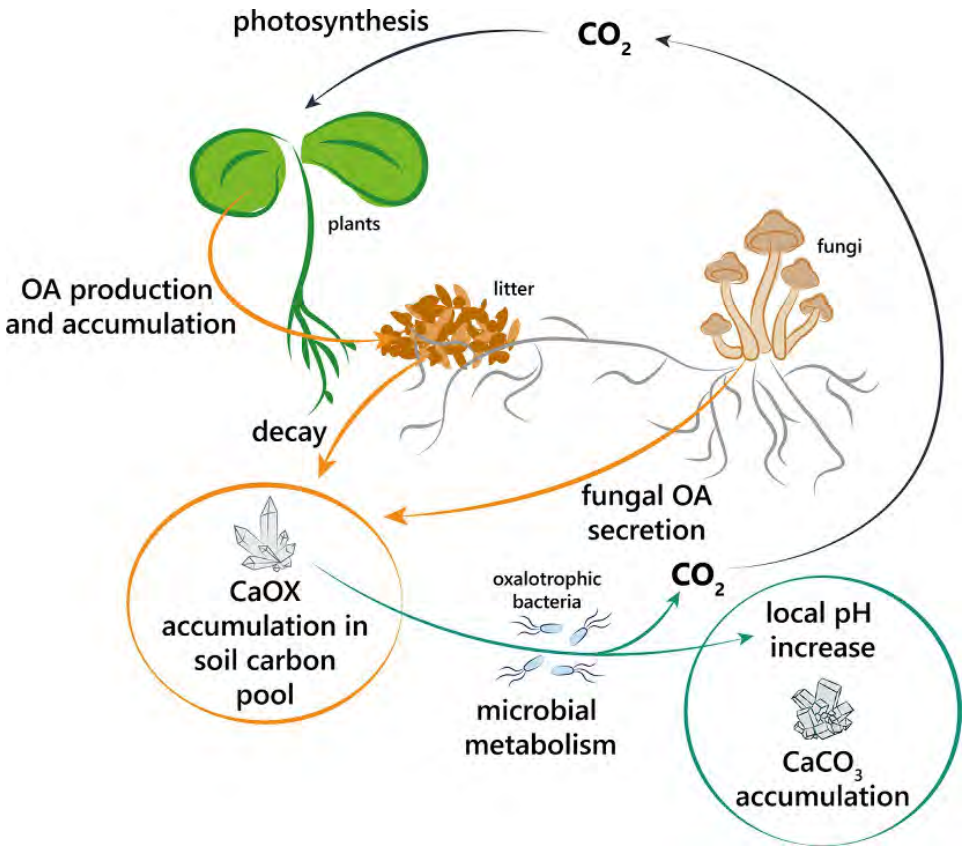

Fig. 5 Oxalate-Carbonate Pathway. Through photosynthesis, plants fix CO_2 and produce the precursors of oxalic acid (OA). Fungi also produce OA and upon decay of plant biomass, calcium oxalate (CaOx) accumulates in the soil. Oxalotrophic bacteria consume oxalic acid and the metabolic products lead to a local increase of pH, which is induced by the exchange of a strong acid (oxalic acid) for a weak one (carbonic acid). This may eventually lead to calcium carbonate precipitation if $pH > 8$. Excess CO_2 is released to the atmosphere and can enter the cycle again.

can eventually promote the precipitation of calcium carbonate ($CaCO_3$) if pH increases above 8 (Aragno & Verrecchia, 2012; Martin et al., 2012; Verrecchia et al., 2006). In this latter process, for each mole of calcium oxalate oxidized, 1 mol of CO_2 is released in the atmosphere, while the other one is sequestered in soils as biomineralized $CaCO_3$. The OCP may therefore act as an efficient carbon sink, as long as calcium originates from carbonate-free minerals (Cailleau et al., 2014; Martin et al., 2012).

One could think that fungi contribute to the OCP only by releasing oxalate into soils through exudates and by fostering the decomposition of plant biomass. However, it seems that fungi may play a more significant role in the oxidation of oxalic acid. Martin et al. (2012) showed that even if bacteria are the main organisms responsible for oxalate oxidation and consequent soil

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alkalinization, their activity is dependent of the presence of fungi. We still do not have a clear understanding of the relationship between bacteria and fungi in the context of the OCP, but Martin et al. (2012) postulated that fungi could protect bacteria from the acidic conditions and help them to colonize the heterogeneous matrix of the soil to reach oxalate crystals. However, there is a lack of knowledge about the importance of oxalotrophic fungi and oxalogenic bacteria in oxalic acid cycling in soils in general and in the OCP in particular.

5.2 BFI and oxalic acid cycling in plants

Root exudates provide various carbon and energy sources for bacteria living in the rhizosphere and the different secreted compounds influence the composition of the microbial community (Jones, 1998). Oxalic acid has been shown to be a major component of the root exudates and to play an important role in the recruitment of bacteria (Dessureault-Rompré, Nowack, Schulin, & Luster, 2007). In *Burkholderia* species, for example, oxalotrophy has been shown to be associated strictly with strains that are beneficial to plant growth, and it is required for the successful colonization of the plant (Kost, Stopnisek, Agnoli, Eberl, & Weisskopf, 2013). As oxalotrophy decreases oxalic acid concentration in both leaves and rhizosphere, it has been postulated that oxalic acid degradation could be a function that protects plants (Kost et al., 2013; Müller et al., 2016). Oxalic acid is a pathogenicity factor of several phytopathogenic fungi and the degradation of this acid could provide protection to the plant by making the environment less favorable to fungi. Indeed, studies on *Arabidopsis thaliana* and *Phaseolus vulgaris* have shown that oxalotrophic bacteria offer protection against pathogenic fungi (Dickman & Chet, 1998; Schoonbeek, Jacquat-Bovet, Mascher, & Métraux, 2007). Dickman and Chet (1998) have found that *Pseudomonas* spp., which degrade oxalic acid, could offer protection for *A. thaliana* against infection from *Sclerotinia sclerotium*. To evaluate the potential of this approach for field applications, experiments with *S. rolfsii*, another oxalic acid-producing soil fungal pathogen, and *P. vulgaris* were conducted. With the addition of oxalotrophic bacteria, the disease incidence in beans was significantly reduced compared to the control by $60 \pm 11\%$ and $75 \pm 9\%$, respectively, for the two strains of bacteria used. Schoonbeek et al. (2007) also investigated the ability of four oxalotrophic bacterial strains that were isolated from agricultural soil samples to protect different crops and *A. thaliana* against the fungal pathogens *Botrytis cinerea* and *S. sclerotium*;

the bacteria increased protection against infection by 30–70%. Therefore, oxalic acid does not only play a major role in plant metabolism, but is also an important feature in microbial recruitment and protection against fungal pathogens.

5.3 BFI and oxalic acid cycling in the human and animal body

In mammals, oxalic acid in the blood can have an exogenous origin from the diet, or an endogenous origin from the metabolism of glycine, glyoxylate and ascorbic acid in the liver (Abratt & Reid, 2010) (Fig. 6). Typically, 20–40% of the oxalic acid originates from common food and beverages, where it is present in high concentrations, including coffee, tea, chocolate, rhubarb, spinach, and other fruits and vegetables (Abratt & Reid, 2010; Libert & Franceschi, 1987; Marengo & Romani, 2008; Stewart, Duncan, & Cave, 2006).

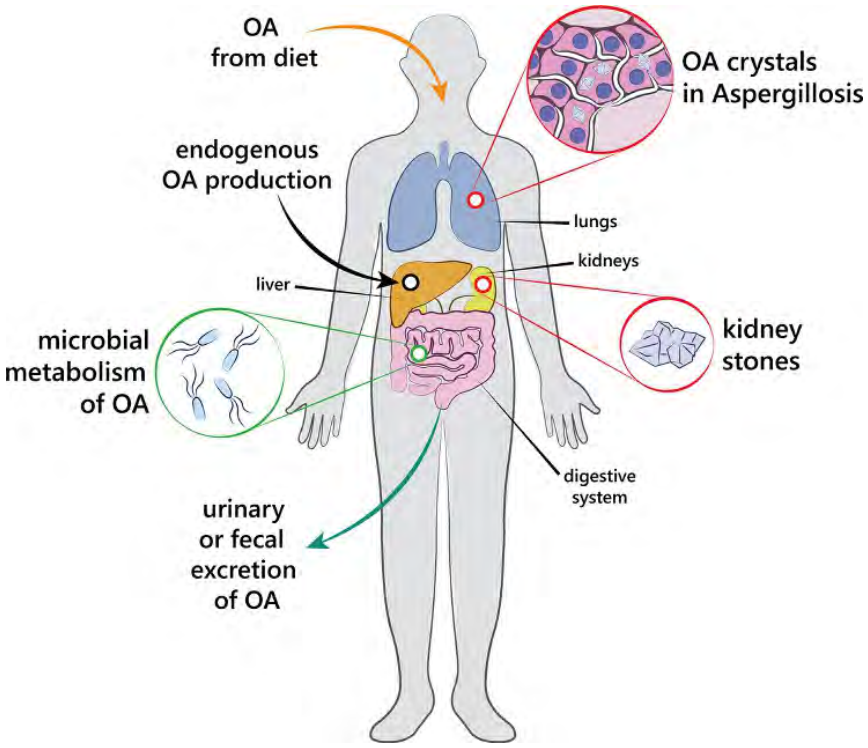

Fig. 6 Implication of BFI in the oxalic acid (OA) cycling in the human body. OA enters the body through the diet or is produced endogenously in the liver. It is then either excreted in the urine or sequestered in the form of calcium oxalate crystals and excreted with the feces. Gastrointestinal oxalate-degrading bacteria, such as *O. formigenes*, can degrade oxalic acid and thus participate to the maintenance of homeostasis. High levels of oxalic acid can lead to hyperoxaluria and the formation of kidney stones. In the case of pulmonary aspergillosis, the presence of calcium oxalate crystals has been reported, suggesting a potential link between oxalic acid and pathogenicity in *Aspergillus* fungi.

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As humans lack the enzymes to degrade oxalic acid, it has to be either excreted or sequestered to maintain homeostasis. It can be absorbed in the kidneys and excreted in the urine or it can form insoluble calcium oxalate and be eliminated in the feces (Abratt & Reid, 2010) (Fig. 6). Moreover, oxalic acid can be degraded by gastrointestinal oxalic acid-degrading bacteria, such as *O. formigenes* (Allison, Cook, Milne, Gallagher, & Clayman, 1986; Stewart et al., 2006) (Fig. 6). A differential oxalic acid movement along the gastrointestinal tract has been demonstrated in humans (Hatch, Freel, & Vaziri, 1993): oxalic acid is absorbed in the distal colon while it is secreted in the ileum, jejunum and proximal colon.

High levels of oxalic acid can be toxic and cause pathologies like hyperoxaluria, a known risk factor for the formation of kidney stones, which are mainly composed of calcium oxalate (Fig. 6). The degradation of oxalic acid by gastrointestinal bacteria like *O. formigenes*, *Lactobacillus* spp. and *Bifidobacterium* spp. is supposed to decrease the rates of oxalic acid absorption and thus provides a potential target for probiotic treatment of hyperoxaluria (Abratt & Reid, 2010). In sheep, *O. formigenes* plays a major role in oxalic acid degradation (Smith, 2002). When they graze in spring pasture fields containing plants with high levels of oxalic acid, sheep die rapidly of renal failure. However, with a gradual exposure to oxalic acid, *O. formigenes*, which is lost from the gut during winter, is able to re-colonize the rumen and degrade oxalic acid, thus regulating the concentration of oxalic acid in kidneys, protecting sheep against disease (Smith, 2002).

Oxalic acid plays an important role in the pathogenicity of plant pathogens, and it might also play a role in human health by human fungal pathogens. However, a link between oxalic acid production and pathogenicity has not been yet reported for human pathogens. Fungi of the *Aspergillus* genus are widespread and some species have been associated with health issues ranging from allergies to life threatening systemic infections (Paulussen et al., 2017). Oxalic acid production by *A. niger* is well known (Payne, Dark, Conway, & Farina, 2017), and several studies reported the presence of calcium oxalate crystals in animal and human lungs in the pulmonary disease aspergillosis (Kauffman, Wilson, & Schwartz, 1984; Kurrein, Path, Green, & Rowles, 1975; Kuwabara & Shibayama, 2012; Maeno, Sasaki, Shibue, Mimura, & Oka, 2015; Muntz, 1999; Oda et al., 2013; Pabuççuoğlu, 2005; Payne et al., 2017). Therefore, oxalic acid could play a role in lung infection. The contribution of opportunistic fungal pathogens to the total oxalic acid pool of the human body and the link between pathogenicity and oxalic acid still need to be investigated.

6. Conclusions

In summary, this review shows that even if a large body of information has been generated in relationship to oxalic acid, there is still some significant gaps of knowledge. Furthermore, this review focuses on plants, bacteria, fungi and animals, but one can expect that oxalic acid is also a relevant metabolite in the case of protists and archaea. Oxalic acid production and its metabolic implications in plants and fungi are fairly well known, while only a few studies have focused on its metabolic implications in bacteria and animals, including humans. Conversely, oxalic acid consumption by bacteria has been extensively studied since the first isolation of an oxalotrophic bacterium. However, the potential ability of fungi to produce energy from oxalic acid remains largely under-investigated. These current knowledge gaps mean that the contribution of organisms such as oxalotrophic fungi or oxalogenic bacteria to oxalate cycling have not yet been investigated in detail. Moreover, as oxalic acid is a compound found ubiquitously in ecosystems, it could play a major role in microbial interactions but its use as a signaling molecule is still poorly understood. A similar knowledge gap exists in our understanding of the role that oxalic acid may play in animal health. Oxalic acid enters the body mainly through the diet and does not seem to have a major metabolic role. However, a balanced gut microbiota seems to be required to efficiently degrade oxalic acid in order to maintain an equilibrium between its uptake and excretion. Although oxalic acid can function as a pathogenicity factor in fungal pathogens of plants, it is still unclear whether this could also be the case for animal pathogens, despite the correlation between the occurrence of pulmonary aspergillosis and the presence of calcium oxalate crystals in the lungs. Therefore, the potential role of oxalic acid in human pathogenic fungi as well as the contribution of the host-associated microbiota in the defense of host still has to be addressed, especially because it may yield new ways to control pathogenic fungi amid the systematic emergence of antifungal resistance.

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References

Abratt, V. R., & Reid, S. J. (2010). Chapter 3: Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease. In A. I. Laskin, S. Sariaslani, & G. M. Gadd (Eds.), *Advances in applied microbiology* (pp. 63–87). Academic Press. https://doi.org/10.1016/S0065-2164(10)72003-7.

Aguilar, C., Urzúa, U., Koenig, C., & Vicuña, R. (1999). Oxalate oxidase from *Ceriporiopsis subvermispora*: Biochemical and cytochemical studies. *Archives of Biochemistry and Biophysics*, *366*, 275–282. https://doi.org/10.1006/abbi.1999.1216.

Albrecht, S., Brandl, H., & Schonfels, C. (1994). Human oxalate-really just an end-product of metabolism? *Angewandte Chemie (International Ed in English)*, *1780*, 1780–1781.

Alexandre, G., Greer, S. E., & Zhulin, I. B. (2000). Energy taxis is the dominant behavior in *Azospirillum brasilense*. *Journal of Bacteriology*, *182*, 6042–6048. https://doi.org/10.1128/JB.182.21.6042-6048.2000.

Allison, M. J., Cook, H. M., Milne, D. B., Gallagher, S., & Clayman, R. V. (1986). Oxalate degradation by gastrointestinal bacteria from humans. *The Journal of Nutrition*, *116*, 455–460.

Anantharam, V., Allison, M. J., & Maloney, P. C. (1989). Oxalate:formate exchange. The basis for energy coupling in oxalobacter. *The Journal of Biological Chemistry*, *264*, 7244–7250.

Aragno, M., & Verrecchia, E. (2012). The oxalate-carbonate pathway: A reliable sink for atmospheric CO_2 through calcium carbonate biomineralization in ferralitic tropical soils. In T. Satyanarayana, A. Prakash, & B. N. Johri (Eds.), *Microorganisms in environmental management: microbes and environment* (pp. 1–819). Springer. https://doi.org/10.1007/978-94-007-2229-3.

Arvieu, J.-C., Leprince, F., & Plassard, C. (2003). Release of oxalate and protons by ectomycorrhizal fungi in response to P-deficiency and calcium carbonate in nutrient solution. *Annals of Forest Science*, *60*, 815–821. https://doi.org/10.1051/forest.

Azcarate-Peril, M. A., Bruno-Bárcena, J. M., Hassan, H. M., & Klaenhammer, T. R. (2006). Transcriptional and functional analysis of oxalyl-coenzyme A (CoA) decarboxylase and formyl-coa transferase genes from *Lactobacillus acidophilus*. *Applied and Environmental Microbiology*, *72*, 1891–1899.

Badri, D. V., & Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant, Cell and Environment*, *32*, 666–681. https://doi.org/10.1111/j.1365-3040.2009.01926.x.

Baetz, A. L., & Allison, M. J. (1990). Purification and characterization of formyl-coenzyme A transferase from *Oxalobacter formigenes*. *Journal of Bacteriology*, *172*, 3537–3540. https://doi.org/10.1128/jb.172.7.3537-3540.1990.

Bassalik, K. (1913). Ueber die Verarbeitung der Oxalsäure durch Bacillus extorquens n. sp., *Jahrbucher fur Wissenschaftliche Botanik*, *53*, 255–302.

Becerra-Castro, C., Kidd, P., Kuffner, M., Prieto-Fernández, Á., Hann, S., Monterroso, C., et al. (2013). Bacterially induced weathering of ultramafic rock and its implications for phytoextraction. *Applied and Environmental Microbiology*, *79*, 5094–5103. https://doi.org/10.1128/AEM.00402-13.

Blackmore, M. A., & Quayle, J. R. (1970). Microbial growth on oxalate by a route not involving glyoxylate carboligase. *The Biochemical Journal*, *118*, 53–59. https://doi.org/10.1042/bj1180053.

Braissant, O., Verrecchia, E. P., & Aragno, M. (2002). Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? *Naturwissenschaften*, *89*, 366–370. https://doi.org/10.1007/s00114-002-0340-0.

Cailleau, G., Mota, M., Bindschedler, S., Junier, P., & Verrecchia, E. P. (2014). Detection of active oxalate-carbonate pathway ecosystems in the Amazon basin: Global implications of a natural potential C sink. *Catena*, *116*, 132–141. https://doi.org/10.1016/j.catena.2013.12.017.

Çalişkan, M. (2000). The metabolism of oxalic acid. *Turkish Journal of Zoology*, *24*, 103–106.

Chandra, T. S., & Shethna, Y. I. (1977). Microbial metabolism of oxalate and one-carbon compounds. *Journal of the Indian Institute of Science*, *59*, 26–52.

Cheng, C., Wang, Q., He, L., & Sheng, X. (2017). Change in mineral weathering behaviors of a bacterium Chitinophaga jiangningensis JN53 under different nutrition conditions. *Journal of Basic Microbiology*, *57*, 293–301. https://doi.org/10.1002/jobm.201600652.

Clark, J., & Haskins, E. (2014). Sporophore morphology and development in the myxomycetes: A review. *Mycosphere*, *5*, 153–170. https://doi.org/10.5943/mycosphere/5/1/7.

Cromack, K., Sollins, P., Graustein, W. C., Speidel, K., Todd, A. W., Spycher, G., et al. (1979). Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus Hysterangium crassum. *Soil Biology and Biochemistry*, *11*, 463–468. https://doi.org/10.1016/0038-0717(79)90003-8.

De Boer, W., Folman, L. B., Summerbell, R. C., & Boddy, L. (2005). Living in a fungal world: Impact of fungi on soil bacterial niche development. *FEMS Microbiology Reviews*, *29*, 795–811.

de Oliveira Ceita, G., Macêdo, J. N. A., Santos, T. B., Alemanno, L., da Silva Gesteira, A., Micheli, F., et al. (2007). Involvement of calcium oxalate degradation during programmed cell death in Theobroma cacao tissues triggered by the hemibiotrophic fungus *Moniliophthora perniciosa*. *Plant Science*, *173*, 106–117. https://doi.org/10.1016/j.plantsci.2007.04.006.

Dessureault-Rompré, J., Nowack, B., Schulin, R., & Luster, J. (2007). Spatial and temporal variation in organic acid anion exudation and nutrient anion uptake in the rhizosphere of Lupinus albus L. *Plant and Soil*, *301*, 123–134. https://doi.org/10.1007/s11104-007-9427-x.

Deveau, A., Bonito, G., Uehling, J., Paoletti, M., Becker, M., Bindschedler, S., et al. (2018). Bacterial-fungal interactions: Ecology, mechanisms and challenges. *FEMS Microbiology Reviews*, *42*, 335–352. https://doi.org/10.1093/femsre/fuy008.

Dickman, M. B., & Chet, I. (1998). Biodegradation of oxalic acid: A potential new approach to biological control. *Soil Biology and Biochemistry*, *30*, 1195–1197.

Dijkhuizen, L., Wiersma, M., & Harder, W. (1977). Energy production and growth of *Pseudomonas oxalaticus* OXl on oxalate and formate. *Archives of Microbiology*, *115*, 229–236. https://doi.org/10.1007/BF00406379.

Duchesne, L. C., Ellis, B. E., & Peterson, R. L. (1989). Disease suppression by ectomycorrhizal fungus *Paxillus involutus*: Contribution of oxalic acid. *Canadian Journal of Botany*, *67*, 2726–2730. https://doi.org/10.1139/b89-351.

Dutton, M. V., & Evans, C. S. (1996). Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. *Canadian Journal of Microbiology*, *42*, 881–895. https://doi.org/10.1139/m96-114.

Fomina, M., Hillier, S., Charnock, J. M., Melville, K., Alexander, I. J., & Gadd, G. M. (2005). Role of oxalic acid overexcretion in transformations of toxic metal minerals by Beauveria caledonica. *Applied and Environmental Microbiology*, *71*, 371–381. https://doi.org/10.1128/AEM.71.1.371-381.2005.

Franceschi, V. R., & Horner, H. T. J. (1980). Calcium oxalate crystals in plants. *The Botanical Review*, *46*, 361–427.

Franceschi, V. R., & Nakata, P. A. (2005). Calcium oxalate in plants: Formation and function. *Annual Review of Plant Biology*, *56*, 41–71. https://doi.org/10.1146/annurev.arplant.56.032604.144106.

Frey, B., Rieder, S. R., Brunner, I., Plötze, M., Koetzsch, S., Lapanje, A., et al. (2010). Weathering-associated bacteria from the damma glacier forefield: Physiological capabilities and impact on granite dissolution. *Applied and Environmental Microbiology*, *76*, 4788–4796. https://doi.org/10.1128/AEM.00657-10.

Oxalic acid and microbial interactions

Gadd, G. M. (1999). Fungal production of citric and oxalic acid: Importance in metal speciation, physiology and biogeochemical processes. *Advances in Microbial Physiology*, *41*, 47–92. https://doi.org/10.1016/S0065-2911(08)60165-4.

Gadd, G. M., Bahri-Esfahani, J., Li, Q., Rhee, Y. J., Wei, Z., Fomina, M., et al. (2014). Oxalate production by fungi: Significance in geomycology, biodeterioration and bioremediation. *Fungal Biology Reviews*, *28*, 36–55. https://doi.org/10.1016/j.fbr.2014.05.001.

Gharieb, M. M., & Gadd, G. M. (1999). Influence of nitrogen source on the solubilization of natural gypsum (CaSO4. 2H2O) and the formation of calcium oxalate by different oxalic and citric acid-producing fungi. *Mycological Research*, *103*, 473–481. https://doi.org/10.1017/S0953756298007382.

Graustein, W. C., Cromack, K. J., & Sollins, P. (1977). Calcium oxalate: Occurrence in soils and effect on nutrient and geochemical cycles. *Science (Washington, D.C.)*, *198*, 1252–1254.

Grąz, M., Jarosz-Wilkołazka, A., & Pawlikowska-Pawlęga, B. (2008). *Abortiporus biennis* tolerance to insoluble metal oxides: Oxalate secretion, oxalate oxidase activity, and mycelial morphology. *Biometals*, *22*, 401. https://doi.org/10.1007/s10534-008-9176-1.

Guggiari, M., Bloque, R., Aragno, M., Verrecchia, E., Job, D., & Junier, P. (2011). Experimental calcium-oxalate crystal production and dissolution by selected wood-rot fungi. *International Biodeterioration and Biodegradation*, *65*, 803–809. https://doi.org/10.1016/j.ibiod.2011.02.012.

Hakala, T. K., Lundell, T., Galkin, S., Maijala, P., Kalkkinen, N., & Hatakka, A. (2005). Manganese peroxidases, laccases and oxalic acid from the selective white-rot fungus Physisporinus rivulosus grown on spruce wood chips. *Enzyme and Microbial Technology*, *36*, 461–468. https://doi.org/10.1016/j.enzmictec.2004.10.004.

Hamel, R., Levasseur, R., & Appanna, V. D. (1999). Oxalic acid production and aluminum tolerance in *Pseudomonas fluorescens*. *Journal of Inorganic Biochemistry*, *76*, 99–104.

Han, Y., Joosten, H. J., Niu, W., Zhao, Z., Mariano, P. S., McCalman, M., et al. (2007). Oxaloacetate hydrolase, the C-C bond lyase of oxalate secreting fungi. *The Journal of Biological Chemistry*, *282*, 9581–9590. https://doi.org/10.1074/jbc.M608961200.

Haq, I. U., Zwahlen, R. D., Yang, P., & van Elsas, J. D. (2018). The response of *Paraburkholderia terrae* strains to two soil Fungi and the potential role of oxalate. *Frontiers in Microbiology*, *9*, 989. https://doi.org/10.3389/fmicb.2018.00989.

Harder, W. (1973). Microbial metabolism of organic C1 and C2 compounds. *Antonie Van Leeuwenhoek*, *39*, 650–652.

Hatch, M., Freel, R. W., & Vaziri, N. D. (1993). Characteristics of the transport of oxalate and other ions across rabbit proximal colon. *Pflügers Archiv/European Journal of Physiology*, *423*, 206–212. https://doi.org/10.1007/BF00374396.

Hervé, V., Junier, T., Bindschedler, S., Verrecchia, E., & Junier, P. (2016). Diversity and ecology of oxalotrophic bacteria. *World Journal of Microbiology and Biotechnology*, *32*, 28. https://doi.org/10.1007/s11274-015-1982-3.

Hess, D., Coker, D. J., Loutsch, J. M., & Russ, J. (2007). Production of oxalates in vitro by microbes isolated from rock surfaces with prehistoric paints in the lower Pecos region, Texas. *Geoarchaeology*, *23*, 3–11. https://doi.org/10.1002/gea.20208.

Jakoby, W. B., & Bhat, J. V. (1958). Microbial metabolism of oxalic acid. *Bacteriological Reviews*, *22*, 75–80.

Jakoby, W. B., Homura, E., & Hayaishi, O. (1956). Enzymatic decarboxylation of oxalic acid. *The Journal of Biological Chemistry*, *222*, 435–446.

Jones, D. L. (1998). Organic acids in the rhizosphere—A critical review. *Plant and Soil*, *205*, 25–44. https://doi.org/10.1023/A:1004356007312.

Just, V. J., Stevenson, C. E. M., Bowater, L., Tanner, A., Lawson, D. M., & Bornemann, S. (2004). A closed conformation of Bacillus subtilis oxalate decarboxylase OxdC provides

evidence for the true identity of the active site. *The Journal of Biological Chemistry*, *279*, 19867–19874. https://doi.org/10.1074/jbc.M313820200.

Kauffman, C. A., Wilson, K. H., & Schwartz, D. B. (1984). Necrotizing pulmonary aspergillosis with oxalosis: Nekrotisierende pulmonale Aspergillose mit oxalose. *Mycoses*, *27*, 535–538. https://doi.org/10.1111/j.1439-0507.1984.tb01984.x.

Kornberg, H. L. (1959). Aspects of terminal respiration in microorganisms. *Annual Review of Microbiology*, *13*, 49–78.

Kornberg, H. L. (1965). Anaplerotic sequences in microbial metabolism. *Angewandte Chemie (International Ed in English)*, *4*, 558–565.

Kost, T., Stopnisek, N., Agnoli, K., Eberl, L., & Weisskopf, L. (2013). Oxalotrophy, a widespread trait of plant-associated Burkholderia species, is involved in successful root colonization of lupin and maize by *Burkholderia phytofirmans*. *Frontiers in Microbiology*, *4*, 1–9. https://doi.org/10.3389/fmicb.2013.00421.

Krieger, C., Calvaruso, C., Morlot, C., Uroz, S., Salsi, L., & Turpault, M. P. (2017). Identification, distribution, and quantification of biominerals in a deciduous forest. *Geobiology*, *15*, 296–310. https://doi.org/10.1111/gbi.12223.

Kritzman, G., Chet, I., & Henis, Y. (1977). The role of oxalic acid in the pathogenic behavior of *Sclerotium rolfsii* Sacc. *Experimental Mycology*, *1*, 280–285. https://doi.org/10.1016/S0147-5975(77)80003-0.

Kuan, I. C., & Tien, M. (1993). Stimulation of Mn peroxidase activity: A possible role for oxalate in lignin biodegradation. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 1242–1246. https://doi.org/10.1073/pnas.90.4.1242.

Kubicek, C. P., Schreferl-Kunar, G., Wöhrer, W., & Röhr, M. (1988). Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. *Applied and Environmental Microbiology*, *54*, 633–637.

Kurrein, F., Path, F. R. C., Green, G. H., & Rowles, S. L. (1975). Localized deposition of calcium oxalate around a pulmonary *Aspergillus niger* fungus ball. *American Journal of Clinical Pathology*, *64*, 556–563. https://doi.org/10.1093/ajcp/64.4.556.

Kuwabara, H., & Shibayama, Y. (2012). Pulmonary aspergilloma with prominent oxalate deposition. *Indian Journal of Pathology & Microbiology*, *55*, 589. https://doi.org/10.4103/0377-4929.107838.

Landeweert, R., Hoffland, E., Finlay, R. D., Kuyper, T. W., & Van Breemen, N. (2001). Linking plants to rocks: Ectomycorrhizal fungi mobilize nutrients from minerals. *Trends in Ecology & Evolution*, *16*, 248–254. https://doi.org/10.1016/S0169-5347(01)02122-X.

Lapeyrie, F. (1988). Oxalate synthesis from soil bicarbonate by the mycorrhizal fungus *Paxillus involutus*. *Plant and Soil*, *110*, 3–8. https://doi.org/10.1007/BF02143532.

Lapeyrie, F., Chilvers, G. A., & Bhem, C. A. (1987). Oxalic acid synthesis by the mycorrhizal fungus Paxillus involtus (Batsh. ex. fr.) fr. *The New Phytologist*, *106*, 139–146. https://doi.org/10.1111/j.1469-8137.1987.tb04797.x.

Lapeyrie, F., Ranger, J., & Vairelles, D. (1991). Phosphate-solubilizing activity of ectomycorrhizal fungi in vitro. *Canadian Journal of Botany*, *69*, 342–346. https://doi.org/10.1139/b91-046.

Libert, B., & Franceschi, V. R. (1987). Oxalate in crop plants. *Journal of Agricultural and Food Chemistry*, *35*, 926–938. https://doi.org/10.1021/jf00078a019.

Maeno, T., Sasaki, M., Shibue, Y., Mimura, K., & Oka, H. (2015). Calcium oxalate in the sputum may aid in the diagnosis of pulmonary aspergillosis: A report of two cases. *Medical Mycology Case Reports*, *8*, 32–36. https://doi.org/10.1016/j.mmcr.2015.01.003.

Mäkelä, M., Galkin, S., Hatakka, A., & Lundell, T. (2002). Production of organic acids and oxalate decarboxylase in lignin-degrading white rot fungi. *Enzyme and Microbial Technology*, *30*, 542–549. https://doi.org/10.1016/S0141-0229(02)00012-1.

Mäkelä, M. R., Hildén, K., & Lundell, T. K. (2010). Oxalate decarboxylase: Biotechnological update and prevalence of the enzyme in filamentous fungi. *Applied Microbiology and Biotechnology*, *87*, 801–814. https://doi.org/10.1007/s00253-010-2650-z.

Marengo, S. R., & Romani, A. M. P. (2008). Oxalate in renal stone disease: The terminal metabolite that just won't go away. *Nature Clinical Practice. Nephrology*, *4*, 368.

Markovich, D., & Aronson, P. S. (2007). Specificity and regulation of renal sulfate transporters. *Annual Review of Physiology*, *69*, 361–375. https://doi.org/10.1146/annurev.physiol.69.040705.141319.

Martin, G., Guggiari, M., Bravo, D., Zopfi, J., Cailleau, G., Aragno, M., et al. (2012). Fungi, bacteria and soil pH: The oxalate-carbonate pathway as a model for metabolic interaction. *Environmental Microbiology*, *14*, 2960–2970. https://doi.org/10.1111/j.1462-2920.2012.02862.x.

Mattevi, A., Obmolova, G., Schulze, E., Kalk, K. H., Westphal, A. H., De Kok, A., et al. (1992). Atomic structure of the cubic core of the pyruvate dehydrogenase multienzyme complex. *Science*, *255*, 1544–1550. https://doi.org/10.1126/science.1549782.

Maxwell, D. P., & Bateman, D. F. (1968). Influence of carbon source and pH on oxalate accumulation in culture filtrates of *Sclerotium rolfsii*. *Phytopathology*, *58*, 1351–1355.

Mela, F., Fritsche, K., de Boer, W., van Veen, J. A., de Graaff, L. H., van den Berg, M., et al. (2011). Dual transcriptional profiling of a bacterial/fungal confrontation: Collimonas fungivorans versus *Aspergillus niger*. *The ISME Journal*, *5*, 1494–1504. https://doi.org/10.1038/ismej.2011.29.

Micales, J. A. (1997). Localization and inducation of oxalate decarboxylase in the brown-rot wood decay fungus Posta placenta. *International Biodeterioration and Biodegradation*, *39*, 125–132.

Moore, D., Robson, G., & Trinci, T. (2011). *21st Century guidebook to fungi*. Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9780511977022.

Müller, D. B., Schubert, O. T., Röst, H., Aebersold, R., & Vorholt, J. A. (2016). Systems-level proteomics of two ubiquitous leaf commensals reveals complementary adaptive traits for phyllosphere colonization. *Molecular & Cellular Proteomics*, *15*, 3256–3269. https://doi.org/10.1074/mcp.M116.058164.

Muntz, F. H. A. (1999). Oxalate-producing pulmonary aspergillosis in an alpaca. *Veterinary Pathology*, *36*, 631–632. https://doi.org/10.1354/vp.36-6-631.

Nakata, P. A. (2003). Advances in our understanding of calcium oxalate crystal formation and function in plants. *Plant Science*, *164*, 901–909. https://doi.org/10.1016/S0168-9452(03)00120-1.

Nakata, P. A. (2011). The oxalic acid biosynthetic activity of *Burkholderia mallei* is encoded by a single locus. *Microbiological Research*, *166*, 531–538. https://doi.org/10.1016/j.micres.2010.11.002.

Nakata, P. A., & He, C. (2010). Oxalic acid biosynthesis is encoded by an operon in *Burkholderia glumae*. *FEMS Microbiology Letters*, *304*, 177–182.

Nguyen, N. H., Song, Z., Bates, S. T., Branco, S., Tedersoo, L., Menke, J., et al. (2016). FUNGuild: An open annotation tool for parsing fungal community datasets by ecological guild. *Fungal Ecology*, *20*, 241–248. https://doi.org/10.1016/j.funeco.2015.06.006.

Oda, M., Saraya, T., Wakayama, M., Shibuya, K., Ogawa, Y., Inui, T., et al. (2013). Calcium oxalate crystal deposition in a patient with Aspergilloma due to *Aspergillus niger*. *Journal of Thoracic Disease*, *5*, E174–E178. https://doi.org/10.3978/j.issn.2072-1439.2013.08.45.

Pabuççuoğlu, U. (2005). Aspects of oxalosis associated with aspergillosis in pathology specimens. *Pathology, Research and Practice*, *201*, 363–368. https://doi.org/10.1016/j.prp.2005.03.005.

Paulussen, C., Hallsworth, J. E., Álvarez-Pérez, S., Nierman, W. C., Hamill, P. G., Blain, D., et al. (2017). Ecology of aspergillosis: Insights into the pathogenic potency of Aspergillus fumigatus and some other Aspergillus species. *Microbial Biotechnology*, *10*, 296–322. https://doi.org/10.1111/1751-7915.12367.

Payne, C. L., Dark, M. J., Conway, J. A., & Farina, L. L. (2017). A retrospective study of the prevalence of calcium oxalate crystals in veterinary aspergillus cases. *Journal of veterinary*

diagnostic investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc., *29*, 51–58. https://doi.org/10.1177/1040638716672254.

Plassard, C., & Fransson, P. (2009). Regulation of low-molecular weight organic acid production in fungi. *Fungal Biology Reviews*, *23*, 30–39. https://doi.org/10.1016/j.fbr.2009.08.002.

Pons, S., Bindschedler, S., Sebag, D., Junier, P., Verrecchia, E., & Cailleau, G. (2018). Bio-controlled soil nutrient distribution under the influence of an oxalogenic-oxalotrophic ecosystem. *Plant and Soil*, 1–16. https://doi.org/10.1007/s11104-018-3573-1.

Punja, Z. K., & Jenkins, S. F. (1984). Influence of medium composition on mycelial growth and oxalic acid production in *Sclerotium rolfsii*. *Mycologia*, *76*, 947–950. https://doi.org/10.2307/3793153.

Quayle, J. R., Keecht, D. B., & Taylor, G. A. (1961). Carbon assimilation by Pseudomonas oxalaticus (OXI) 4. metabolism of oxalate in cell-free extracts of the organism grown on oxalate. *The Biochemical Journal*, *78*, 225–236. https://doi.org/10.1042/bj0780225.

Rio, M. C. S., de Oliveira, B. V., de Tomazella, D. P. T., Silva, J. A. F., & Pereira, G. A. G. (2008). Production of calcium oxalate crystals by the basidiomycete Moniliophthora perniciosa, the causal agent of witches broom disease of cacao. *Current Microbiology*, *56*, 363–370. https://doi.org/10.1007/s00284-007-9091-7.

Ruan, Z. S., Anantharam, V., Crawford, I. T., Ambudkar, S. V., Rhee, S. Y., Allison, M. J., et al. (1992). Identification, purification, and reconstitution of OxlT, the oxalate:formate antiport protein of *Oxalobacter formigenes*. *The Journal of Biological Chemistry*, *267*, 10537–10543.

Rudnick, M. B., Veen, J. A., & Boer, W. (2015). Oxalic acid: A signal molecule for fungus-feeding bacteria of the genus Collimonas? *Environmental Microbiology Reports*, 7, 709–714. https://doi.org/10.1111/1758-2229.12290.

Ruijter, G. J. G., van de Vondervoort, P. J. I., & Visser, J. (1999). Oxalic acid production by aspergillus Niger: An oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. *Microbiology*, *145*, 2569–2576. https://doi.org/10.1099/00221287-145-9-2569.

Sahin, N. (2003). Oxalotrophic bacteria. *Research in Microbiology*, *154*, 399–407. https://doi.org/10.1016/S0923-2508(03)00112-8.

Schmalenberger, A., Duran, A. L., Bray, A. W., Bridge, J., Bonneville, S., Benning, L. G., et al. (2015). Oxalate secretion by ectomycorrhizal *Paxillus involutus* is mineral-specific and controls calcium weathering from minerals. *Scientific Reports*, *5*, 1–14. https://doi.org/10.1038/srep12187, 12187.

Schneider, K., Skovran, E., & Vorholt, J. A. (2012). Oxalyl-coenzyme A reduction to glyoxylate is the preferred route of oxalate assimilation in *Methylobacterium extorquens* AM1. *Journal of Bacteriology*, *194*, 3144–3155. https://doi.org/10.1128/JB.00288-12.

Schoonbeek, H., Jacquat-Bovet, A.-C., Mascher, F., & Métraux, J.-P. (2007). Oxalate-degrading bacteria can protect *Arabidopsis thaliana* and crop plants against *Botrytis cinerea*. *Molecular Plant-Microbe Interactions*, *20*, 1535–1544. https://doi.org/10.1094/MPMI-20-12-1535.

Shimada, M., Ma, D. B., Akamatsu, Y., & Hattori, T. (1994). A proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. *FEMS Microbiology Reviews*, *13*, 285–295. https://doi.org/10.1111/j.1574-6976.1994.tb00049.x.

Sidhu, H., Ogden, S. D., Lung, H. Y., Luttge, B. G., Baetz, A. L., & Peck, A. B. (1997). DNA sequencing and expression of the formyl coenzyme A transferase gene, frc, from *Oxalobacter formigenes*. *Journal of Bacteriology*, *179*, 3378–3381. https://doi.org/10.1128/jb.179.10.3378-3381.1997.

Simon, A. (2016). *Highways and subways: A story of fungi and bacteria in soils*. Neuchâtel: University of Neuchâtel, Faculty of Science.

Oxalic acid and microbial interactions

Smith, L. (2002). The many roles of oxalate in nature. *Transactions of the American Clinical and Climatological Association*, *113*, 1–20.

Stewart, C. S., Duncan, S. H., & Cave, D. R. (2006). *Oxalobacter formigenes* and its role in oxalate metabolism in the human gut. *FEMS Microbiology Letters*, *230*, 1–7. https://doi.org/10.1016/S0378-1097(03)00864-4.

Strobel, B. W. (2001). Influence of vegetation on low-molecular-weight carboxylic acids in soil solution—A review. *Geoderma*, *99*, 169–198. https://doi.org/10.1016/S0016-7061(00)00102-6.

Tamer, A. Ü., & Aragno, M. (1980). Isolement, caractérisation et essai d'identification de bactéries capables d'utiliser l'oxalate comme seule source de carbone et d'énergie. *Bulletin de la Société Neuchâteloise des Sciences Naturelles*, *103*, 91–104.

Tsao, G. T. (1963). Production of oxalic acid by a wood-rotting fungus. *Applied Microbiology*, *11*, 249–254.

Turroni, S., Vitali, B., Bendazzoli, C., Candela, M., Gotti, R., Federici, F., et al. (2007). Oxalate consumption by lactobacilli: Evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in Lactobacillus acidophilus. *Journal of Applied Microbiology*, *103*, 1600–1609. https://doi.org/10.1111/j.1365-2672.2007.03388.x.

Uren, N. C. (2018). Calcium oxalate in soils, its origins and fate—A review. *Soil Research*, *56*, 443. https://doi.org/10.1071/SR17244.

van Kan, J. A. L. (2006). Licensed to kill: The lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science*, *11*, 247–253. https://doi.org/10.1016/j.tplants.2006.03.005.

van Kan, J. A. L., Shaw, M. W., & Grant-Downton, R. T. (2014). Botrytis species: Relentless necrotrophic thugs or endophytes gone rogue? *Molecular Plant Pathology*, *15*, 957–961. https://doi.org/10.1111/mpp.12148.

Verrecchia, E. P., Braissant, O., & Cailleau, G. (2006). The oxalate-carbonate pathway in soil carbon storage: The role of fungi and oxalotrophic bacteria. In *Fungi in biogeochemical cycles* (pp. 289–310). Cambridge University Press. https://doi.org/10.1017/CBO9780511550522.013. 9780521845.

Wang, T., Egbert, A. L., Abbiati, T., Aronson, P. S., & Giebisch, G. (1996). Mechanisms of stimulation of proximal tubule chloride transport by formate and oxalate. *The American Journal of Physiology*, *271*, F446–F450. https://doi.org/10.1152/ajprenal.1996.271.2.F446.

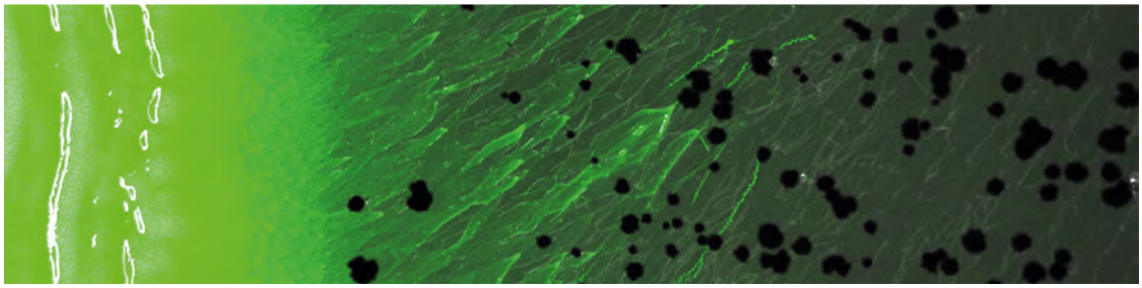
Watanabe, T., Hattori, T., Tengku, S., & Shimada, M. (2005). Purification and characterization of NAD-dependent formate dehydrogenase from the white-rot fungus *Ceriporiopsis subvermispora* and a possible role of the enzyme in oxalate metabolism. *Enzyme and Microbial Technology*, *37*, 68–75. https://doi.org/10.1016/j.enzmictec.2005.01.032.

Watanabe, T., Shitan, N., Suzuki, S., Umezawa, T., Shimada, M., Yazaki, K., et al. (2010). Oxalate efflux transporter from the Brown rot fungus Fomitopsis palustris. *Applied and Environmental Microbiology*, *76*, 7683–7690.

Yadav, A. N., Sharma, D., Gulati, S., Singh, S., Dey, R., Pal, K. K., et al. (2015). Haloarchaea endowed with phosphorus solubilization attribute implicated in phosphorus cycle. *Scientific Reports*, *5*, 1–10. https://doi.org/10.1038/srep12293.

Yang, J., Tewari, J. P., & Verma, P. R. (1993). Calcium oxalate crystal formation in Rhizoctonia solani AG 2-1 culture and infected crucifer tissue: Relationship between host calcium and resistance. *Mycological Research*, *97*, 1516–1522. https://doi.org/10.1016/S0953-7562(09)80227-X.

CHAPTER 3



Low Molecular Weight Organic Acids (LMWOAs) & Bacterial-Fungal Interactions (BFI) – Screening & Preliminary experiments

Foreword

In this chapter, we tested the impact of trophic conditions (medium composition) on the interactions between fungi and bacteria and discussed the implications of the outcome of these interactions for the development of a potential biocontrol strategy.

The main findings of this chapter are:

- Over the four fungal strains tested (*A. niger*, *B. cinerea*, *F. oxysporum* and *T. rossicum*), *A. niger* was the only fungus that produced consistently LMWOAs in both solid media tested and produced oxalic acid as the sole LMWOA in both liquid media tested. It has therefore been selected as model human fungal pathogen.

- *C. necator* and *C. oxalaticus* controlled the pH and growth of *A. niger* in co-cultures on WYA + BP culture medium, whereas *P. putida* did not have any effect.

- Oxalogenic and non-oxalogenic fungi, here *A. niger* and *T. rossicum*, respectively, seem to interact differently with oxalotrophic and non-oxalotrophic bacteria, i.e. *C. necator* and *C. oxalaticus*, and *P. putida*, respectively. *A. niger* outcompetes all three bacteria tested on MA. When the amount of nutrients is 10x less concentrated (MA 1/10), oxalotrophic bacteria limit sporulation of *A. niger*, whereas *P. putida* is outcompeted. On R2A, all three bacteria outcompete *A. niger*, with *C. oxalaticus* showing a stronger *A. niger* growth inhibition. For the non-oxalogenic fungus (*T. rossicum*), none of the three bacteria seem to have any influence on the growth.

3. Low Molecular Weight Organic Acids (LMWOAs) & Bacterial-Fungal Interactions (BFI) – Screening & Preliminary experiments

Fabio Palmieri[1], Saskia Bindschedler[1], Pilar Junier[1].

[1] Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland.

Abstract

In fungi, production of low molecular weight organic acids (LMWOAs), and more particularly oxalic acid (OA), has important roles in processes as diverse as pathogenesis, competition, mineral weathering or lignocellulose degradation. More recently, LMWOAs have been shown to play a pivotal role in the interaction between fungi and bacteria, especially in soils. Several factors can influence LMWOAs' production, such as the available carbon and nitrogen sources, the pH or the presence of divalent cations, such as for instance Ca^{2+} or Mn^{2+}. Given the central role of LMWOA on the interaction between fungi and bacteria, these same factors might in turn affect the outcome of such interactions. Therefore, this chapter aims at identifying a fungal model with relevance to human health based on its ability to produce OA (or other relevant LMWOA) in different media and testing the impact of trophic conditions on the outcome of interaction with bacteria. By using a fast screening method based on a cultural assay and Ultra-High-Performance Liquid Chromatography analysis (UHPLC), we assessed LMWOA production in four different fungal strains, i.e. *Aspergillus niger*, *Botrytis cinerea*, *Fusarium oxysporum* and *Trichoderma rossicum*. We showed that among all fungi tested, only *A. niger* consistently produced OA (and more generally LMWOAs) in all media tested. We thus selected it as a relevant model for human health. This model was contrasted with *T. rossicum*, a fungus that did not produce LMWOAs under any of the conditions tested. We showed that the C/N ratio influence the outcome of interactions between *A. niger* and oxalotrophic (*Cupriavidus necator* and *Cupriavidus oxalaticus*) and non-oxalotrophic bacteria (*Pseudomonas putida*). The medium with a high C/N ratio medium (malt) favored fungal growth, while the low C/N ratio medium (Reasoner'2) favored bacterial growth. On the contrary, *T. rossicum* (non-oxalogenic) and all three bacterial species co-existed in all media tested. This study shows the importance of understanding the factors that influence LMWOA production by fungal pathogens, and thus shape their interactions with bacteria when developing a biocontrol approach.

3.1. Introduction

Low molecular weight organic acids (LMWOA), such as for instance oxalate, acetate or citrate, are commonly produced by plants and fungi (1, 2). An extensive study by Liaud *et al.* (3), which screened 66 filamentous fungal strains for LMWOA production, showed that different fungal strains produced a wide and diverse array of LMWOAs. In fungi, LMWOAs participate in various processes, ranging from pathogenesis, competition and mineral weathering, to heavy metal detoxification or lignocellulose degradation (4, 5). This is particularly true in the case of oxalic acid (OA). OA is one of the most common LMWOAs secreted by fungi (4, 5). Depending on the environmental pH, OA readily converts into oxalate (dianion) and forms diverse salts with divalent cations, such as Ca^{2+}, Mg^{2+}, Fe^{2+}, or Cu^{2+}. Therefore, metal-oxalate salts are widespread in the environment, with calcium oxalate (CaOx) being the most common in terrestrial ecosystems (6).

Phytopathogenic fungi, such as for instance *Sclerotinia sclerotiorum* or *Botrytis cinerea,* are known to use OA as a pathogenicity factor (7, 8). These pathogens secrete OA to acidify the host tissues and to weaken the cell wall structure (by chelating calcium ions), thus facilitating infection (4). Moreover, OA was also shown to inhibit the plant defenses and induce programmed cell death (4, 9). While the role of OA in the pathogenicity of plant fungal pathogens is well known, this is not the case for human pathogens. However, several clinical reports have shown the presence of CaOx in the lungs of animals and humans infected by *Aspergillus* spp., suggesting a potential role of OA also in the pathogenesis of opportunistic animal pathogens (10-17).

Aside its role in the pathogenicity, OA has also been shown to have a pivotal role in the interactions of fungi with bacteria (5). Indeed, OA is used as a signaling molecule by bacteria to localize their fungal partner and establish different types of trophic interactions (18-20). OA, as well as other LMWOAs such as for instance acetic acid, citric acid, or malic acid, can also be used as sole carbon and energy sources by bacteria (1). Although many bacterial species are able to metabolize most LMWOAs, OA can only be metabolized by a limited number of bacterial species, belonging to the phyla *Actinobacteria*, *Firmicutes* and *Proteobacteria*, called oxalotrophic bacteria (1, 21). Due to the low molecular weight and high oxidation state of OA, the growth yield of oxalotrophy, i.e. OA degradation, is quite low (1). Despite the stability and low solubility of metal-oxalates (for instance, the Ksp of CaOx monohydrate is 2.32×10^{-9}) (22, 23), they rarely accumulate in the environment. This has been suggested to be the result of metabolization by oxalotrophic bacteria, indicating that in spite of its theoretical low energetic yield, oxalotrophy in the environment is very active (1).

The trophic interaction between oxalogenic fungi and oxalotrophic bacteria constitutes the basis of an

important biogeochemical process called the oxalate-carbonate pathway (OCP) (24). The OCP, which involves plants, fungi and bacteria, results in the conversion of biomass into minerals (25). Plants and fungi produce oxalate as a by-product of their metabolism (photosynthesis or respiration, respectively). The decay of plant organic matter and the *in-situ* production of fungal oxalate, results in the accumulation of CaOx in soils. This soil CaOx pool is then consumed by oxalotrophic bacteria. Oxalate degradation is associated with a local pH increase (24, 26, 27), due to the conversion of a strong acid into a weaker one (oxalic acid versus carbonic acid; pKa = 1.25 and 4.14, respectively). In presence of calcium ions in the soil solution, this change in soil pH eventually leads to the precipitation of calcite ($CaCO_3$, if the pH increases above 8). The impact of this pathway on soil pH has been highlighted in acidic tropical soils (24, 25), and it is suggested that the OCP could act as a long-term terrestrial carbon sink (28).

Several abiotic factors can influence the production of OA and LMWOAs (4, 29). These factors include the available carbon and nitrogen sources (30-32), the presence of inorganic phosphorus (31), and pH (33). The production of OA is also favored by the presence of divalent cations, such as Ca^{2+} (34) or Mn^{2+}, which is a cofactor of the oxaloacetate hydrolase (OAH), one of the enzymes involved in its biosynthesis (35). Although media composition is known to influence and to trigger differential production of LMWOA (30, 31), its influence in the outcome of interacting fungi and bacteria is poorly known. Oxalate production has been shown to be affected by C/N ratio and the nitrogen sources for a wide range of Basidiomycetes (4). Moreover, a high C/N ratio has been shown to be required for a high/optimal oxalic acid production by the fungus *A. niger* (31). Additionally, a high C/N ratio has been shown to favor fungal growth, whereas a low C/N ratio has been shown to favor bacterial growth (36, 37). The aim of this study was thus to first **identify a fungal model with relevance to human health based on the ability to produce OA (or LMWOAs)** in different media, and second to **test the impact of environmental (trophic) conditions on the interaction between fungi and bacteria**. Accordingly, our hypothesis is that **trophic conditions have an effect on LMWOA production, as well as on the interaction with bacteria**. More specifically, a **high C/N ratio will favor OA production, as well as fungal growth** over bacterial growth when co-cultured together. On the contrary, a **low C/N ratio will decrease OA production and favor bacterial growth** over fungal growth. To test this hypothesis, we first assessed the production of OA and other LMWOAs by selected fungal strains, *Aspergillus niger*, *Botrytis cinerea*, *Fusarium oxysporum* and *Trichoderma rossicum*. We used two complementary approaches: a culture medium assay with a colorimetric pH-indicator, and Ultra-High-Performance Liquid Chromatography. *A. niger* was found to consistently produce OA in all media tested. As *Aspergillus* spp. are known to infect human lungs, *A. niger* was selected as fungal model with relevance to human health. In contrast, *T. rossicum* did not produce LMWOAs under any of the conditions tested. After that, we performed

confrontations assays of the selected fungal strains with two strains of oxalotrophic bacteria, *Cupriavidus oxalaticus* and *Cupriavidus necator*, and one non-oxalotrophic bacterial strain, *Pseudomonas putida*, in order to investigate the effect of these bacterial strains on the production of LMWOAs in the selected fungal strains. Finally, the outcome of bacterial-fungal interactions between *A. niger* (oxalogenic) and *T. rossicum* (non-oxalogenic), and both *Cupriavidus* strains and *P. putida*, were assessed on agar culture media differing in their nutrient content.

3.2. Materials & Methods

3.2.1. Model organisms

Based on the literature review presented in Chapter 2, we selected a series of fungal species (Table 1) for which the production of oxalic acid has been reported in *in vitro* studies (8, 35, 38). In addition, we included a fungal species for which the production of LMWOA was not reported, *T. rossicum*, as a negative control. Fungi were routinely cultured on Malt Agar medium (MA, Table 3).

Table 1. Fungal species selected to test for the production of low molecular weight organic acids (LMWOA).

Code	LAMUN Coll. N°	Species	Ecology	Origin
An	NEU M8	*Aspergillus niger*	Opportunistic animal pathogen	(39)
Bc	NEU M23	*Botrytis cinerea*	Opportunistic plant pathogen	Unknown
Fo	NEU M47	*Fusarium oxysporum* MUCL 53649 *	Opportunistic plant and animal pathogen	UCL Belgium collection
Tr	NEU M135	*Trichoderma rossicum*	Saprotrophic and mycophagous fungus	(40)

* BLAST on NCBI & UNITE databases on 14/09/2020: *Fusarium* sp. (100% identity), *Fusarium culmorum* (99.60% identity) ; *Fusarium* sp., *Fusarium culmorum*, *Gibberella zeae/Fusarium graminearum*

In order to test the interactions of the selected fungi with bacteria, we used the bacterial strains indicated in Table 2. All three model bacterial strains are tagged with a fluorescent marker gene (*gfp* or *mCherry*), which is integrated in the genomic DNA and thus expressed constitutively (Table 2). *Pseudomonas putida* KT2440 was kindly provided by Dr. Arnaud Dechesne (Technical University of Denmark). *Cupriavidus necator* JMP289 was kindly provided by Prof. Jan van der Meer (University of Lausanne). *C. oxalaticus* Ox1 was tagged in-house using insertion with a MiniTn7 system. Bacterial strains were routinely cultured on Nutrient Agar (23g NA in 1L deionized water, Carl Roth, Karlsruhe, Germany) medium. *P. putida* KT2440 is non-oxalotrophic, whereas both *Cupriavidus* strains (*C. necator* JMP289 & *C. oxalaticus* Ox1) are oxalotrophic.

Table 2. Model bacterial species used

Code	LAMUN Coll. N°	Species	Fluorescent tag	References
Pp	NEU 1264	*Pseudomonas putida* KT2440	GFP	(41)
Cn	NEU 1286	*Cupriavidus necator* JMP289	GFP	(42)
Co	NEU 1287	*Cupriavidus oxalaticus* Ox1	mCherry	(43)

3.2.2. Inoculation design

In order to assess the interactions between fungi and bacteria, confrontation assays were performed. To do so, the mycelium of the respective fungus was sampled from an agar plate using the wider end of a sterile Pasteur pipette. This agar plug was inoculated in the center of a fresh Petri dish containing the selected confrontation medium. Bacteria were refreshed on NA, and the plates were incubated overnight at 30°C. The bacterial inoculum was sampled with a sterile inoculation loop and streaked as a line on both sides of the fungal inoculum (Fig. 1).

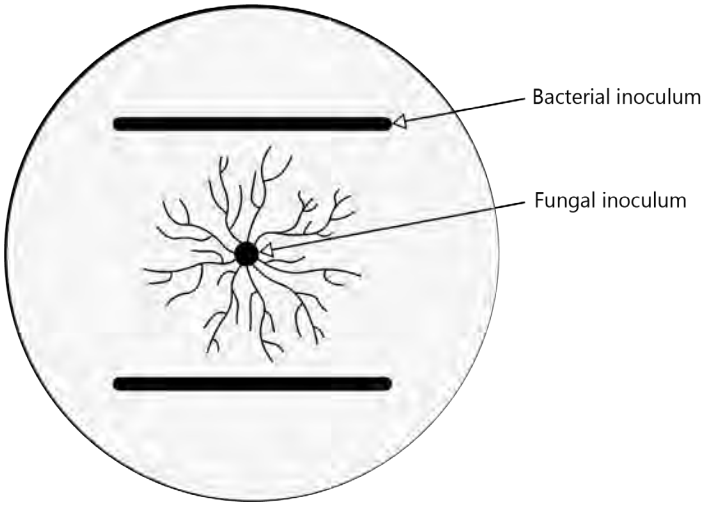


Fig. 1. Inoculation design. The fungus is inoculated in the center of the plate, and the bacteria are streaked as a line on both sides of the fungal inoculum.

3.2.3. Media used

The media used for the LMWOAs detection and the confrontation tests are presented in Table 3.

Table 3. List of media used

Medium	Composition	References
Angle + BP	Composition in **Supp. Mat.** 10 mg/L of bromocresol purple was added before autoclaving.	(44)
MA (Malt Agar)	12 g of malt extract (Sios Homebrewing GmbH, Wald, Switzerland), 15 g agar (Biolife Italiana, Milano, Italy), per liter of deionized (DI) water	
MA 1/10	1.2 g of malt extract (Sios Homebrewing GmbH, Wald, Switzerland), 15 g agar (Biolife Italiana, Milano, Italy), per liter of deionized (DI) water For liquid malt 1/10, no agar was added.	

Medium	Composition	References
R2A (Reasoner's 2 Agar)	0.5 g yeast extract, 0.5 g Bacto Peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g Na-pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \times 7H_2O$, 15 g agar, per liter of Milli-Q® water For liquid R2 medium, no agar was added.	(45)
WYA + BP (Water Yeast Agar)	1 g K_2HPO_4, 5 g NaCl, 0.1 g yeast extract, 10 mg bromocresol purple, 20 g agar, per liter of Milli-Q® water, pH 6.5.	(19)

3.2.4. LMWOAs detection by colorimetric pH indicator-based Petri dish assays

LMWOAs were detected by a colorimetric culture-based assay using WYA and Angle media supplemented with bromocresol purple (WYA + BP and Angle + BP, respectively). The selected fungal strains were inoculated at the center of a Petri dish with an agar plug sampled from an agar growth plate using the wider end of a sterile Pasteur pipette in three different plates for each culture medium. After one week of incubation at room temperature, images of Petri plates were taken, and the area corresponding to the LMWOAs secretion extent was calculated using the ImageJ software (version 2.0.0-rc-69/1.52p). The presence of typical bi-pyramidal shaped CaOx crystals in the Angle + BP cultures was assessed by observing a thin slice of agar medium sampled at the edge of the fungal colony and stained with lactophenol cotton blue with a Leica DM4 B optical microscope connected to a Leica DFC7000 T camera.

3.2.5. LMWOAs detection by Ultra-High Performance Liquid Chromatography (UHPLC)

For the detection of LMWOAs by UHPLC, fungal liquid cultures were made by inoculating an agar plug sampled from an agar growth plate using the wider end of a sterile Pasteur pipette in a 10 mL-liquid culture containing either malt 1/10 or Reasoner's 2 liquid media. The cultures were incubated at room temperature during two weeks under agitation (120 rpm). Then, three times 1mL of liquid culture (pseudoreplication) was sampled and 500 µl of 30 mM H_2SO_4 were added to each of them, in order to obtain 20 mM H_2SO_4 final concentration. The samples were incubated at 60°C for two hours in order to dissolve precipitated metal oxalate crystals, and then centrifuged at 3000 g for 10 min. All the samples were filtered at 0.22 µm (13mm syringe filters, PTFE, hydrophilic) and 200 µl were added into HPLC vials with 250 µl conical inserts. UHPLC (Ultimate 3000 RS-Dionex, Thermo Fisher Scientific, MA, USA) was coupled with DAD detector set at 210 ± 2 nm. Five µL of this sample was injected onto a Prevail™ organic acid column (5 µm particle size, 150 x 4.6 mm, Grace Davison Discovery Sciences, Deerfield, IL, USA) at a constant temperature of 40°C. The mobile phase consisted of 50 mM phosphate buffer adjusted to pH 2.5 with phosphoric acid with a flow rate of 1 mL/min. Pure oxalic acid, malic acid, acetic acid, citric acid, succinic acid, and formic acid (Merck KGaA, Darmstadt, Germany) were identified by the retention time and were quantified with an external standard curve with five calibration points (0.2 to 5 mg/mL).

3.3. Results & Discussion

3.3.1. Production of LMWOAs in different trophic conditions

Given that trophic conditions are known to influence the production of LMWOAs in fungi, we investigated the production of OA and other LMWOAs by the selected fungal strains in different culture media.

3.3.1.1. pH indicator-based cultural assay

We first conducted a culture-based assay by using two different solid culture media supplemented with the pH indicator Bromocresol purple (BP). Water Yeast Agar (WAY + BP) is a medium that has been used by Mela *et al.* (19) to test organic acid production. Angle medium has a composition similar to the soil solution in terms of ionic concentrations of nutrients (44), but the addition of BP (Angle + BP) should make it also suitable to visually assess the production of LMWOA. LMWOA production was detected in *A. niger* and *B. cinerea* in both media tested (Fig. 2A, WYA; Fig. 2B, Angle + BP), confirming previous reports using pH indicator-containing agar culture media (19, 46, 47). Moreover, crystals showing a characteristic bipyramidal shape of CaOx were visible in the cultures of *A. niger* and *B. cinerea* (Fig. 2B, insets), suggesting the presence of oxalic acid in the exudates of both strains. In contrast, *F. oxysporum* MUCL 53649 appear to produce LMWOAs solely in Angle medium (Fig. 2B). Moreover, no CaOx crystals were detected in the cultures of *F. oxysporum* MUCL 53649, suggesting this fungal strain produced other LMWOAs in these conditions. Finally, *T. rossicum* did not produce any organic acids in any of the media tested.

The higher amount of LMWOAs produced by *A. niger* in Angle medium as compared to the WYA medium (Fig. 2B vs. 2A) can be explained by the presence of glucose in the former. WYA only contains yeast extract as carbon source for fungal growth. Indeed, higher concentrations of glucose were shown to increase OA production in *A. niger* (31). This also seems to be the case for *F. oxysporum*, whereas it was not for *B. cinerea*.

3.3.1.2. UHPLC analysis

We then performed a UHPLC analysis as a complementary approach for quantifying and identifying the LMWOA produced in the selected fungal strains. Among the six different LMWOAs tested (i.e. oxalic, malic, acetic, citric, succinic, and formic acids, oxalic acid), oxalic acid was the sole LMWOA produced in large quantities by *A. niger* in both liquid media tested (malt 1/10 and Reasoner's 2) (Fig. 3). However, *A. niger* is also reported to produce citric acid (29, 35, 48-50), which was not detected in our case. Nevertheless, this is not surprising as *A. niger* is reported to produce citric acid only when the carbon

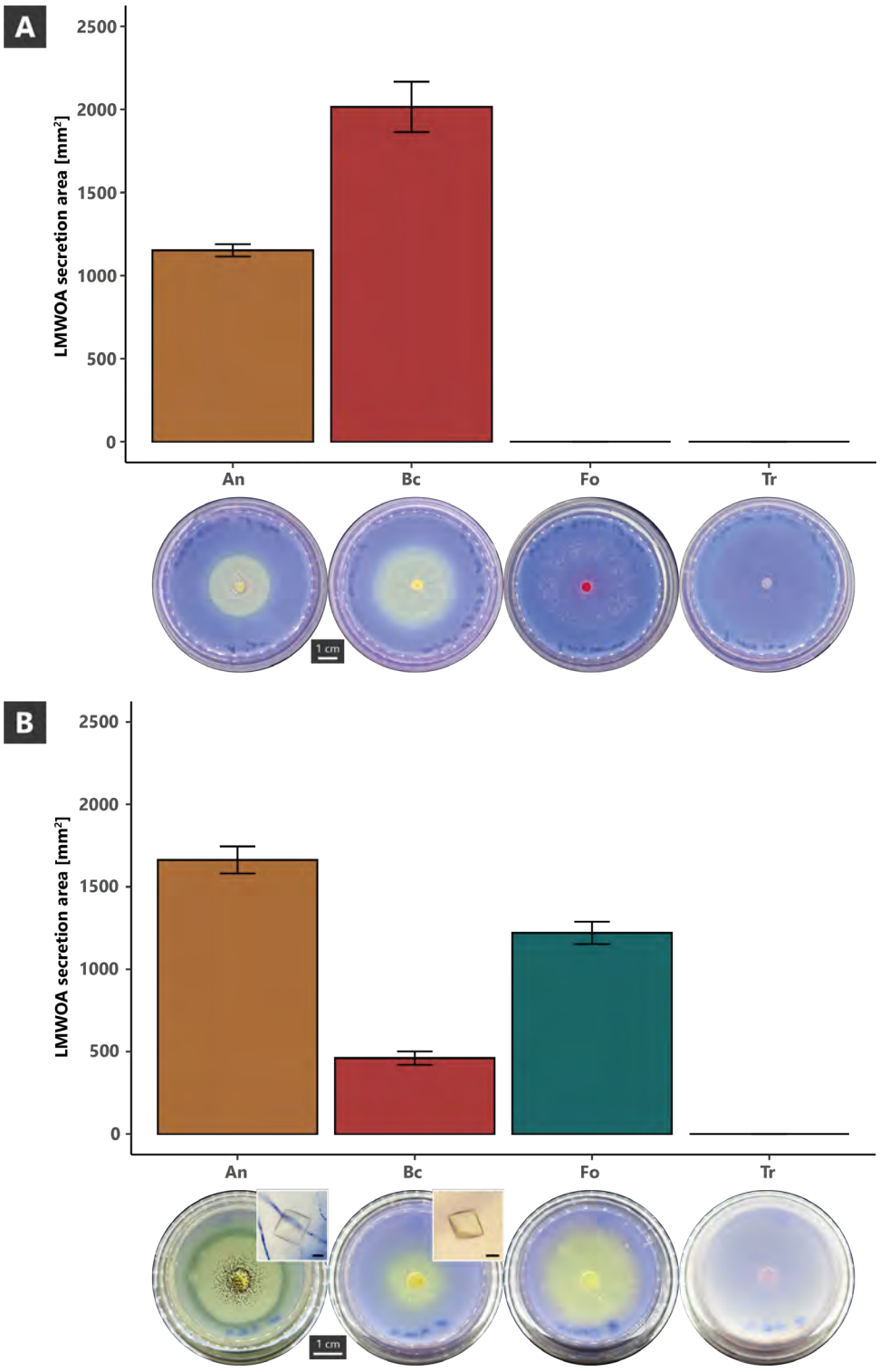


Fig. 2. Evaluation of the production of LMWOAs on nutrient agar media supplemented with bromocresol purple by the selected fungal strains. (A) WYA + BP. (B) Angle +BP. Bar graphs represent the mean LMWOA secretion area of three replicates ± SD. The images of Petri dishes were taken after 1 week of incubation. The production of LMWOA is detected by a change in the color of the medium using the pH indicator Bromocresol purple. A purple color indicates a pH above 6, yellow indicates a pH below 6. The small insets at the right upper corner of the Petri dishes in (B) show a CaOx crystal with its typical bipyramidal shape. Scale bar = 10µm. An = *A. niger*, Bc = *B. cinerea*, Fo = *F. oxysporum*, Tr = *T. rossicum*.

source is provided in excess (> 50 g/L) and the pH is under 3 (51, 52). Neither of these two conditions were tested here. In addition, citric acid production was shown to occur when the biosynthesis pathway of oxalic acid is genetically suppressed (35). Moreover, *A. niger* was shown to use citric acid as carbon source for oxalic acid production when glucose (53, 54), or sucrose (55), are no longer available in the culture medium. Consequently, the absence of citric acid in the culture medium of *A. niger* might simply be the result of timing (too late in the time-course of the culture), because of its reuse as carbon source. Temporality thus needs to be considered in the assessment of the production of LMWOAs by a given fungal strain. Another explanation could be the intra-specific variations in the LMWOAs production profile of different strains of the same species. Indeed, Liaud *et al.* (3) showed that different strains of *A. niger* have different organic acid production profiles, even though all strains were cultured in the same liquid culture medium. This could be the result of ecological adaptation of the strains to their environment of origin.

In the case of *B. cinerea*, oxalic acid, as well as large quantities of malic acid (2.47 ± 0,07 mg/L, data not shown), were detected only in Reasoner's 2 liquid medium (Fig. 3), confirming previous report (47, 56). Concerning *F. oxysporum*, no oxalic acid or any other LMWOA were detected (Fig. 3), in spite of the fact that we highlighted LMWOA production in Angle + BP (Fig. 2), and the reported ability of this fungal strain to produce oxalic acid (38). These discrepancies in the presented results might be either due to a problem during sample preparation, detection during the analysis, or to the composition of the medium. Indeed, different culture media were used for both analyses, i.e. WYA + BP and Angle + BP for the pH indicator-based cultural assay and liquid malt 1/10 and Reasoner's 2 for the UHPLC analysis, which could explain differential LMWOA production. Additionally, these differences might also be attributed to strain-specific variations in the LMWOAs production profiles. Furthermore, the acidification of the Angle + BP medium could also be due to another acidifying compound secreted by our *F. oxysporum* strain. Indeed, *F. oxysporum* and other *Fusarium* spp. are known to produce fusaric acid, a mycotoxin that has been shown to contribute to pathogenesis in plants by acting as a metal chelator, enhancing ROS (reactive oxygen species) production, and altering membrane permeability (57-60). However, we did not attempt to measure fusaric acid in this study, something for which a specific sample preparation is required (61). Finally, as expected, no OA or any other LMWOAs were detected in the case of *T. rossicum* (Fig. 3). However, this is not necessarily the case of all *Trichoderma* spp. Indeed, *T. asperellum* 302 has been shown to produce OA (20).

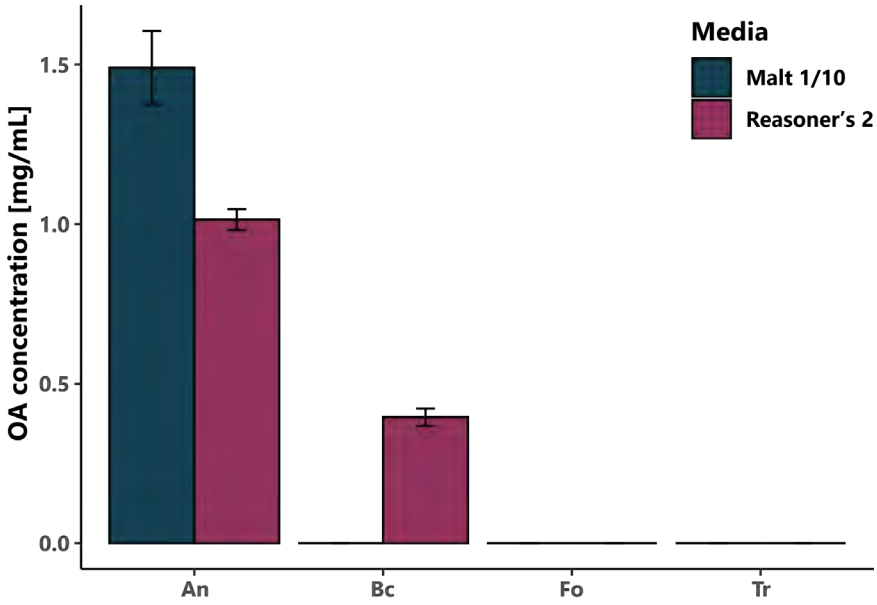


Fig. 3. Quantification of OA by UHPLC. OA was quantified in two different media (liquid malt 1/10 and Reasoner's 2) to trigger differential production depending on the nutrient conditions. Bars represent the mean of three replicates ± SD. An = *A. niger*, Bc = *B. cinerea*, Fo = *F. oxysporum*, Tr = *T. rossicum*.

We also confirmed that the C/N ratio influence the production of LMWOAs, which is in accordance with what was stated in Dutton *et al.* (4). Indeed, compared to liquid malt 1/10 medium, liquid Reasoner's 2 medium contains multiple sources of organic nitrogen (yeast extract, peptone, casamino acids), making it a nitrogen-rich medium. A higher C/N ratio (malt 1/10) seems to favor OA production in *A. niger*, whereas it seems to be the contrary in the case of *B. cinerea*, with the higher OA (and malic acid) being measured in the lower C/N ratio culture condition (Reasoner's 2).

To summarize this part, we showed that *A. niger* consistently produced LMWOAs across the culture media tested. The same was observed for *B. cinerea*, except for Malt 1/10 in which it did not produce any LMWOAs. Concerning *F. oxysporum*, our strain only produced LMWOAs in solid Angle medium. Finally, *T. rossicum* did not produce organic acids in any of the media tested.

3.3.2. Bacterial-fungal interactions and pH control by oxalotrophic bacteria

We then performed confrontation assays by co-culturing the selected fungal strains, i.e. *A. niger*, *B. cinerea*, *F. oxysporum* and *T. rossicum*, with the non-oxalotrophic bacterium *P. putida*, and both oxalotrophic strains – *C. necator* and *C. oxalaticus* – in Angle + BP in order to investigate the potential pH and growth control exerted by oxalotrophic bacteria on the fungal strains based on their ability to produce OA (Fig. 4). Both *Cupriavidus* strains seemed to exert a strong pH control when confronted with *B. cinerea*. This also seemed to be the case for *A. niger*, but to a lesser extent. Moreover, *P. putida* seemed to contribute to the acidification of the medium. In the case of *F. oxysporum*, the oxalotrophic bacterial strains do

not seem to have any effect on growth or pH of the medium. However, *P. putida* appears to have a stimulatory effect on *F. oxysporum* growth, as it seems to have colonized the entire plate. Moreover, *P. putida* seems to stimulate the production of LMWOAs by *F. oxysporum*, as almost the entire plate turned acidic (yellow).

After that, we performed confrontations of *A. niger* with the three bacteria strains on WYA + BP (Fig. 5). We confirmed the ability of oxalotrophic bacterial strains to control the pH of the medium, and thus LMWOA production, as well as to control the growth of *A. niger*, whereas *P. putida* does not. This phenotype of pH

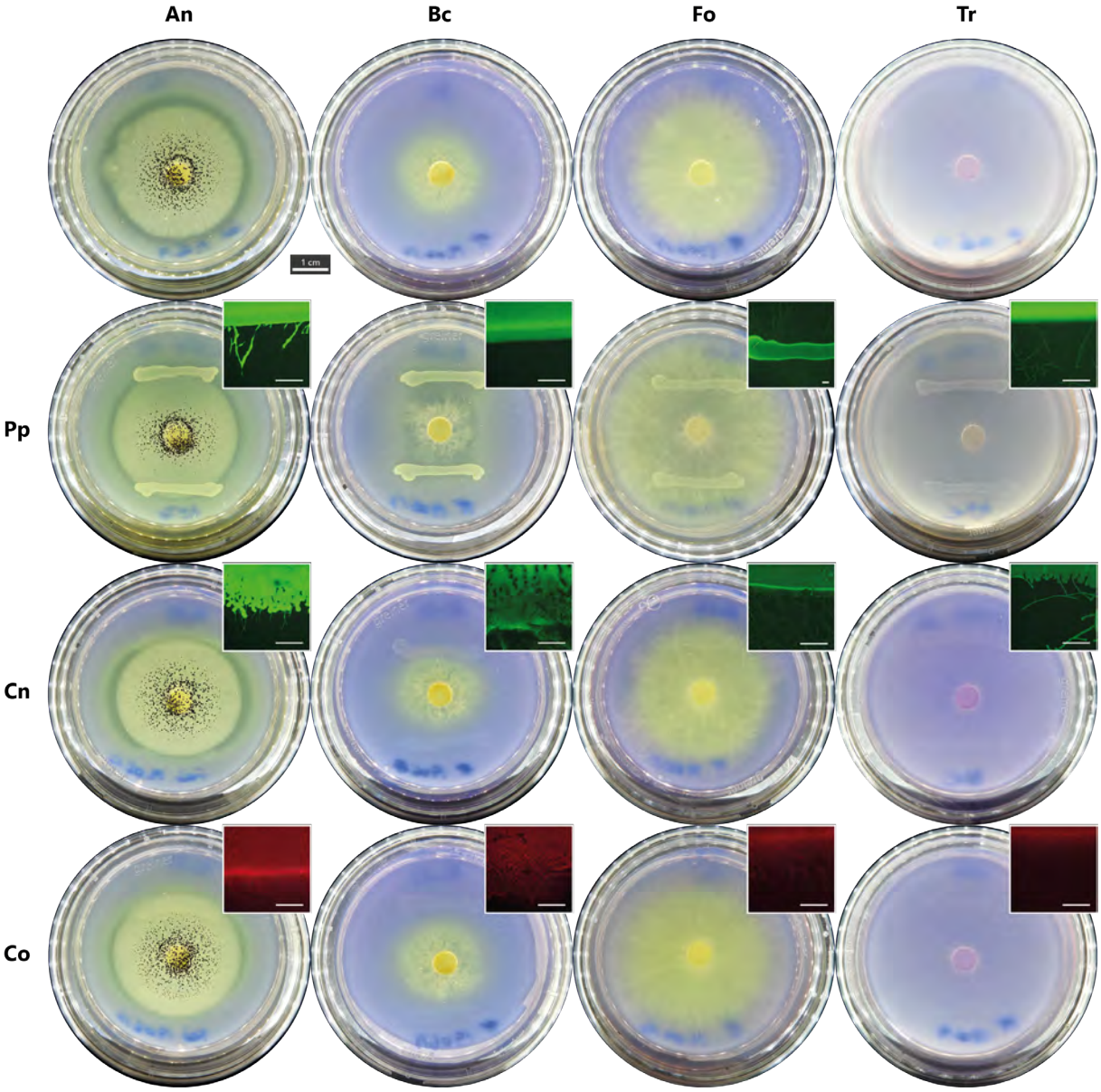


Fig. 4. Bacterial-fungal confrontation co-cultures between the selected fungal strains and bacteria. Pictures of the different fungal strains – i.e. *A. niger* (An), *B. cinerea* (Bc), *F. oxysporum* (Fo) and *T. rossicum* (Tr) – cultured on Angle + BP, in co-cultures with the selected bacterial strains – i.e. *P. putida* (Pp), *C. necator* (Cn) and *C. oxalaticus* (Co) – after 1 week of incubation. Bacterial inocula correspond to two 2µl-drops of bacterial suspension in physiological water (9g/L NaCl) at an OD_{550} of 0.6 (approx. 10^9 bacteria per mL). The inserts show the fluorescence of the bacterial strains. Scale bar = 500µm, except for Fo + Pp and Fo + Cn where it is 1000µm. The production of LMWOAs is detected by a change in the color of the medium using the pH indicator Bromocresol purple. A purple color indicates a pH above 6, yellow indicates a pH below 6.

control observed in confrontation cultures between *A. niger* and the oxalotrophic bacteria *C. necator* and *C. oxalaticus* on WYA + BP confirmed a previous study by Mela *et al.* (19) who reported pH and growth control of *A. niger* by *Collimonas fungivorans*, an oxalate-degrading bacterium in confrontation assay on WYA + BP. A transcriptomic analysis revealed that oxalate-degradation-related genes were upregulated when *C. fungivorans* was co-cultured in confrontation with *A. niger* (19). However, this bacterial species was shown to be unable to grow on oxalate (18, 19). Transcriptomics analyses would be necessary in order to investigate the expression of oxalotrophy-related genes in *C. necator* and *C. oxalaticus* in the co-cultures with *A. niger*. Oxalotrophic bacteria are known to control the growth of *B. cinerea* and thus confer protection to *Arabidopsis thaliana* (8, 62). However, to our knowledge, no such antagonistic interactions have been reported between *F. oxysporum* and oxalotrophic bacteria. Finally, *T. rossicum* has been shown to interact positively with oxalotrophic bacteria by letting them disperse on its fungal mycelium in order to access nutrients such as CaOx in soils (40).

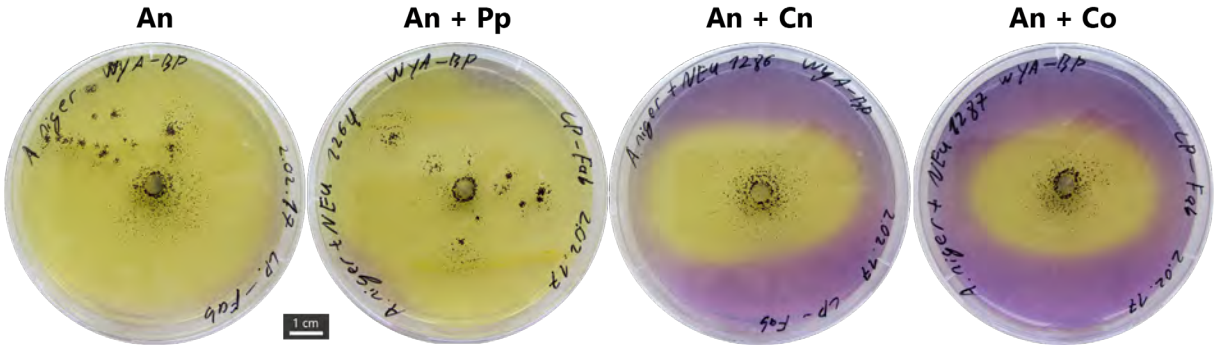


Fig. 5. Co-culture of *A. niger* with *P. putida*, *C. necator* and *C. oxalaticus* on WYA + BP. Pictures were taken after 1 week of incubation. When co-cultured with *A. niger*, both *Cupriavidus* strains seem to control pH and thus LMWOA production, whereas *P. putida* does not show any effect on the pH of the medium. An = *A. niger*, Pp = *P. putida*, Cn = *C. necator*, Co = *C. oxalaticus*.

3.3.3. Bacterial-Fungal interactions in different trophic conditions: interaction of an oxalogenic and a non-oxalogenic fungus with oxalotrophic and non-oxalotrophic bacteria

As shown above by the UHPLC analysis, trophic conditions trigger differential LMWOA production, with a medium with a higher C/N ratio (malt 1/10) inducing more oxalic acid production than a lower C/N ratio medium (Reasoner's 2). Moreover, we also showed that oxalotrophic bacteria (*C. necator* and *C. oxalaticus*) were able to control fungal growth and the pH of the medium when co-cultured with *A. niger* (oxalogenic fungus), while no specific fungal growth or pH control occurred when oxalotrophic bacteria were co-cultured with *T. rossicum* (non-oxalogenic fungus). We thus performed confrontation assays between *A. niger* or *T. rossicum*, and *P. putida* (non-oxalotrophic), *C. necator*, or *C. oxalaticus* (both oxalotrophic) on three culture differing in their nutrient content.

On MA, *A. niger* seemed to outcompete all three bacterial strains tested (conidia production visible on the bacterial inoculum). This is visible in the inserts, which appear black (no fluorescence), which suggests all three bacteria did not survive the interaction. On MA 1/10, *P. putida* is outcompeted by the fungus, whereas both *Cupriavidus* strains limit sporulation. On R2A however, all three bacteria strains seemed to outcompete the fungus, with *C. oxalaticus* having a stronger effect on limiting the growth of *A. niger* (Fig. 6). For *T. rossicum*, the three bacterial strains tested did not seem to have any visible effect on fungal growth in either of the media tested (Fig. 7). These results suggest that the outcome of the interaction depends on the nutrient content of the culture medium, and on the ability of the fungus to produce LMWOA. However, as explained above R2A is rather a low C/N ratio medium, as it contains multiple sources of organic nitrogen. In the case of MA 1/10, the C/N ratio should be similar to the one of MA, but the differences observed in the outcome of interaction could be explained by the lower quantity of nutrients compared to the full-strength agar medium (MA). Indeed, Leite *et al.* (37) showed that high C/N ratio favors fungi, whereas low C/N ratio favors bacteria. However, more strains need to be tested in order to affirm that.

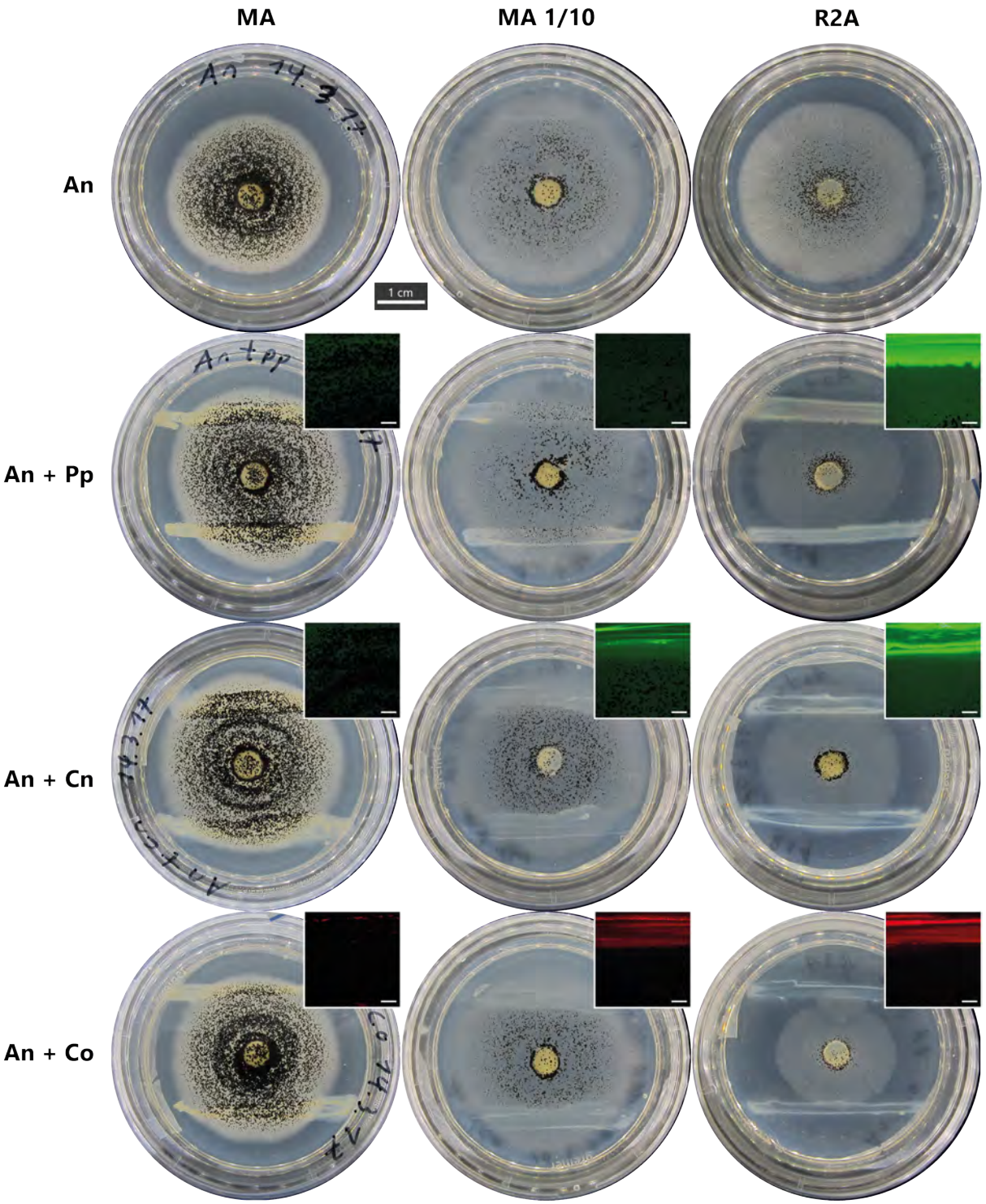


Fig. 6. Interaction between *A. niger* and the oxalotrophic and non-oxalotrophic bacteria on different culture media. Pictures of the plates were taken after 1 week of incubation. The inserts show the fluorescence of the bacterial strains. Scale bar = 2000µm. On MA, *A. niger* seem to outcompete all three bacteria tested. Indeed, the lack of fluorescence confirms bacterial death. On MA 1/10, *P. putida* is outcompeted by the fungus, whereas both *Cupriavidus* strains limit its sporulation. On R2A, all three bacteria tested seem to outcompete the fungus, with *C. oxalaticus* having a stronger effect on limiting the growth of *A. niger*. An = *A. niger*, Pp = *P. putida*, Cn = *C. necator*, Co = *C. oxalaticus*.

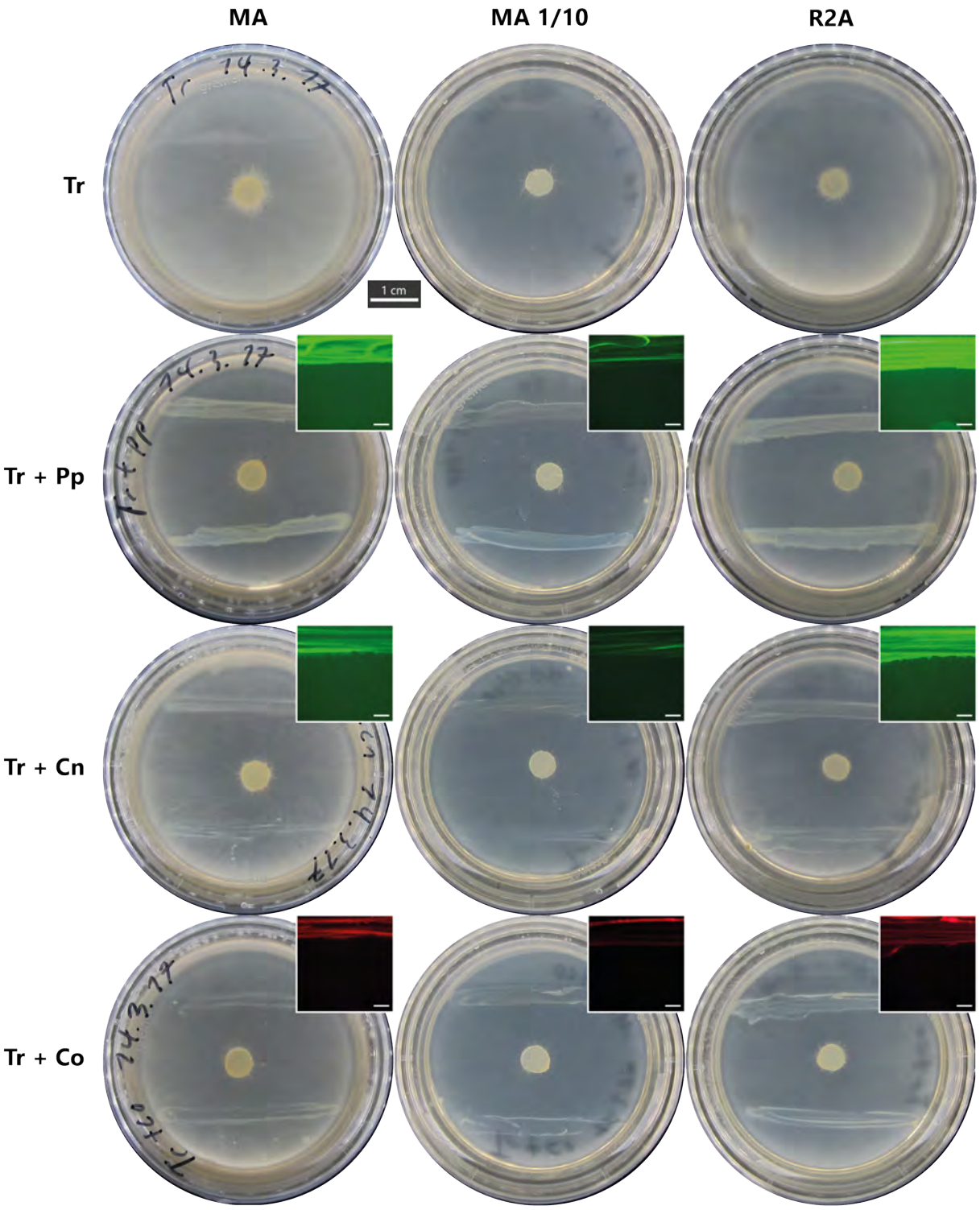


Fig. 7. Interaction between T. rossicum and the oxalotrophic and non-oxalotrophic bacteria on different culture media. Pictures of the plates were taken after 1 week of incubation. The inserts show the fluorescence of the bacterial strains. Scale bar = 2000µm. All three bacteria tested do not seem to have any visible effect on fungal growth in either of the media tested. Tr = *T. rossicum*, Pp = *P. putida*, Cn = *C. necator*, Co = *C. oxalaticus*.

3.4. Conclusions

This preliminary chapter aimed at identifying a fungal model with relevance to human health based on the ability to produce oxalic acid (or LMWOAs) in different media, and to test the impact of environmental (trophic) conditions on the interaction between fungi and bacteria. First, we showed that *A. niger* consistently produced LMWOAs in all the media tested and produced significant amounts of oxalic acid in liquid malt 1/10 and R2. We thus selected *A. niger* as fungal model relevant for human health. Indeed, fungi from the genus *Aspergillus* are known to cause pulmonary aspergillosis in immunocompromised patients. *F. oxysporum* would have been another potential candidate, however, despite its reported ability to produce oxalic acid, we did not detect oxalic acid, or other LMWOAs in any of the media used. We could also see that oxalic acid production (and more generally total LMWOA), depend on the trophic conditions, i.e., medium composition. However, we could not identify the exact component influencing the production of oxalic acid in the fungal strains we tested. Indeed, a more systematic experimental design with the use of a mineral minimal medium allowing the individual control of all components, such as the carbon and nitrogen sources, as well as the initial pH, would have been necessary in order to test the influence of different medium components on the LMWOA production profile by the selected fungal strains. Moreover, we could also show the effect of oxalotrophic bacteria on organic acid production of *A. niger*, confirming a previous report. Furthermore, we also showed the effect of trophic conditions on the interaction of fungi, i.e. oxalogenic as *A. niger*, and non-oxalogenic as *T. rossicum*, with oxalotrophic (*C. necator* & *C. oxalaticus*), and non-oxalotrophic (*P. putida*) bacteria. More specifically, in the case of the oxalogenic fungus *A. niger*, high C/N ratio medium, such as in MA, was shown to favor fungal growth over bacterial growth, while low C/N ratio medium (R2A), was shown to favor bacteria over fungi. In the intermediate C/N ratio medium, i.e. MA 1/10, oxalotrophic bacterial growth was favored over the fungus, while fungal growth was favored over the non-oxalotrophic bacterium. In the case of the non-oxalogenic fungus *T. rossicum*, interactions with the three bacteria tested were beneficial in all three media tested. These differences in outcome of the interactions of *A. niger* with bacteria might be due to differential production of oxalic acid in the three different media tested. These findings highlight the importance of understanding and choosing the conditions in which the bacteria are favored over the pathogenic fungal strain when it comes to the design of a biocontrol model system. However, all these hypotheses have to be confirmed in further experiments, where the pH, as well as the exact profile and quantity of LMWOAs, are quantified in all media, in the presence or not of the different bacterial strains. In addition, transcriptomics experiments are needed in order to understand the mechanistic basis of these interactions. To conclude, understanding the factors that shape bacterial-fungal interactions and co-evolution in soils is key for the development of a biocontrol strategy aiming to limit the growth of a fungal pathogen.

Acknowledgments

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References

1. Braissant O, Verrecchia EP, Aragno M. Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? Naturwissenschaften. 2002;89(8):366-70.

2. Adeleke R, Nwangburuka C, Oboirien B. Origins, roles and fate of organic acids in soils: A review. South African Journal of Botany. 2017;108:393-406.

3. Liaud N, Giniés C, Navarro D, Fabre N, Crapart S, Gimbert IH, et al. Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi. Fungal Biology and Biotechnology. 2014;1(1).

4. Dutton MV, Evans CS. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology. 1996;42(9):881-95.

5. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. In: Gadd GM, Sariaslani S, editors. 106: Academic Press; 2019. p. 49-77.

6. Gadd GM. Fungal Production of Citric and Oxalic Acid: Importance in Metal Speciation, Physiology and Biogeochemical Processes. Advances in Microbial Physiology. 1999;41:47-92.

7. Cessna SG, Sears VE, Dickman MB, Low PS. Oxalic acid, a pathogenicity factor for Sclerotinia sclerotiorum, suppresses the oxidative burst of the host plant. Plant Cell. 2000;12(11):2191-9.

8. Schoonbeek H-j, Jacquat-Bovet A-C, Mascher F, Métraux J-P. Oxalate-degrading bacteria can protect Arabidopsis thaliana and crop plants against Botrytis cinerea. Molecular plant-microbe interactions. 2007;20(12):1535-44.

9. de Oliveira Ceita G, Macêdo JNA, Santos TB, Alemanno L, da Silva Gesteira A, Micheli F, et al. Involvement of calcium oxalate degradation during programmed cell death in Theobroma cacao tissues triggered by the hemibiotrophic fungus Moniliophthora perniciosa. Plant Science. 2007;173(2):106-17.

10. Yi Y, Cho SY, Lee DG, Jung JI, Park YJ, Lee KY. Invasive Pulmonary Aspergillosis Due to Aspergillus awamori: Role of Calcium Oxalate Crystal Precipitation Mimicking Mucormycosis. 2020. p. 409-11.

11. Ghio AJ, Peterseim DS, Roggli VL, Piantadosi CA. Pulmonary oxalate deposition associated with Aspergillus niger infection. An oxidant hypothesis of toxicity. Am Rev Respir Dis. 1992;145(6):1499-502.

12. Kauffman CA, Wilson KH, Schwartz DB. Necrotizing Pulmonary Aspergillosis with Oxalosis: Nekrotisierende pulmonale Aspergillose mit Oxalose. Mycoses. 1984;27(11):535-8.

13. Kurrein F, Path FRC, Green GH, Rowles SL. Localized deposition of calcium oxalate around a pulmonary Aspergillus niger fungus ball. American Journal of Clinical Pathology. 1975;64(4):556-63.

14. Maeno T, Sasaki M, Shibue Y, Mimura K, Oka H. Calcium oxalate in the sputum may aid in the diagnosis of pulmonary aspergillosis: A report of two cases. Medical Mycology Case Reports. 2015;8:32-6.

15. Muntz FHA. Oxalate-producing pulmonary aspergillosis in an alpaca. Veterinary Pathology. 1999;36(6):631-2.

16. Pabuççuoğlu U. Aspects of oxalosis associated with aspergillosis in pathology specimens. Pathology Research and Practice. 2005;201(5):363-8.

17. Payne CL, Dark MJ, Conway JA, Farina LL. A retrospective study of the prevalence of calcium oxalate crystals in veterinary Aspergillus cases. Journal of Veterinary Diagnostic Investigation. 2017;29(1):51-8.

18. Rudnick MB, van Veen JA, de Boer W. Oxalic acid: A signal molecule for fungus-feeding bacteria of the genus Collimonas? Environmental Microbiology Reports. 2015;7(5):709-14.

19. Mela F, Fritsche K, de Boer W, van Veen JA, de Graaff LH, van den Berg M, et al. Dual transcriptional profiling of a bacterial/fungal confrontation: Collimonas fungivorans versus Aspergillus niger. ISME Journal. 2011;5(9):1494-504.

20. Haq IU, Zwahlen RD, Yang P, van Elsas JD. The Response of Paraburkholderia terrae Strains to Two Soil Fungi and the Potential Role of Oxalate. Frontiers in Microbiology. 2018;9(989).

21. Hervé V, Junier T, Bindschedler S, Verrecchia E, Junier P. Diversity and ecology of oxalotrophic bacteria. World Journal of Microbiology and Biotechnology. 2016;32(2):1-7.

22. Certini G, Corti G, Ugolini FC. Vertical trends of oxalate concentration in two soils under Abies alba from Tuscany (Italy). Journal of Plant Nutrition and Soil Science. 2000;163(2):173-7.

23. Hofmann BA, Bernasconi SM. Review of occurrences and carbon isotope geochemistry of oxalate minerals: implications for the origin and fate of oxalate in diagenetic and hydrothermal fluids. Chemical Geology. 1998;149(1-2):127-46.

24. Martin G, Guggiari M, Bravo D, Zopfi J, Cailleau G, Aragno M, et al. Fungi, bacteria and soil pH: The oxalate-carbonate pathway as a model for metabolic interaction. Environmental Microbiology. 2012;14(11):2960-70.

25. Cailleau G, Braissant O, Verrecchia EP. Turning sunlight into stone: The oxalate-carbonate pathway in a tropical tree ecosystem. Biogeosciences. 2011;8(7):1755-67.

26. Braissant O, Cailleau G, Aragno M, Verrecchia EP. Biologically induced mineralization in the tree Milicia excelsa (Moraceae): its causes and consequences to the environment. Geobiology. 2004;2(1):59-66.

27. Jayasuriya GC. The isolation and characteristics of an oxalate-decomposing organism. Journal of general microbiology. 1955;12(3):419-28.

28. Cailleau G, Mota M, Bindschedler S, Junier P, Verrecchia EP. Detection of active oxalate-carbonate pathway ecosystems in the Amazon Basin: Global implications of a natural potential C sink. Catena. 2014;116:132-41.

29. Plassard C, Fransson P. Regulation of low-molecular weight organic acid production in fungi. 2009. p. 30-9.

30. Punja ZK, Jenkins SF. Influence of Medium Composition on Mycelial Growth and Oxalic Acid Production in Sclerotium rolfsii. Mycologia. 1984;76(5):947-50.

31. Gharieb MM. Nutritional effects on oxalic acid production and solubilization of gypsum by Aspergillus niger. Mycological Research. 2000;104(5):550-6.

32. Brunner I, Goren A, Schlumpf A. Patterns of organic acids exuded by pioneering fungi from a glacier forefield are affected by carbohydrate sources. Environmental Research Letters. 2014;9(2).

33. Vrabl P, Fuchs V, Pichler B, Schinagl CW, Burgstaller W. Organic Acid Excretion in Penicillium ochrochloron Increases with Ambient pH. Front Microbiol. 2012;3:121.

34. Guggiari M, Bloque R, Aragno M, Verrecchia E, Job D, Junier P. Experimental calcium-oxalate crystal production and dissolution by selected wood-rot fungi. International Biodeterioration & Biodegradation. 2011;65(6):803-9.

35. Ruijter GJG, van de Vondervoort PJI, Visser J. Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology. 1999;145(9):2569-76.

36. de Vries FT, Hoffland E, van Eekeren N, Brussaard L, Bloem J. Fungal/bacterial ratios in grasslands with contrasting nitrogen management. Soil Biology and Biochemistry. 2006;38(8):2092-103.

37. Leite MFA, Pan Y, Bloem J, Berge HT, Kuramae EE. Organic nitrogen rearranges both structure and activity of the soil-borne microbial seedbank. Scientific Reports. 2017;7(1):42634-.

38. Swain MR, Ray RC. Oxalic acid production by Fusarium oxysporum Schlecht and Botryodiplodia theobromae Pat., post-harvest fungal pathogens of yams (Dioscorea rotundata L.) and detoxification by Bacillus subtilis CM1 isolated from culturable cowdung microflora. Archives Of Phytopathology And Plant Protection. 2009;42(7):666-75.

39. Imeria Ferro K. The impact of oxalogenic plants on soil carbon dynamics - formation of a millennium carbon storage as calcium carbonate. Neuchâtel: University of Neuchâtel; 2012.

40. Bravo D, Cailleau G, Bindschedler S, Simon A, Job D, Verrecchia E, et al. Isolation of oxalotrophic bacteria able to disperse on fungal mycelium. FEMS Microbiology Letters. 2013;348(2):157-66.

41. Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VAP, et al. Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440. Environmental Microbiology. 2002;4(12):799-808.

42. Sentchilo V, Czechowska K, Pradervand N, Minoia M, Miyazaki R, Van Der Meer JR. Intracellular excision and reintegration dynamics of the ICEclc genomic island of Pseudomonas knackmussii sp. strain B13. Molecular Microbiology. 2009;72(5):1293-306.

43. Şahin N, Işik K, Tamer AÜ, Goodfellow M. Taxonomic position of 'Pseudomonas oxalaticus' strain Ox1(T) (DSM 110(T)) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia oxalatica comb, nov. Systematic and Applied Microbiology. 2000;23(2):206-9.

44. Angle JS, McGrath SP, Chaney RL. New Culture Medium Containing Ionic Concentrations of Nutrients Similar to Concentrations Found in the Soil Solution. Applied and Environmental Microbiology. 1991;57(12):3674.

45. Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology. 1985;49(1):1-7.

46. Foster JW, Davis H. Detection and Occurrence of Acid-Producing Fungi. Bulletin of the Torrey Botanical Club. 1949;76(3):174-6.

47. Yin Y, Wu S, Chui C, Ma T, Jiang H, Hahn M, et al. The MAPK kinase BcMkk1 suppresses oxalic acid biosynthesis via impeding phosphorylation of BcRim15 by BcSch9 in Botrytis cinerea. PLoS Pathog. 2018;14(9):e1007285.

48. Show PL, Oladele KO, Siew QY, Aziz Zakry FA, Lan JCW, Ling TC. Overview of citric acid production from Aspergillus niger. 2015. p. 271-83.

49. Sturm EV, Frank-Kamenetskaya O, Vlasov D, Zelenskaya M, Sazanova K, Rusakov A, et al. Crystallization of calcium oxalate hydrates by interaction of calcite marble with fungus Aspergillus Niger. American Mineralogist. 2015;100(11-12):2559-65.

50. Li Z, Bai T, Dai L, Wang F, Tao J, Meng S, et al. A study of organic acid production in contrasts between two phosphate solubilizing fungi: Penicillium oxalicum and Aspergillus niger. Sci Rep. 2016;6:25313.

51. Karaffa L, Kubicek CP. Aspergillus niger citric acid accumulation: do we understand this well working black box? Appl Microbiol Biotechnol. 2003;61(3):189-96.

52. Kobayashi K, Hattori T, Honda Y, Kirimura K. Oxalic acid production by citric acid-producing Aspergillus niger overexpressing the oxaloacetate hydrolase gene oahA. J Ind Microbiol Biotechnol. 2014;41(5):749-56.

53. Müller HM. Oxalate accumulation from citrate by Aspergillus niger - I. Biosynthesis of oxalate from its ultimate precursor. Archives of Microbiology. 1975;103(1):185-9.

54. Müller HM, Frosch S. Oxalate accumulation from citrate by Aspergillus niger - II. Involvement of the tricarboxylic acid cycle. Archives of Microbiology. 1975;104(1):159-62.

55. Walaszczyk E, Podgorski W, Janczar-Smuga M, Dymarska E. Effect of medium pH on chemical selectivity of oxalic acid biosynthesis by Aspergillus niger W78C in submerged batch cultures with sucrose as a carbon source. Chem Zvesti. 2018;72(5):1089-93.

56. Verhoeff K, Leeman M, Peer R, Posthuma L, Schot N, Eijk GW. Changes in pH and the Production of Organic Acids During Colonization of Tomato Petioles by Botrytis cinerea. Journal of Phytopathology. 1988;122(4):327-36.

57. Bacon CW, Porter JK, Norred WP, Leslie JF. Production of fusaric acid by Fusarium species. Applied and Environmental Microbiology. 1996;62(11):4039-43.

58. Venter SL, Steyn PJ, Steyn HSF. Production of fusaric acid by Fusarium oxysporum. Potato Research. 1996;39(1):79-83.

59. López-Díaz C, Rahjoo V, Sulyok M, Ghionna V, Martín-Vicente A, Capilla J, et al. Fusaric acid contributes to virulence of Fusarium oxysporum on plant and mammalian hosts. Molecular Plant Pathology. 2018;19(2):440-53.

60. Simonetti E, Roberts IN, Montecchia MS, Gutierrez-Boem FH, Gomez FM, Ruiz JA. A novel Burkholderia ambifaria strain able to degrade the mycotoxin fusaric acid and to inhibit Fusarium spp. growth. Microbiol Res. 2018;206:50-9.

61. Gapillout I, Milat ML, Blein JP. Effects of fusaric acid on cells from tomato cultivars resistant or susceptible toFusarium oxysporum f. sp.Lycopersici. European Journal of Plant Pathology. 1996;102(2):127-32.

62. Marina M, Romero FM, Villarreal NM, Medina AJ, Gárriz A, Rossi FR, et al. Mechanisms of plant protection against two oxalate-producing fungal pathogens by oxalotrophic strains of Stenotrophomonas spp. Plant Molecular Biology. 2019;100(6):659-74.

Supplementary Material

Angle medium

Modified from Angle et. al, 1991

Modifications:

- Preparation of a 10x concentrated stock solution.
- Replacement of yeast extract by vitamin solution SV-1.

Angle stock solution 10x

Tris-HCl 0.5M pH 7	400 mL
Fe-EDTA solution	1.1 mL
Trace element solution SL-6 10x	10 mL
KH_2PO_4 0.1% w/v	6.8 mL
KOH 1M	5 mL
NH_4NO_3	2 g
$CaSO_4 \cdot 2H_2O$	8.725 g
$MgCl_2$	4.06 g
Milli-Q® H_2O	ad 1000 mL

Keep at 4°C.

Note: Stir well before using!

Tris-HCl 0.5M pH 7

Tris	60.57 g
Milli-Q® H_2O	900 mL

Adjust to pH 7 by adding approx. 17 mL HCl concentrated and then continue drop by drop.
Add Milli-Q® H_2O ad 1000 mL. Adjust to pH 7 with HCl.

Fe-EDTA solution

Na_2EDTA	14 mg
$FeSO_4 \cdot 7H_2O$	50 mg
H_2SO_4 concentrated	5 µl
Milli-Q® H_2O	10 mL

KH_2PO_4 0.1%

KH_2PO_4	0.1 g
Milli-Q® H_2O	100 mL

KOH 1M

KOH	2.8 g
Milli-Q® H_2O	50 mL

Trace element solution SL-6 10x (modified from Aragno et Schlegel, 1992)

$ZnSO_4 \cdot 7H_2O$	100 mg
$MnCl_2 \cdot 4\ H_2O$	30 mg
H_3BO_3	300 mg
$CoCl_2 \cdot 6H_2O$	200 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$NiCl_2 \cdot 6H_2O$	20 mg
$Na_2MoO_4 \cdot 2H_2O$	30 mg
Milli-Q® H_2O	qsp. 1 L

Adjust to pH 3-4.

***Vitamin solution SV-1* (Aragno's recipe)**

Biotine (Vit. H)	0.2 mg
Acide nicotinique (Vit. B)	2 mg
Thiamine (Vit. B1)	1 mg
p-aminobenzoate (Vit. Bx)	1 mg
Ca-panthotenate (Vit. B5)	0.5 mg
Pyridoxine (Vit. B6)	5 mg
Cyanocobalamine (Vit. B12)	1 mg
Acide folique	1 mg
Riboflavine (Vit. B2)	5 mg
Milli-Q® H_2O	qsp. 100 mL

Filter-sterilize through 0.2 µm. Keep at 4°C.

12.5% glucose solution

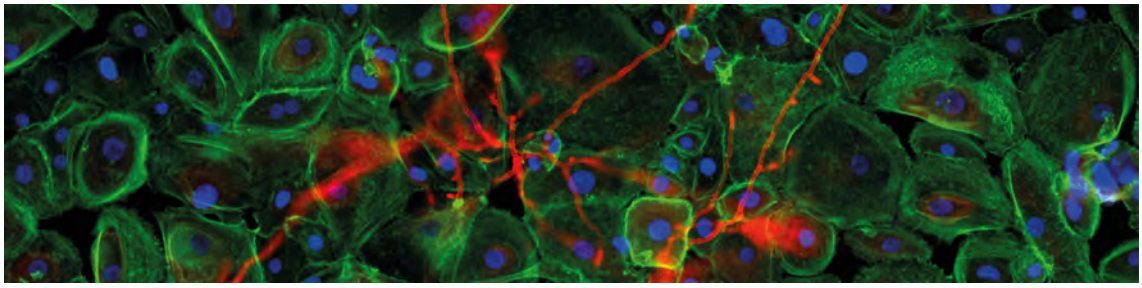
Glucose	12.5 g
Milli-Q® H_2O	100 ml

Filter-sterilize through 0.2 µm. Keep at 4°C.

Preparation of the Angle media

For 1000 mL of Angle medium, take 100 mL of Angle stock solution 10x and add Milli-Q® H2O ad 991 mL. Adjust to pH 7. Autoclave. Before pouring the medium, add 1/1000 of filter-sterilized vitamin solution SV-1 (1 mL/L), and 8 mL 12.5% glucose solution.

CHAPTER 4



Biocontrol of *Aspergillus niger* in 3D-lung cell tissues by oxalotrophic bacteria

Foreword

In this chapter, we assess the biocontrol potential of oxalotrophic bacteria over *A. niger* in *in-vitro* Petri dishes and 3D-lung cell tissues and we demonstrate for the first time the principle of environmental interference by which oxalotrophic bacteria interfere with the pH manipulation exerted by *A. niger* through oxalic acid production by consuming oxalic acid.

The key findings of this chapter are:

- Infection of 3D-bronchial cell tissues (Transwell® and bronchioles-on-a-chip) by *A. niger* led to changes in three key environmental parameters: we observed a significant drop in pH that was accompanied by a drop in soluble calcium and soluble oxalic acid concentrations. We also observed the presence of calcium oxalate crystals, most probably explaining the drop in calcium and oxalic acid concentrations observed due to the sequestration of calcium ions by oxalic acid. Moreover, infection by *A. niger* also induced a strong cytopathic effect resulting in the complete destruction of the bronchial tissues.

- pH, calcium and oxalic acid levels were statistically indistinguishable from cells alone or cells + bacteria controls when *C. oxalaticus* was co-inoculated with *A. niger* conidia, indicating most probably inhibition of *A. niger* conidia germination. Moreover, calcium oxalate crystals were not visible anymore when cells were co-inoculated with *C. oxalaticus* and *A. niger*.

4. Biocontrol of *Aspergillus niger* in 3D-lung cell tissues by oxalotrophic bacteria

Fabio Palmieri[1], Ilona Palmieri[1], Nourine Noormamode[1], Aislinn Estoppey[1], M. Omar Ishak[2], Julia M. Kelliher[2], Armelle Vallat[3], Rashi Iyer[4], Saskia Bindschedler[1], Karen Davenport[2], Patrick S. G. Chain[2], Jennifer Foster Harris[2], Pilar Junier[1].

[1] Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland. [2] Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA. [3] Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Neuchâtel, Switzerland. [4] Analytics, Intelligence and Technology Division, Los Alamos National Laboratory, Los Alamos, NM, USA.

Abstract

Aspergillus fungi are opportunistic pathogens that affect a large number of people worldwide. Many aspects of *Aspergillus* spp. pathogenesis toward humans are known, but their ability to enhance their infectious potential by manipulating the environmental pH of its host has not been considered yet. In this study, we tested the hypothesis that by producing oxalic acid, *Aspergillus niger* can manipulate pH during lung infection and thus, interfering with this process could limit pathogenicity. To test this hypothesis, we co-cultured *A. niger* with oxalotrophic bacteria in increasingly complex testing systems (Petri dishes and 3D-cell cultures systems). In *in vitro* tests, oxalotrophic bacteria limit oxalic acid production and suppressed the pH shift induced by *A. niger*. In 3D-cell cultures (Transwells® and Bronchioles-on-a-chip), *A. niger* also modified pH, Ca^{2+} and oxalic acid concentrations. Co-inoculation with as little as 10 cells of the oxalatrophic bacterium strongly inhibited the germination and development of *A. niger* and returned each of the three parameters to the baseline physiological values of uninfected cells. This biocontrol interaction between oxalotrophic bacteria and oxalate-producing *A. niger* could represent a paradigm shift in the fight against opportunistic fungal pathogens, where the host environment is rendered less permissive to fungal development.

4.1. Introduction

Fungal diseases are estimated to kill more than 1.5 million people every year (1, 2). Over the last three decades, the increase in the number of at-risk individuals has correlated with an intensification in the burden of fungal disease on human health (3). As the size of at-risk populations (e.g. immunosuppressed patients) is expected to keep increasing in the future (2), tackling fungal pathogenesis is urgent. However, only a very limited number of antifungal drugs are used nowadays to control fungal pathogens. Because of this restricted chemical arsenal, the same classes of molecules are used in human health, animal husbandry, and agriculture, leading to the rise, and rapid spread, of resistance in fungal pathogens that can affect both plant and animal hosts (4). In spite of this, tackling fungal diseases has been largely neglected up to now (5).

The most prevalent fungal pathogens affecting humans are airborne opportunists such as *Aspergillus* spp., *Cryptococcus* spp., *Pneumocystis* spp., and human-associated commensals like *Candida albicans* (6, 7). These fungal species are responsible for approximately 90% of the deaths due to fungal infection (1). The ecology of all these organisms plays a very significant role in their ability to transition to pathogenic lifestyles. For instance, the remarkable plasticity in the ecology and stress-response of *Aspergillus* spp. is believed to form the basis of its success as an opportunistic pathogen (8, 9).

Although many aspects of the ecology of *Aspergillus* spp. have been connected to pathogenicity (9), its ability to manipulate pH via the secretion of low molecular weight organic acids (LMWOA), and in particular, oxalic acid, has been largely ignored in the context of human pathogenesis. Several clinical reports have shown the presence of calcium oxalate (CaOx) crystals in pulmonary aspergillosis in animals and humans (10-15). Oxalic acid and oxalate crystals are thought to directly cause damage to the host tissues (including pulmonary blood vessels), and to generate free radicals which can harm cells indirectly (14). A recent case report of invasive pulmonary aspergillosis in a 69-year old man with lymphoma and pneumonia indicated the presence of CaOx crystals around blood vessels and within the blood vessel walls. This suggests a potential mechanical role of oxalate crystals in the angioinvasion of *Aspergillus* (15). However, a link between oxalic acid production and pathogenicity has not yet been made in fungi from this genus or in any other fungal human pathogen. On the contrary, oxalic acid production has been widely acknowledged as a pathogenicity factor in fungal plant pathogens, such as *Sclerotinia sclerotiorum* and *Botrytis cinerea* (16, 17) where pH manipulation and calcium chelation plays a direct role in pathogenesis (18-20). Both acidification and cation complexation in the local environment is exploited by plant pathogens to weaken the cell wall structure, facilitate infection, inhibit plant defenses, and induce programmed cell death (19, 21).

Oxalic acid is a ubiquitous compound in the environment and is thought to have a central role in fungal metabolism (19). Its production and consumption by microorganisms have been directly associated with pH regulation in soil (22). In such ecosystems, oxalic acid is often found complexed with divalent cations, especially calcium (23). Despite its chemical stability (K_{sp} CaOx monohydrate = 2.32×10^{-9}), CaOx rarely accumulates (24, 25). This is because oxalate is used by soil oxalotrophic bacteria as carbon and energy sources. The transformation of a strong organic acid into a weaker one (oxalic acid *versus* carbonic acid; pK_a = 1.25 and 4.14, respectively), leads to a local increase in soil pH. This overall process has been coined out in the oxalate-carbonate pathway (26). Moreover, oxalic acid plays a key role in bacterial:fungal interactions acting as a signaling cue by bacteria in order to localize fungi and to establish different types of trophic interactions with them (27-29).

In this study we wanted to evaluate whether the metabolic processes associated to oxalic acid during *Aspergillus* spp. infection could parallel those occurring during its natural cycling in the oxalate-carbonate pathway in soils. Indeed, humans can be seen as a complex ecosystem governed by the same ecological principles affecting any other ecosystem (30). Thus, the ability of oxalotrophic bacteria to degrade oxalic acid produced by *Aspergillus* spp., and control the subsequent pH manipulation and calcium chelation, would result in a mechanism to control biologically this opportunistic fungal pathogen (Fig. 1). To test our hypothesis, we selected *Aspergillus niger* as a model. This organism is regarded as a safe relative of the more infectious *Aspergillus fumigatus* (31), and has been extensively studied for its ability to produce oxalic acid (32, 33). It is also a known agent of aspergillosis in humans, being responsible for 5% of the cases (9, 34). We first confirmed that *A. niger* secretes oxalic acid and assessed the effect of co-cultivation with oxalotrophic bacteria on pH and oxalic acid concentration, and on the inhibition of *A. niger*'s growth. This was done *in vitro* and on human bronchial epithelial cell (HBEC) cultures using two complementary 3D-cell cultures systems (Transwell® inserts and bronchioles-on-a-chip (BoC)).

4.2. Materials and Methods

4.2.1. Bacterial and Fungal cultures

All bacterial and fungal strains come from the collection of the Laboratory of Microbiology of the University of Neuchâtel (LAMUN; Table 1). *P. putida* KT2440 was kindly provided by Dr. Arnaud Dechesne (Technical University of Denmark). *C. necator* JMP289 was kindly provided by Prof. Jan van der Meer (University of Lausanne). *C. oxalaticus* Ox1 was tagged in-house using insertion with a MiniTn7 system. Table 2 summarizes all the media used. Bacterial strains were routinely cultured on NA medium. *Aspergillus niger* was routinely cultured on MA medium. PDA was used for *A. niger* conidia production. BHIA was used to

have mycelium-only colony edge without any conidia in order to prevent unwanted conidia dispersal during confrontations with bacteria.

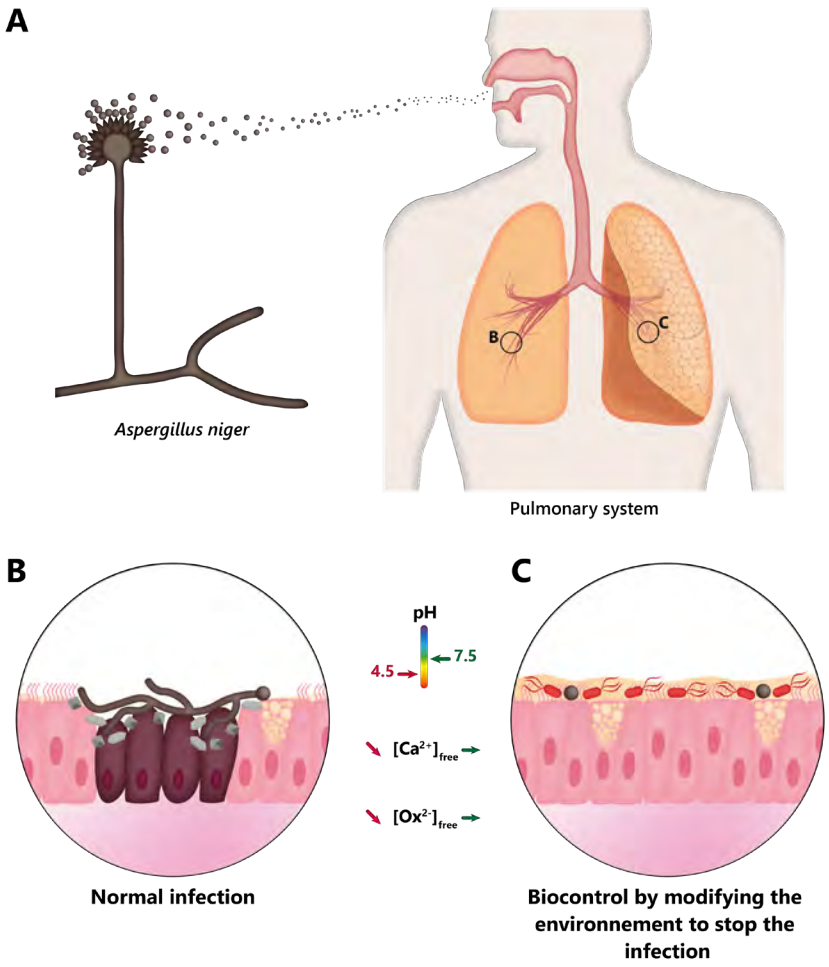


Fig. 1. Schematic summary of the proposed strategy to control *Aspergillus niger* infection by introducing oxalotrophic bacteria to modify the *A. niger* environmental niche. (A) *A. niger* conidia (depicted in dark grey) arrive in the respiratory system through breathing. (B) During a normal infection process in a susceptible host, *A. niger* modifies the environment by secreting oxalic acid (or oxalate Ox^{2-}) which decreases pH and chelates free calcium in the form of CaOx (depicted in light grey) crystals. This results in the infection of the host's tissue. (C) The biocontrol strategy proposed here takes advantage of the ability of oxalotrophic bacteria (cells depicted in red) to consume CaOx and thus reestablish physiological pH and free calcium concentrations.

Table 1. Bacterial and fungal strains used

Code	Strain #	Species	Fluorescent tag	References
Pp	NEU 1264	*Pseudomonas putida* KT2440	GFP	(56)
Cn	NEU 1286	*Cupriavidus necator* JMP289	GFP	(57)
Co	NEU 1287	*Cupriavidus oxalaticus* Ox1	mCherry	(58)
An	NEU M8	*Aspergillus niger*	-	(59)

All the bacterial and fungal strains used in this study come from the collection of the Laboratory of Microbiology of the University of Neuchâtel.

Table 2. Culture media recipes

Medium	Composition	References
ALI (Air-Liquid Interface)	1:1 of DMEM/F12 (Cat.# 11320033, Thermo Fisher Scientific) and LHC Basal Medium (Cat.# 12677019, Thermo Fisher Scientific)	(40)
BHIA (Brain Heart Infusion Agar	37 g Brain Heart Broth (Sigma-Aldrich, Darmstadt, Germany), 15 g agar (Biolife Italiana, Milano, Italy), per liter of deionized (DI) water	
MA (Malt Agar)	12 g of malt extract (Sios Homebrewing GmbH, Wald, Switzerland), 15 g agar (Biolife Italiana, Milano, Italy), per liter of deionized (DI) water	
MA 1/10	1.2 g of malt extract (Sios Homebrewing GmbH, Wald, Switzerland), 15 g agar (Biolife Italiana, Milano, Italy), per liter of deionized (DI) water For liquid malt 1/10, no agar was added.	
NA (Nutrient Agar)	23 g NA (Carl Roth, Karlsruhe, Germany), per liter of deionized (DI) water	
PDA (Potato Dextrose Agar)	39 g PDA (Carl Roth, Karlsruhe, Germany), per liter of deionized (DI) water	
R2A (Reasoner's 2 Agar)	0.5 g yeast extract, 0.5 g Bacto Peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g Na-pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 15 g agar, per liter of Milli-Q® water For liquid R2 medium, no agar was added.	(60)
WYA + BP (Water Yeast Agar)	1 g K_2HPO_4, 5 g NaCl, 0.1 g yeast extract, 10 mg bromocresol purple, 20 g agar, per liter of Milli-Q® water	(28)

4.2.2. LMWOA detection by UHPLC and by colorimetric pH indicator-based Petri dish assay

For the UHPLC analysis, 500 µl of 30 mM H_2SO_4 were added to 1 mL of a two-week liquid culture in malt 1/10, Reasoner's 2, and ALI liquid media in triplicate, to obtain 20 mM H_2SO_4 final concentration in order to obtain a low pH for the extraction of LMWOAs and to dissolve any precipitated crystals. The samples were incubated at 60°C for two hours to dissolve precipitated metal oxalate crystals, and then centrifuged at 3000 g for 10 min. All the samples were filtered at 0.22 µm (13mm syringe filters, PTFE, hydrophilic) and 200 µl were added into HPLC vials with 250 µl conical inserts. UHPLC (Ultimate 3000 RS-Dionex, Thermo Fisher Scientific, USA) was coupled with DAD detector set at 210 ± 2 nm. A 5 µL of sample was injected onto a Prevail™ organic acid column (5 µm particle size, 150 x 4.6 mm, Grace Davison Discovery Sciences, USA) with the temperature kept at 40°C. The mobile phase consisted of 50 mM phosphate buffer adjusted to pH 2.5 with phosphoric acid with a flow rate of 1 mL/min. Pure oxalic acid (Merck, Germany) was identified by the retention time and was quantified by an external standard curve, linear regression from five calibration points (0.2 to 5 mg/mL). For the culture-based assay, WYA supplemented with bromocresol purple (WYA+BP) was used as a pH indicator-containing medium. After one week of incubation at room temperature (RT), the presence of typical bi-pyramidal shaped CaOx crystals was assessed by observing a thin slice of agar medium sampled at the edge of the colony and stained with lactophenol cotton blue under a Leica DM4 B optical microscope connected to a Leica DFC7000 T camera.

4.2.3. Confrontation assays on solid media

Confrontations assays were performed between *A. niger* and *P. putida*, *C. necator* and *C. oxalaticus* (Table 1), on three culture media (MA 1/10, R2A and WYA+BP). A plug coming from the apical part of an actively growing *A. niger* colony was sampled using the wider end of a Pasteur pipette and inoculated in the center of the plates. The bacterial strains were inoculated from fresh plates as opposite lines on either side of the fungal inoculum. Plates were incubated at RT for 20 days, and pictures of the plates at 20 days were taken. Pictures of the bacterial inocula were taken using a Nikon SMZ18 epifluorescence stereoscope, connected to a Nikon DS-Ri2 camera, in order to assess the viability of the bacterial strains thanks to constitutively expressed fluorescent proteins.

4.2.4. Growth tests and confrontation assay in Air-Liquid Interface (ALI) medium

Bacterial growth in ALI medium was tested for 3 days at RT. To produce conidial suspensions, *A. niger* was cultured on PDA for 10 days at RT. Conidia were harvested using Dulbecco's Phosphate Buffer Saline (DPBS) supplemented with 0.01% (v/v) Tween 80. Harvested conidia were washed three times with DPBS following centrifugation at 2000xg for 5 min at RT. Finally, conidia were resuspended in 2 mL DPBS and quantified with an Improved Neubauer counting chamber. Two µL of 16'000 conidia/bacterial cell per µL suspensions were inoculated in 200 µL ALI medium. To test mycelial growth in the different media, small agar plugs (approximately 3x3 mm) coming from the edge of a colony of *A. niger* on BHIA were used. The 96-well plate was incubated at RT for 7 days and growth was visually assessed.

Confrontation of *A. niger* with *C. oxalaticus* was performed in 100-mm Corning® tissue culture plate containing 12 mL ALI medium to allow the fungus to attach during growth. The fungal inoculum was taken from the apical part of an active colony grown on BHIA by using the wider end of a Pasteur pipette. For the confrontation assay, the fungal inoculum was placed in the medium after addition and mixing of 100 µL inoculum of an overnight culture of *C. oxalaticus* in ALI medium. A fungus-only plate was used as control. The plates were incubated at RT for 7 days. Oxalic acid concentration was quantified by using the Oxalic Acid Colorimetric Assay Kit (Sigma-Aldrich, Germany), following the manufacturer instructions.

4.2.5. Preparation and sterilization of the bronchiole-on-a-chip (BoC)

The chip was assembled as described in Hsieh et al. (53). Each unit of the culture platform was fabricated by using a layer-by-layer stacking technique (54). The devices were designed using Solid Edge 2D software (ST9, Siemens PLM Software), and each layer of the prelaminated polymeric sheet was obtained using a CO_2 laser cutter (Universal Laser System). The prelaminated polymeric sheets were combined with biocompatible adhesive tapes (9122, 3M Company) with PMMA (1.5 and 3 mm thick) or PET (0.1

and 0.25mm thick). After cutting, each layer was aligned and assembled using a seam roller to complete the devices. The culture chip includes a Y-shaped apical and a basal part separated by a porous PET membrane (pore size = 0.4µm) prepared as described in Arefin *et al.* (55). The PET membrane was sandwiched between two PET sheets using adhesive transfer tape to create the cell culture surface. This allows nutrients to pass from the media to the cells through the porous membrane. The open design of the tissue chip makes cell seeding procedure easy and accessible.

For sterilization, each chip was placed in a 100 mm Petri dish and sterilized with 5% H_2O_2 solution for 1 h. The chips were then rinsed 2-3 times with sterile deionized water for 15 min between each rinse. Once all liquid was removed, the chips were let dry overnight under a laminar flow hood. The next day, the inlet and outlet of the chip were connected with a sterile tubing and rinsed 3 more times with sterile deionized water as explained before. After the last rinse, 200 µl sterile DPBS was added in the channel (apical part) and 5 mL in the basolateral part (bottom part) of the chip, and the chip was placed in a humidified incubator at 37°C with 5% CO_2 overnight. The next morning, peroxide contamination was checked in each chip using a CG8+ i-STAT cartridge (Abbott, USA). Rinses with sterile deionized water and overnight incubation with sterile DPBS were repeated until peroxide was no longer detected.

4.2.6. Primary normal human bronchial epithelial cell culture

Primary normal human bronchial epithelial cells (Lifeline Cell Technology, USA) were expanded in a T-75 cell culture flask with vent cap (Corning, USA) in BronchiaLife™ B/T complete medium (Lifeline Cell Technology, USA) supplemented with 0.5% Phenol Red solution (Sigma-Aldrich, USA, 15 mg/L final concentration) to 70-80% confluence in a humidified incubator at 37°C with 5% CO_2. Culture medium was changed every other day. Cells were used until passage 2 for all experiments. Cells were harvested by trypsinization with 0.05% Trypsin / 0.02% EDTA (Lifeline Cell Technology, USA), followed by the addition of Trypsin Neutralizing buffer (Lifeline Cell Technology, USA), and counted using a hemocytometer after centrifugation at 100xg for 5 min and resuspension of the cell pellet in BronchiaLife™ medium.

Cells were seeded at a density of $3x10^4$ cells/well in 200 µl BronchiaLife™ medium for submerged undifferentiated tissue culture in 96-well plates, and $5x10^4$ cells and $8.6x10^4$ cells in 200 µL BronchiaLife™ medium for air-lifted differentiated tissue culture in the apical side of Transwell® inserts in 24-well plates (Corning, USA) and BoC devices, respectively. Transwell® inserts and BoC were first coated with collagen (30 µg/mL) prior seeding of the cells in order to allow proper cell attachment onto the porous membrane, as described in Arefin *et al.* (55). 600 µL of BronchiaLife™ medium was added to the basolateral side of Transwell® inserts in the 24-well plates and 3 mL in the basolateral side of the BoC device. 96-well

plates, Transwell® inserts, and BoC devices were placed in a humidified incubator at 37°C with 5% CO_2 for 2-3 days until confluence and formation of a monolayer of bronchial cells. For differentiated bronchial cell tissues (Transwells® and BoCs), cells were shifted to air-liquid interface by removing carefully the BronchiaLife™ medium from the apical side and replacing it by Air-Liquid Interface (ALI) Epithelial Differentiation Medium (Lifeline Cell Technology, USA) supplemented with 0.5% Phenol Red solution (Sigma-Aldrich, USA, 15 mg/L final concentration). The same was done for the medium on the basolateral side. Finally, the medium on the apical side was removed and the inserts and devices were placed in a humidified incubator at 37°C with 5% CO_2 for 21 days. Medium was changed every other day as described previously. The cultures were observed daily using an EVOS™ XL Core bright field inverted microscope (Thermo Fisher Scientific, USA).

4.2.7. Determination of conidial and bacterial load and confrontation assay on submerged undifferentiated bronchial epithelial cell cultures

In order to determine the optimal conidial and bacterial load to be used for confrontation on bronchial tissue cultures, increasing conidial and bacterial loads were tested to assess their effect on the morphology of bronchial epithelial cells in submerged cultures. *A. niger* was cultured on PDA for 7 days at 37°C in order to produce conidia. *A. niger* conidia were then harvested as already described. Finally, conidia were resuspended in 2 mL DPBS and quantified with an Improved Neubauer counting chamber. Bacteria were cultured in BronchiaLife™ medium at 37°C overnight and quantified with an Improved Neubauer counting chamber. A stock suspension of *A. niger* conidia was made at 10^6 conidia/mL that was diluted further to obtain suspensions at $5x10^5$ to 5×10^3 and 10^3 conidia/mL. The same was done for *C. oxalaticus* from a stock suspension at 10^5 bacterial cells/mL diluted until 10^3 bacterial cells/mL. 10 µL of each suspension was added to submerged undifferentiated bronchial tissue in a 96-well plate in order to have 10^4 to 10 conidia/well (200 µL) for *A. niger*, and 10^3 to 10 bacterial cells/well (200 µL) for *C. oxalaticus*. The plates were placed in a humidified incubator at 37°C with 5% CO_2 for 24h. For the confrontations assay, cells were infected with 10 or 500 *A. niger* conidia and were put in confrontation with 10 bacterial cells. The plate was placed in a humidified incubator at 37°C with 5% CO_2 for 72h. After incubation, cells were fixed, stained (actin and nucleus), and imaged as described below (Immunofluorescence staining).

4.2.8. Confrontations on differentiated bronchial tissues in Transwell® inserts and BoC devices

Differentiated bronchial tissues in Transwell® inserts and in BoC devices were infected with 10 µL of 10^3 conidia or bacterial cell to get 10 conidia or bacterial cells per inserts or devices. Fungus and bacteria

were co-inoculated for the confrontation and controls with only medium, cells, fungus or bacteria were included. Each condition was done in triplicate for the Transwell® inserts and one unique replicate for each condition was done for the BoC devices. All Transwell® inserts and BoC devices were incubated in a humidified incubator at 37°C with 5% CO_2 for 72h.

4.2.9. Immunofluorescence staining

Undifferentiated and differentiated bronchial tissues (Transwell® and BoCs) were fixed with 100 µL 4% paraformaldehyde in DPBS for 15 min at RT. Cells were then rinsed 3 times with 200 µL DPBS, with 2 min waiting time between each rinse. Cells were permeabilized with 100 µL 0.5% Triton X-100 in DPBS for 15 min at RT and rinsed 3 times with 200 µL DPBS, with 2 min waiting time between each rinse. After that, cells were blocked with 100 µL 3% BSA in DPBS for 1h at RT. Anti-Mucin 5AC mouse monoclonal antibody (Abcam, USA, Cat.# ab218466) was prepared in DPBS (1/100). The actin stain (ActinGreen™ 488 ReadyProbes™ Reagent) and the nuclei counterstain (NucBlue™ Live ReadyProbes™ Reagent) were added to the same buffer (2 drops/mL and 1 drop/mL, respectively). Anti-Aspergillus rabbit polyclonal antibody (Abcam, Cat.# ab20419) was also added (1/200) in the staining buffer for the conditions where *A. niger* conidia were inoculated. Fixed cells were incubated with 100 µL buffer containing the stains and Anti-Mucin 5AC and Anti-Aspergillus antibodies overnight at 4°C. The next day, fixed cells were washed 3 times with DPBS and secondary antibodies were applied. Goat anti-Mouse IgG antibody (1/250) conjugated with Alexa Fluor 546 (Thermo Fisher Scientific, USA, Cat.# A-11003,) directed against Anti-Mucin 5AC antibody and Goat anti-Rabbit IgG antibody (1/500) conjugated with Alexa Fluor 594 (Thermo Fisher Scientific, USA, Cat.# A-11012) directed against Anti-Aspergillus antibody were prepared in DPBS. Fixed cells were incubated with 100 µL buffer containing the secondary antibodies overnight at 4°C. The following day, fixed cells were once again washed 3 times with DPBS and the membranes from the inserts were carefully cut out with a sharp knife. The membranes were mounted on a glass slide using Fluoromount-G™ Mounting Medium (Thermo Fisher Scientific, USA) and imaged with a Zeiss Axio Observer Z1 fluorescence inverted microscope (Carl Zeiss AG, Germany).

4.2.10. pH measurements and quantification of calcium, oxalic acid and lactate dehydrogenase (LDH)

pH measurements of the culture medium after 72h incubation were done directly after taking the samples out of the incubator using pH-indicator strips (Merck, Germany) for the Transwell® inserts samples, and CG8+ i-STAT cartridges for the BoC devices, as the pH change of the culture medium indicator was less visible. Free calcium (Calcium Colorimetric Assay, Sigma-Aldrich, Germany), free oxalic acid (Oxalic Acid

Colorimetric Assay Kit), and LDH (ScienCell Research Laboratories, USA) were quantified in the culture medium using colorimetric assay kits following the manufacturer instructions.

4.2.11. Statistical analyses

Statistical significance of the data from the confrontations on differentiated bronchial tissues in Transwell® inserts (3 replicates, n = 3) was tested with unpaired two-tailed Student t-tests in Microsoft® Excel (Version 16.37). The statistical significance threshold was set to 5%.

4.3. Results

4.3.1. Detection of oxalic acid produced by *Aspergillus niger* in different culture conditions

Since the metabolism of fungi can change significantly depending on the nutritional conditions of the growth medium, we first tested whether our fungal strain produced oxalic acid only or a mixture of different organic acids in culture media differing in their trophic conditions. While *A. niger* is known to produce high amounts of oxalic acid, it is also well known to produce other LMWOA such as citric acid (32, 35-37). An Ultra-High-Performance Liquid Chromatography (UHPLC) analysis revealed that in the conditions we tested, *A. niger* produced oxalic acid, only (Fig. S1A). The absence of other organic acids is not surprising as the conditions for other LMWOA production are highly specific (e.g. carbon concentration above 50 g/L carbon and pH < 3; (38, 39) and are not provided in the conditions we tested. Acidification and the presence of CaOx crystals could also be detected in water yeast agar (WYA) (Fig. S1B). We thus concluded that our *A. niger* strain consistently produced oxalic acid and acidified the pH of its medium under laboratory growth conditions.

4.3.2. Confrontation assays between *A. niger* and selected bacterial strains

We assessed how the presence of oxalotrophic bacteria impacted *A. niger* growth and pH evolution in different growth media. Two oxalotrophic bacteria (*Cupriavidus necator* and *C. oxalaticus*) and one non-oxalotrophic bacterium (*Pseudomonas putida*) were used in confrontation assays. In malt agar diluted 10 times (MA 1/10), *A. niger* colonized the entire Petri dish, including the area in which the bacterial inocula were applied. All three inoculated bacteria did not survive in the area of interaction with the fungus (Fig. S2A). In Reasoner's 2 agar (R2A), *A. niger* mycelia did not colonize the area beyond the barrier formed by the bacterial inocula, but some hyphae were still able to grow beyond the bacterial inoculation zone and develop into microcolonies in the co-culture with *P. putida* (Fig. S2B). In WYA, mycelial growth was also restricted to the area delimited by the inocula in the co-culture with the two oxalotrophic bacteria.

On the other hand, *P. putida* did not survive the interaction with the fungus, which instead, colonized the entire plate. Moreover, in WYA medium, which contained a pH indicator, the acidification of the medium by the fungus grown alone or in co-culture with the non-oxalotrophic bacterium was clearly visible. In contrast, in the co-cultures with oxalotrophic bacteria, the pH of the medium did not change (Fig. S2C). The control over fungal growth was particularly remarkable in the case of *C. oxalaticus* (Fig. 2). Given the impact of the medium on the control of fungal growth, we repeated the confrontation assays in Air-Liquid Interface (ALI) medium, which is the medium used for differentiation of lung cells (40). The non-oxalotrophic bacterial model (*P. putida*) acidified the medium when grown alone, while the oxalotrophic bacteria (*C. necator* and *C. oxalaticus*) did not acidify the medium (Fig S3A). *C. oxalaticus* was found to not only control mycelial growth but also inhibit conidia germination (Fig. S3B). Moreover, the presence of *C. oxalaticus* in co-culture with the fungus stabilized the pH of the culture medium at a neutral pH (Fig. S3C), consistent with decreased oxalic acid concentration in the medium (Fig. S3D). We therefore conclude that oxalotrophic bacteria have a significant inhibitory effect on *A. niger* growth in all media tested.

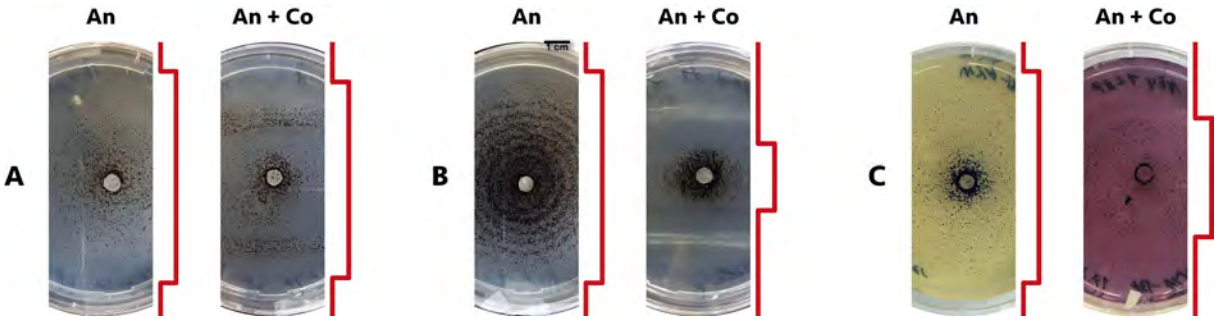


Fig. 2. Comparison of the growth of *Aspergillus niger* (An) alone and in confrontation with *Cupriavidus oxalaticus* (Co) in different culture media. The red line next to each picture represents the extent of *A. niger* growth. On MA 1/10 (A), there is no significant growth inhibition of *A. niger*, as it kills *C. oxalaticus*. *A. niger* growth is highly restricted to the center of the plate when co-cultured with *C. oxalaticus* on R2A (B). The growth inhibition of *A. niger* when co-cultured with *C. oxalaticus* is less pronounced on WYA + BP. Moreover, the presence of C. oxalaticus revert the pH of the medium to a neutral value (C). A yellow color indicates an acidic pH <6.

4.3.3. Establishment of a dose-response curve on submerged cultures

In order to obtain evidence that oxalotrophic bacteria control the growth of *A. niger* in an animal-free lung infection model that is compatible with the 3R principles of animal experimentation (41), we set up experiments on human bronchial epithelial cell (HBEC) cultures. We established a dose-response curve for increasing conidial and/or bacterial loads on HBECs in submerged cultures, to identify the optimal load to perform experiments in Transwells® and BoC systems. After 24 h, the overall size, shape and integrity of the lung cells changed at an absolute load of 500 conidia and above. The HBECs shrank in size due to actin agglomeration. Moreover, from a conidial load of >=1000, fungal growth also had an adverse effect on tissue integrity (Fig. S4). The same experiment was performed with *C. oxalaticus* and *P.*

putida. A strong morphological change was induced by a total load of 500 bacterial cells and above for the former (Fig. S5), and as little as 10 cells for the latter (Fig. S6), which was not used further. The HBECs co-cultured with *C. oxalaticus* became rounder, and actin agglomeration increased compared with the cells-only control (Fig. S5).

To analyze the effect of co-culturing *A. niger* with the oxalotrophic bacterium on HBECs integrity, we performed a test with 10 and 500 conidia confronted with 10 bacterial cells. After 72h, *A. niger* induced morphological changes (size reduction and actin agglomeration), with a stronger effect for 500 conidia, confirming the results obtained at 24h. With the co-inoculation of as few as 10 *C. oxalaticus* cells, the morphology of the HBECs was similar to the morphology of HBECs of the bacteria-only control, suggesting the inhibition of fungal development (Fig. S7). We concluded that a conidial and bacterial load of 10 conidia/cells was ideal to monitor the interaction of *A. niger* and *C. oxalaticus* in differentiated HBECs in Transwells® and BoC systems.

4.3.4. Biocontrol assay of *A. niger* bronchial cells infection by *C. oxalaticus*

After establishing a dose-response curve on HBECs in submerged cultures, the effect of inoculation of 10 *A. niger* conidia alone or in co-culture with 10 *C. oxalaticus* cells was assessed in differentiated bronchial tissue in Transwell® inserts and BoC systems. In the presence of the fungus alone, changes in three key environmental factors were observed: pH, Ca^{2+} concentration, and concentration of soluble oxalic acid. The pH dropped from 7.5 down to 4.5 in Transwells® and from 7.3 to 6.8 in BoC systems (Fig. 3A). Ca^{2+} concentrations changed from 1 mM to around 0.2 mM in both culture systems (Fig. 3B). The level of soluble oxalic acid produced by *A. niger* dropped from 500 µM (cells alone) to 75 µM (cells inoculated with the fungus) (Fig. 3C). In contrast, pH, Ca^{2+} and free oxalic acid levels were statistically indistinguishable when oxalotrophic bacteria were co-cultured with the fungus compared to the controls with lung cells alone or with bacteria. In addition, CaOx crystals were observed in the cultures in which the fungus developed (Fig. 3D), but not when the fungus was co-cultured with oxalotrophic bacteria (Fig. 3E). This suggests that the lower levels of soluble oxalic acid measured in the treatment with the fungus were likely the result of complexation of oxalic acid and Ca^{2+}, and corroborates the pH and Ca^{2+} concentration data. Moreover, the absence of CaOx crystals when the fungus was in co-culture with *C. oxalaticus* agrees with the pH, Ca^{2+} concentration and soluble oxalic concentrations measured. These results validated our hypothesis that oxalotrophic bacteria can be used to manipulate the microenvironment created by *A. niger*. In addition to the changes in the environmental parameters measured above, we also observed a cytopathic effect when conidia of *A. niger* developed into mycelia (Fig. 4A and B). This cytopathic effect resulted in the destruction of the bronchial epithelium. Lactate dehydrogenase (LDH) activity were

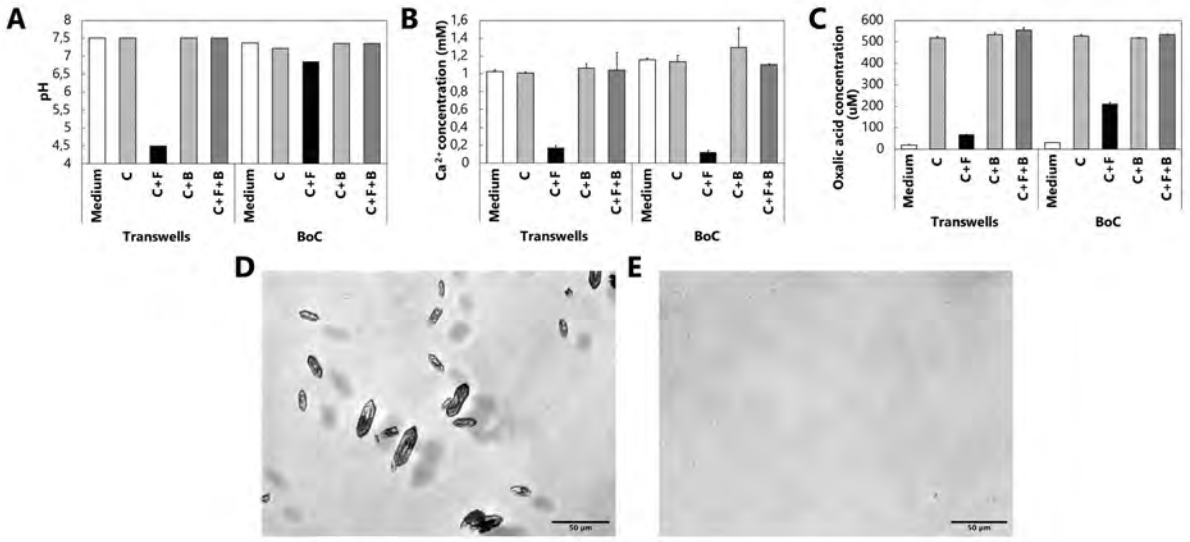


Fig. 3. Influence of the interaction between *Aspergillus niger* and the oxalotrophic bacterium *Cupriavidus oxalaticus* on environmental parameters of differentiated bronchial tissue in Transwell® inserts and bronchiole-on-a-chip (BoC) devices. In the presence of the fungus, the pH (A) decreases, as compared to all other treatments. This pH decrease is correlated with a drastic decrease in the concentration of free Ca^{2+} (B) (*p*-value between C and C+F = 8,951 × 10^{-7}). Free oxalic acid concentrations were lower in the presence of the fungus, compared with the basal level secreted by the bronchial cells (C) (*p*-value between C and C+F = 4,587 × 10^{-8}**)**. These results are supported by the detection of CaOx crystals in the presence of the fungus (D). In the co-culture with the oxalotrophic bacterium, pH, free Ca^{2+} and free oxalic acid concentrations return to physiological levels, and this was concomitant with the absence of crystals (E) (*p*-values between C+F and C+F+B for free Ca^{2+} and free oxalic acid = 0,002 and 1,415 × 10^{-7}, respectively). C: lung cells; C+F: lung cells + fungus; C+B: lung cells + bacteria; C+F+B: lung cells + fungus + bacteria. For A, B, C, the results represent the mean + sd of three independent measurements for the Transwell® inserts for each condition (three biological replicates, n = 3). For A, pH results for the BoC devices represent a unique measurement per condition (one replicate, n = 1). For B and C, Ca^{2+} and oxalic acid results for the BoC devices represents the mean + sd of two measurements per condition (one replicate).

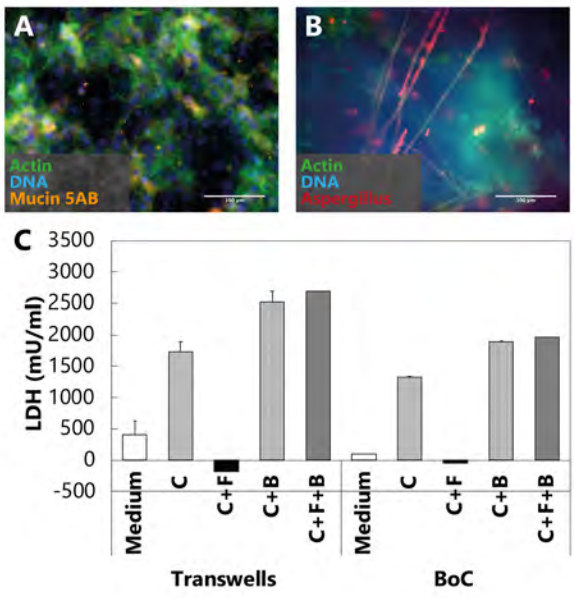


Fig. 4. Cytopathic effect of *Aspergillus niger* on differentiated bronchial tissue and lactate dehydrogenase (LDH) measurements of the co-culture between *A. niger* and *Cupriavidus oxalaticus* in Transwell® inserts and bronchiole-on-a-chip (BoC) devices. (A) Control showing healthy differentiated bronchial epithelial cells. (B) Bronchial epithelial cells infected with *A. niger*. (C) LDH leakage was measured as a proxy for cell damage. In the presence of the fungus, no LDH has been detected, probably because of the destruction of the tissue by *A. niger* (B). *C. oxalaticus* cause significantly more LDH leakage that the basal LDH level of control cells (p-value = 0,004). C: lung cells; C+F: lung cells + fungus; C+B: lung cells + bacteria; C+F+B: lung cells + fungus + bacteria. For C, the results represent the mean + sd of three independent measurements for the Transwell® inserts for each condition (three biological replicates, n = 3). LDH results for the BoC devices represents the mean + sd of three measurements per condition (one replicate, n = 1).

elevated in response to the presence of the foreign oxalotrophic bacteria (Fig. 4C), something that needs to be addressed for any future therapeutic application.

4.3.5. Genomic potential for oxalic acid production in other *Aspergillus* spp.

We performed a genomic screening of orthologous genes to the oxaloacetate acetylhydrolase (*oahA*) and the oxalate/formate antiporter (genes involved in oxalic acid production in *A. niger*) in genomes available in the *Aspergillus* Genome Database (AspGD). The genomic screening revealed that orthologs of both genes are found in multiple *Aspergillus* spp. and are highly conserved across diverse species (Fig. 5). In the case of the orthologs to the oxalate/formate antiporter, they were conserved to a lesser extent (Fig. S8). This genomic analysis confirmed that diverse *Aspergillus* spp. possess the genes necessary to produce and secrete oxalic acid.

4.4. Discussion

Here we present a biological interaction between *A. niger* and oxalotrophic bacteria that results in the biological control of *A. niger*, preventing infection in 3D-lung cell tissues (Transwells® and BoC). The direct consequence of acidification through oxalic acid production by *A. niger* was the decrease in free Ca^{2+} and subsequent precipitation of CaOx crystals. CaOx crystals are well known to occur in lung tissues upon infection by *A. niger* (10, 11, 13). Presumably, by consuming the oxalate produced by *A. niger*, the oxalotrophic bacterial species *C. oxalaticus* blocks the subsequent decrease in pH and formation of CaOx crystals observed in the absence of the bacterium. To obtain a direct confirmation of the role of oxalic acid in the manipulation of pH during lung infection, the use of a non-oxalate-producer *A. niger* mutant would be indispensable. Such mutants (*oahA* gene) are described in the literature and exhibited a decreased acidification of the culture medium and reduced extracellular protease activity (32, 42). After multiple failed attempts to obtain the published mutants by addressing the corresponding scientific teams, we attempted to construct a non-oxalate-producer mutant of our *A. niger* strain using CRISPR-Cas9 gene editing. However, this was unsuccessful due to multiple targets of the sgRNA probes and thus could not be included in this study.

Oxalate-degrading bacteria are known inhabitants of the human gut, where they perform the key function of degrading dietary oxalate (43). These species have also been used as probiotics for the treatment of hyperoxaluria (high oxalate in urine) and the management of kidney stones (43, 44). While oxalate-degrading bacteria are well characterized in the gut, this is not the case of the lung. Although considered sterile for a long time, the lung is now known to harbor a diverse microbiota (45, 46). Oxalate-degrading capabilities have been previously reported in strains of the genera *Lactobacillus* (47), *Streptococcus* (48),

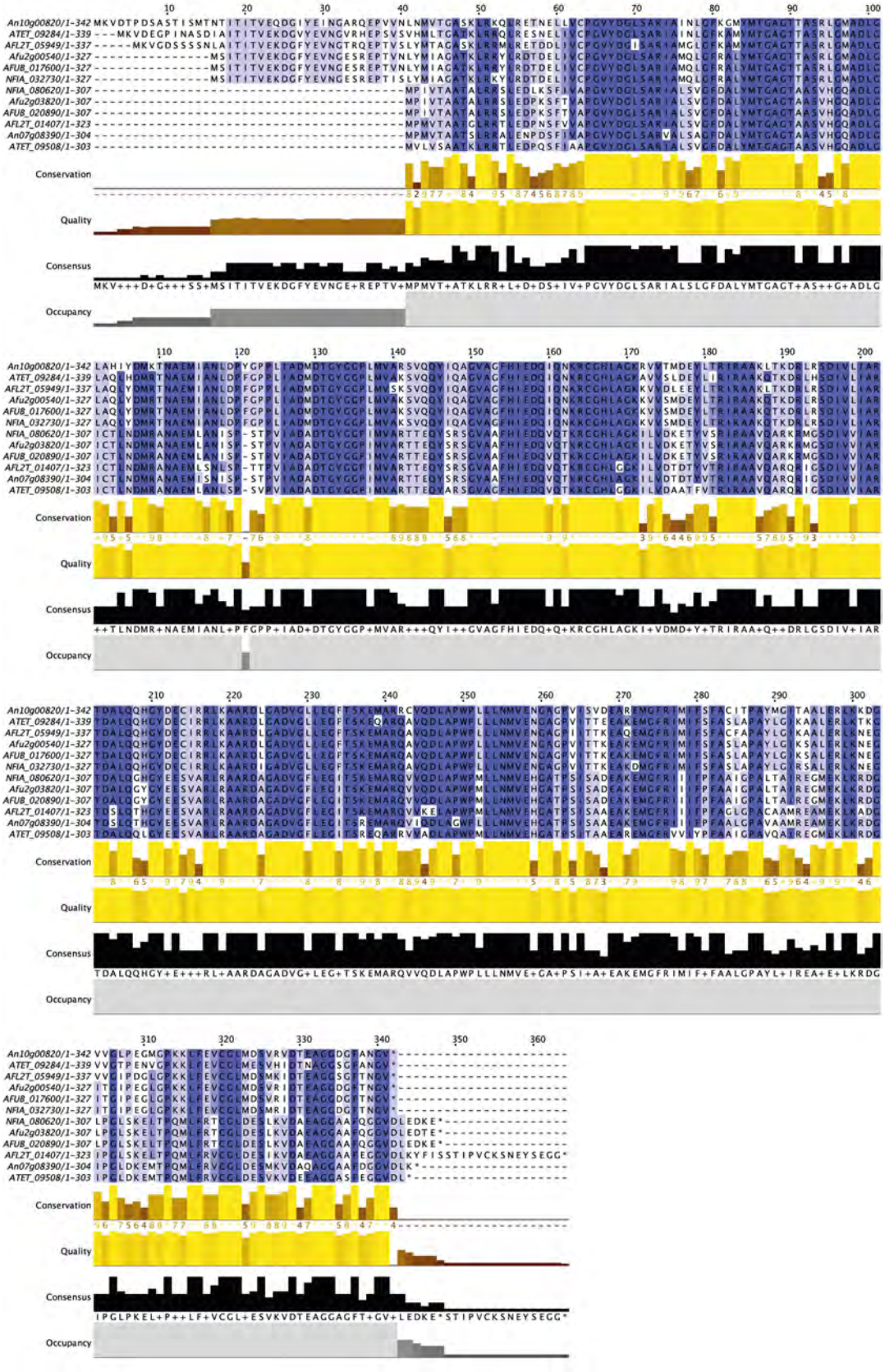


Fig. 5. Genomic screening of the oxaloacetate acetylhydrolase (OAH) in other *Aspergillus* spp. Multiple sequence alignment of the protein sequences orthologous to the OAH of *A. niger* CBS 513.88 (GenBank accession number CAD99195.1) revealed they were well conserved across diverse species, as indicated by an intense purple color of the amino acids. Multiple sequence alignments were performed using the MUSCLE protein alignment algorithm in Jalview (version 2.11.1.2). An = *A. niger* CBS 513.88, ATET = *A. terreus* NIH2624, AFL2T = *A. flavus* NRRL 3357, Afu = *A. fumigatus* Af293, AFUB = *A. fumigatus* A1163, NFIA = *Neosartorya fisheri* NRRL 181 (formerly *A. fisheri*).

Prevotella (49, 50) and *Veillonella* (50), all of which are reported as components of the lung microbiota. However, assessing the oxalotrophic potential of the lung microbiome is something that still need to be accomplished.

The genomic analysis of multiple *Aspergillus* spp. suggests that oxalotrophy could also be relevant to other *Aspergillus* causing pulmonary aspergillosis (5). The presence of CaOx crystals during infection by *A. fumigatus* has been reported in the literature (8, 11, 14, 51). Accordingly, we found orthologs of the oxaloacetate acetylhydrolase (OAH) and the oxalate/formate antiporter of *A. niger* in the genomes of two well characterized model *A. fumigatus* strains Af293 and A1163 (52), suggesting the production of oxalic acid by this pathogen and the potential of using oxalotrophic bacteria in fungal species more relevant for human health. To conclude, the results presented here represent a stepping stone towards developing an alternative approach to control the development of oxalate-producing *Aspergillus* spp. based on the manipulation of the lung environment using bacterial:fungal interactions.

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References

1. Fisher MC, Gurr SJ, Cuomo CA, Blehert DS, Jin H, Stukenbrock EH, et al. Threats posed by the fungal kingdom to humans, wildlife, and agriculture. mBio. 2020;11(3).

2. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases—estimate precision. 2017.

3. Ramirez-Ortiz ZG, Means TK. The role of dendritic cells in the innate recognition of pathogenic fungi (A. fumigatus, C. neoformans and C. albicans). 2012. p. 635-46.

4. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. 2018. p. 739-42.

5. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden killers: Human fungal infections. 2012. p. 165rv13-rv13.

6. Brunke S, Mogavero S, Kasper L, Hube B. Virulence factors in fungal pathogens of man. 2016. p. 89-95.

7. Pfaller MA, Diekema DJ. Rare and emerging opportunistic fungal pathogens: Concern for resistance beyond Candida albicans and Aspergillus fumigatus. 2004. p. 4419-31.

8. Kousha M, Tadi R, Soubani AO. Pulmonary aspergillosis: A clinical review. 2011. p. 156-74.

9. Paulussen C, Hallsworth JE, Álvarez-Pérez S, Nierman WC, Hamill PG, Blain D, et al. Ecology of aspergillosis: insights into the pathogenic potency of Aspergillus fumigatus and some other Aspergillus species. Microbial Biotechnology. 2017;10(2):296-322.

10. Kurrein F, Path FRC, Green GH, Rowles SL. Localized deposition of calcium oxalate around a pulmonary Aspergillus niger fungus ball. American Journal of Clinical Pathology. 1975;64(4):556-63.

11. Maeno T, Sasaki M, Shibue Y, Mimura K, Oka H. Calcium oxalate in the sputum may aid in the diagnosis of pulmonary aspergillosis: A report of two cases. Medical Mycology Case Reports. 2015;8:32-6.

12. Muntz FHA. Oxalate-producing pulmonary aspergillosis in an alpaca. Veterinary Pathology. 1999;36(6):631-2.

13. Oda M, Saraya T, Wakayama M, Shibuya K, Ogawa Y, Inui T, et al. Calcium oxalate crystal deposition in a patient with Aspergilloma due to Aspergillus niger. Journal of thoracic disease. 2013;5(4):E174-8.

14. Payne CL, Dark MJ, Conway JA, Farina LL. A retrospective study of the prevalence of calcium oxalate crystals in veterinary Aspergillus cases. Journal of Veterinary Diagnostic Investigation. 2017;29(1):51-8.

15. Yi Y, Cho SY, Lee DG, Jung JI, Park YJ, Lee KY. Invasive Pulmonary Aspergillosis Due to Aspergillus awamori: Role of Calcium Oxalate Crystal Precipitation Mimicking Mucormycosis. 2020. p. 409-11.

16. Cessna SG, Sears VE, Dickman MB, Low PS. Oxalic acid, a pathogenicity factor for Sclerotinia sclerotiorum, suppresses the oxidative burst of the host plant. Plant Cell. 2000;12(11):2191-9.

17. Schoonbeek H-j, Jacquat-Bovet A-C, Mascher F, Métraux J-P. Oxalate-degrading bacteria can protect Arabidopsis thaliana and crop plants against Botrytis cinerea. Molecular plant-microbe interactions. 2007;20(12):1535-44.

18. Lehner A, Meimoun P, Errakhi R, Madiona K, Barakate M, Bouteau F. Toxic and signalling effects of oxalic acid. Canadian Journal of Microbiology. 2008;3(September):746-8.

19. Dutton MV, Evans CS. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology. 1996;42(9):881-95.

20. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. In: Gadd GM, Sariaslani S, editors. 106: Academic Press; 2019. p. 49-77.

21. de Oliveira Ceita G, Macêdo JNA, Santos TB, Alemanno L, da Silva Gesteira A, Micheli F, et al. Involvement of calcium oxalate degradation during programmed cell death in Theobroma cacao tissues triggered by the hemibiotrophic fungus Moniliophthora perniciosa. Plant Science. 2007;173(2):106-17.

22. Graustein WC, Cromack K, Sollins P. Calcium oxalate: Occurrence in soils and effect on nutrient and geochemical cycles. Science. 1977;198(4323):1252-4.

23. Martin G, Guggiari M, Bravo D, Zopfi J, Cailleau G, Aragno M, et al. Fungi, bacteria and soil pH: The oxalate-carbonate pathway as a model for metabolic interaction. Environmental Microbiology. 2012;14(11):2960-70.

24. Hofmann BA, Bernasconi SM. Review of occurrences and carbon isotope geochemistry of oxalate minerals: implications for the origin and fate of oxalate in diagenetic and hydrothermal fluids. Chemical Geology. 1998;149(1-2):127-46.

25. Certini G, Corti G, Ugolini FC. Vertical trends of oxalate concentration in two soils under Abies alba from Tuscany (Italy). Journal of Plant Nutrition and Soil Science. 2000;163(2):173-7.

26. Braissant O, Cailleau G, Aragno M, Verrecchia EP. Biologically induced mineralization in the tree Milicia excelsa (Moraceae): its causes and consequences to the environment. Geobiology. 2004;2(1):59-66.

27. Rudnick MB, van Veen JA, de Boer W. Oxalic acid: A signal molecule for fungus-feeding bacteria of the genus Collimonas? Environmental Microbiology Reports. 2015;7(5):709-14.

28. Mela F, Fritsche K, De Boer W, Van Veen JA, De Graaff LH, Van Den Berg M, et al. Dual transcriptional profiling of a bacterial/fungal confrontation: Collimonas fungivorans versus Aspergillus niger. ISME Journal. 2011;5(9):1494-504.

29. Haq IU, Zwahlen RD, Yang P, van Elsas JD. The Response of Paraburkholderia terrae Strains to Two Soil Fungi and the Potential Role of Oxalate. Frontiers in Microbiology. 2018;9(989).

30. Dethlefsen L, McFall-Ngai M, Relman DA. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature. 2007;449(7164):811-8.

31. Schuster E, Dunn-Coleman N, Frisvad J, Van Dijck P. On the safety of Aspergillus niger - A review. 2002. p. 426-35.

32. Ruijter GJG, van de Vondervoort PJI, Visser J. Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology. 1999;145(9):2569-76.

33. Cameselle C, Bohlmann JT, Núñez MJ, Lema JM. Oxalic acid production by Aspergillus niger. Bioprocess Engineering. 1998;19(4):247-52.

34. Person AK, Chudgar SM, Norton BL, Tong BC, Stout JE. Aspergillus niger: An unusual cause of invasive pulmonary aspergillosis. Journal of Medical Microbiology. 2010;59(7):834-8.

35. Plassard C, Fransson P. Regulation of low-molecular weight organic acid production in fungi. 2009. p. 30-9.

36. Show PL, Oladele KO, Siew QY, Aziz Zakry FA, Lan JCW, Ling TC. Overview of citric acid production from Aspergillus niger. 2015. p. 271-83.

37. Sturm EV, Frank-Kamenetskaya O, Vlasov D, Zelenskaya M, Sazanova K, Rusakov A, et al. Crystallization of calcium oxalate hydrates by interaction of calcite marble with fungus Aspergillus Niger. American Mineralogist. 2015;100(11-12):2559-65.

38. Karaffa L, Kubicek CP. Aspergillus niger citric acid accumulation: do we understand this well working black box? Appl Microbiol Biotechnol. 2003;61(3):189-96.

39. Kobayashi K, Hattori T, Honda Y, Kirimura K. Oxalic acid production by citric acid-producing Aspergillus niger overexpressing the oxaloacetate hydrolase gene oahA. J Ind Microbiol Biotechnol. 2014;41(5):749-56.

40. Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH. Well-differentiated human airway epithelial cell cultures. Methods in molecular medicine. 2005;107:183-206.

41. Kendall LV, Owiny JR, Dohm ED, Knapek KJ, Lee ES, Kopanke JH, et al. Replacement, Refinement, and Reduction in Animal Studies With Biohazardous Agents. ILAR journal. 2018;59(2):177-94.

42. Han Y, Joosten HJ, Niu W, Zhao Z, Mariano PS, McCalman M, et al. Oxaloacetate hydrolase, the C-C bond lyase of oxalate secreting fungi. Journal of Biological Chemistry. 2007;282(13):9581-90.

43. Abratt VR, Reid SJ. Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease. 72: Academic Press; 2010. p. 63-87.

44. Hoppe B, Von Unruh G, Laube N, Hesse A, Sidhu H, editors. Oxalate degrading bacteria: New treatment option for patients with primary and secondary hyperoxaluria?2005/11//.

45. Enaud R, Prevel R, Ciarlo E, Beaufils F, Wieërs G, Guery B, et al. The Gut-Lung Axis in Health and Respiratory Diseases: A Place for Inter-Organ and Inter-Kingdom Crosstalks. 2020.

46. Mitchell AB, Oliver BGG, Glanville AR. Translational aspects of the human respiratory virome. 2016. p. 1458-64.

47. Turroni S, Vitali B, Bendazzoli C, Candela M, Gotti R, Federici F, et al. Oxalate consumption by lactobacilli: Evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in Lactobacillus acidophilus. Journal of Applied Microbiology. 2007;103(5):1600-9.

48. Miller AW, Dearing D. The metabolic and ecological interactions of oxalate-degrading bacteria in the mammalian gut. 2013. p. 636-52.

49. Ticinesi A, Nouvenne A, Chiussi G, Castaldo G, Guerra A, Meschi T. Calcium oxalate nephrolithiasis and gut microbiota: Not just a gut-kidney axis. a nutritional perspective. 2020.

50. Suryavanshi MV, Bhute SS, Jadhav SD, Bhatia MS, Gune RP, Shouche YS. Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures. Scientific Reports. 2016;6(1).

51. Pabuççuoğlu U. Aspects of oxalosis associated with aspergillosis in pathology specimens. Pathology Research and Practice. 2005;201(5):363-8.

52. Bertuzzi M, van Rhijn N, Krappmann S, Bowyer P, Bromley MJ, Bignell EM. On the lineage of Aspergillus fumigatus isolates in common laboratory use. Medical Mycology. 2020:myaa075-myaa.

53. Hsieh HL, Nath P, Huang JH. Multistep Fluidic Control Network toward the Automated Generation of Organ-on-a-Chip. ACS Biomaterials Science and Engineering. 2019;5(9):4852-60.

54. Lin CK, Hsiao YY, Nath P, Huang JH. Aerosol delivery into small anatomical airway model through spontaneous engineered breathing. Biomicrofluidics. 2019;13(4):044109-.

55. Arefin A, McCulloch Q, Martinez R, Martin SA, Singh R, Ishak OM, et al. Micromachining of Polyurethane Membranes for Tissue Engineering Applications. ACS Biomaterials Science and Engineering. 2018;4(10):3522-33.

56. Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VAP, et al. Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440. Environmental Microbiology. 2002;4(12):799-808.

57. Sentchilo V, Czechowska K, Pradervand N, Minoia M, Miyazaki R, Van Der Meer JR. Intracellular excision and reintegration dynamics of the ICEclc genomic island of Pseudomonas knackmussii sp. strain B13. Molecular Microbiology. 2009;72(5):1293-306.

58. Şahin N, Işik K, Tamer AÜ, Goodfellow M. Taxonomic position of 'Pseudomonas oxalaticus' strain Ox1(T) (DSM 110(T)) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia oxalatica comb, nov. Systematic and Applied Microbiology. 2000;23(2):206-9.

59. Imeria Ferro K. The impact of oxalogenic plants on soil carbon dynamics - formation of a millennium carbon storage as calcium carbonate. Neuchâtel: University of Neuchâtel; 2012.

60. Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology. 1985;49(1):1-7.

Supplementary Material

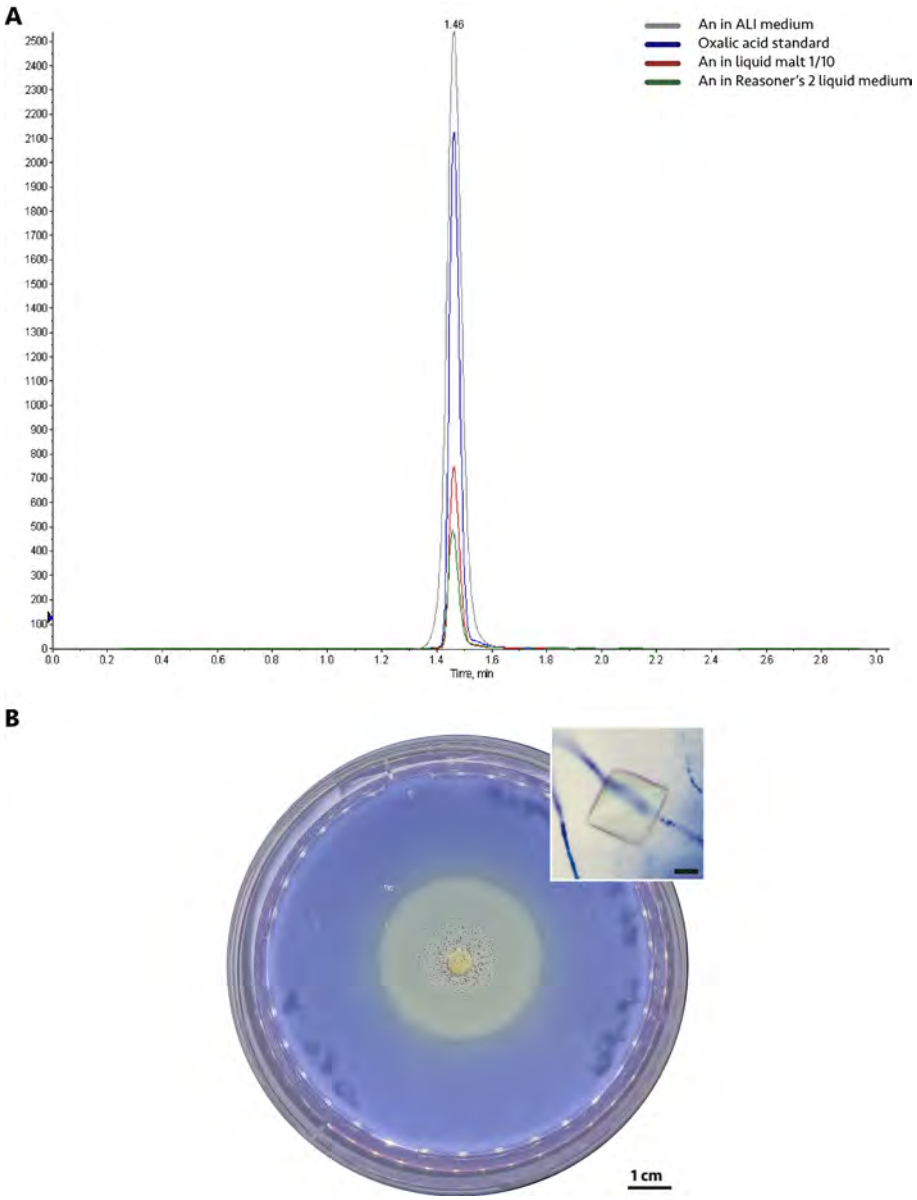


Fig. S1. Detection of oxalic acid production by *Aspergillus niger* in different culture media. (A) Ultra-High-Performance Liquid Chromatography (UHPLC) analysis confirmed the production of oxalic acid (blue chromatogram) by *A. niger* in Air-Liquid Interface (ALI, gray chromatogram), liquid malt 1/10 (red chromatogram) and liquid reasoner's 2 media (green chromatogram). (B) Production of low molecular weight organic acids (LMWOA) by *A. niger* in a water yeast agar (WYA) plate supplemented with bromocresol purple as pH indicator. A yellow color indicates an acidic pH. The presence of characteristic bipyramidal shaped calcium oxalate crystals in the agar medium confirmed the production of oxalic acid by *A. niger* in this medium. Scale bar in the insert = 10 µm.

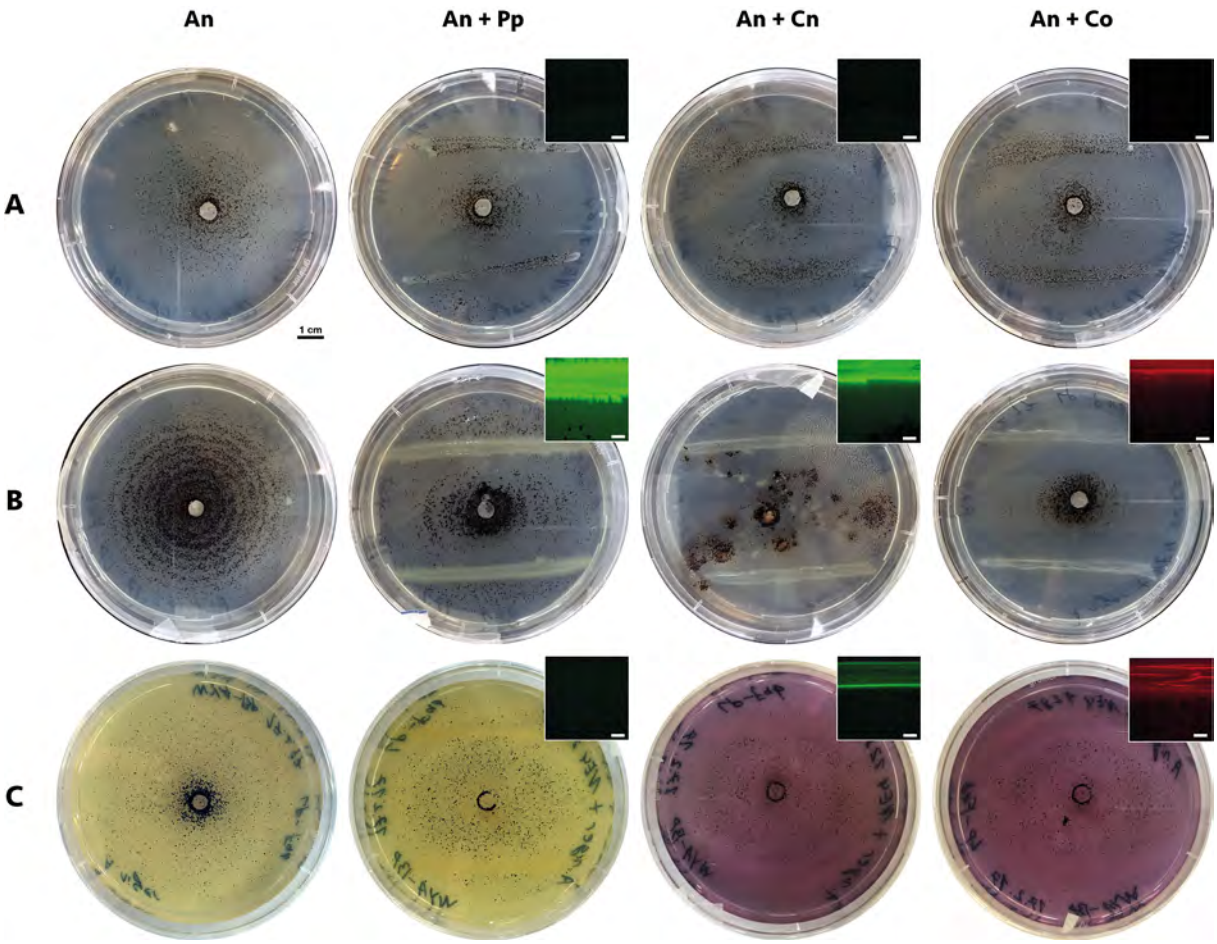


Fig. S2. Interaction between *A. niger* (An), the non-oxalotrophic bacterium *P. putida* (Pp) and the oxalotrophic bacteria *C. necator* (Cn) and *C. oxalaticus* (Co) on different culture media. Growth (and pH) inhibition depends on the nutrient medium. (A) In medium favoring the fungus (malt agar diluted 1/10 -MA 1/10-), the bacteria are all killed, regardless of their metabolism. Indeed, the lack of fluorescence (see inserts) confirms bacterial death. (B) In medium favoring the bacteria (R2A), oxalotrophic bacteria seem to control fungal growth, in contrast to the non-oxalotrophic strain. (C) In a poor-nutrient medium (WYA supplemented with Bromocresol Purple pH indicator), the oxalotrophic bacteria seem to control the LMWOA production by the fungus, as showed by the color reversal of the pH indicator (a yellow color indicates an acidic pH <6). As confirmed by the epifluorescence stereoscope observation, both oxalotrophic strains are alive, whereas the non-oxalotrophic one is dead. Scale bars in inserts: 2000µm.

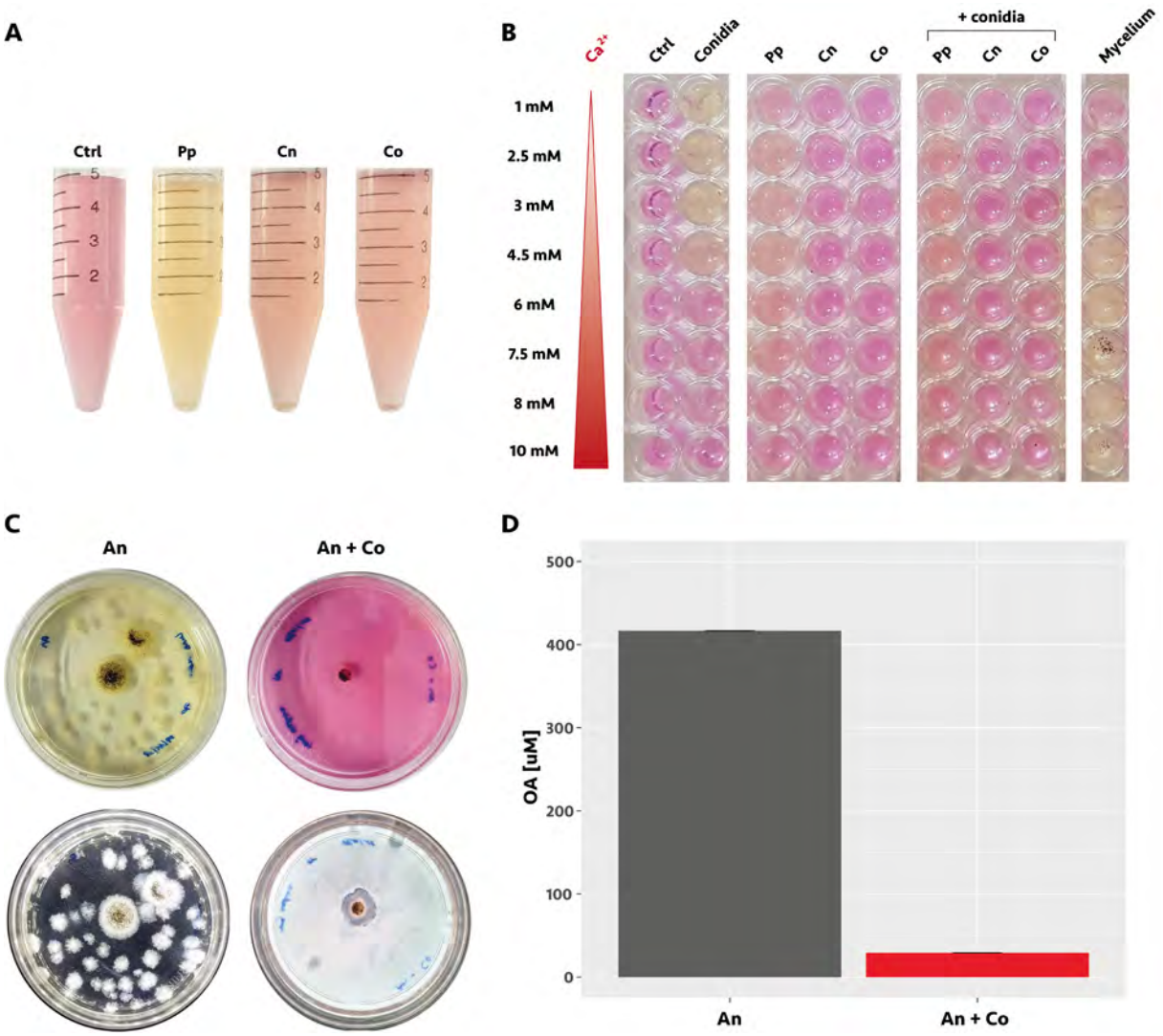


Fig. S3. Growth tests and confrontation assay of *Aspergillus niger* and *C. oxalaticus* in Air-Liquid Interface (ALI) medium. (A). The oxalotrophic bacteria (*Cupriavidus necator* –Cn- and *C. oxalaticus* –Co-) developed in ALI medium but without inducing a change in pH in contrast to non-oxalotrophic bacteria (*Pseudomonas putida* –Pp-). pH indicator= phenol red. (B). Development of the same bacterial species indicated in A, as well as *A. niger* inoculated as conidia or mycelium on ALI medium with increasing concentrations of calcium. Calcium concentration and the mode of inoculation (fungal conidia versus mycelium) has an effect on fungal development and control of fungal growth by bacteria. While conidia germination was inhibited at 6 mM Ca^{2+} and above, mycelial growth was stimulated from 3-10 mM, but inhibited at the lower Ca^{2+} concentrations. Growth of *P. putida* was also affected by Ca^{2+} (inhibition above 8 mM), but increasing Ca^{2+} concentrations did not affect growth of the oxalotrophic bacteria. When conidia were co-inoculated with the three bacterial species indicated above, all the bacteria appear to partially inhibit germination, but the effect on the stabilization of the medium pH was only observed with the two oxalotrophic bacteria. (C) The top row shows the difference in pH (yellow for acidic pH and pink for neutral) for both treatments: fungus alone (An) and fungus in co-culture with the bacterium (An+Co). The bottom row shows images of same plates on the top, but in this case, a light source was used from below the plate to better visualize the growth of the fungus in both conditions. (D) Oxalic acid concentration decreased by close to 90% in presence of the bacterium (An+Co) as compared to the control with the fungus alone (An).

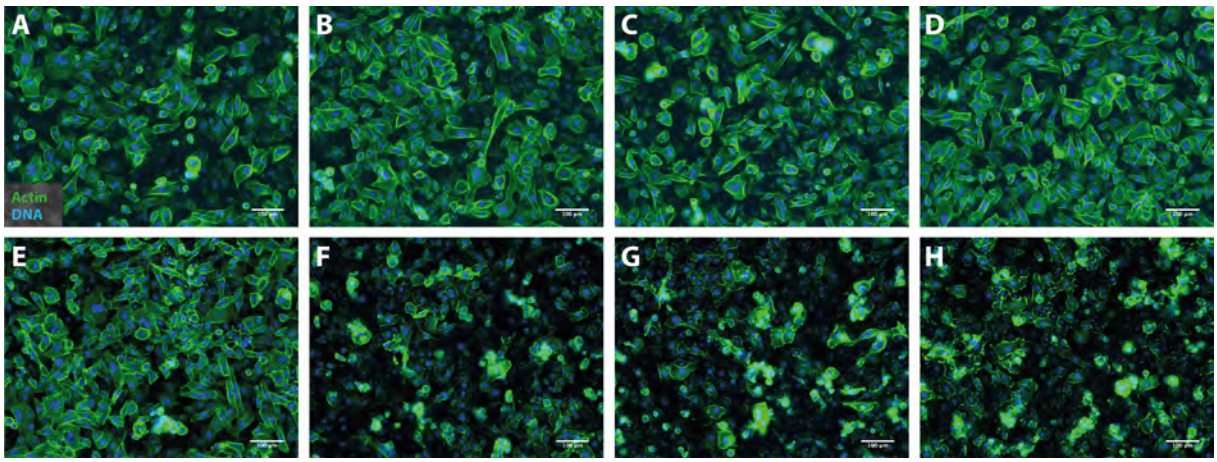


Fig. S4. Effect of increasing *A. niger* conidial load on the morphology of bronchial epithelial cells. (A) Control cells. (B) 10 conidia. (C) 50 conidia. (D) 100 conidia. (E) 500 conidia. (F) 1000 conidia. (G) 5000 conidia. (H) 10'000 conidia. After 24h incubation, an increase in cell damage with increasing conidial load was observed. Damage was visible from a conidial load of 500. Damaged cells began to shrink, and actin got more agglomerated, compared to the cells-only control. From a conidial load of 1000 and on, fungal growth has an adverse effect on tissue integrity. Culture medium volume was 200 µl/well. Scale bars = 100 µm.

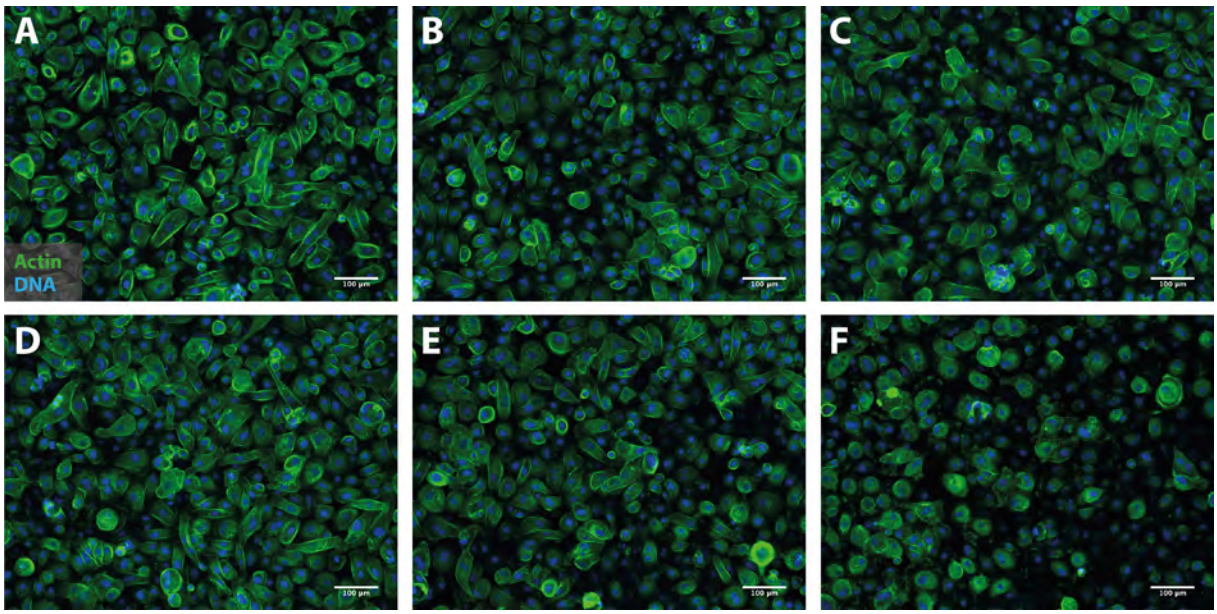


Fig. S5. Effect of increasing cell load of *C. oxalaticus* on the morphology of bronchial epithelial cells. (A) Control cells. (B) 10 bacterial cells. (C) 50 bacterial cells. (D) 100 bacterial cells. (E) 500 bacterial cells. (F) 1000 bacterial cells. After 24h incubation, an increase in cell damage with increasing bacterial cell load was observed. As few as 10 bacterial cells have already an impact on cell morphology. Indeed, cells became rounder, and actin got more agglomerated, compared to the cells-only control. However, the cytopathic effect observed in the presence of *A. niger* was less pronounced (Figure S4). Culture medium volume was 200 µl/well. Scale bars = 100 µm.

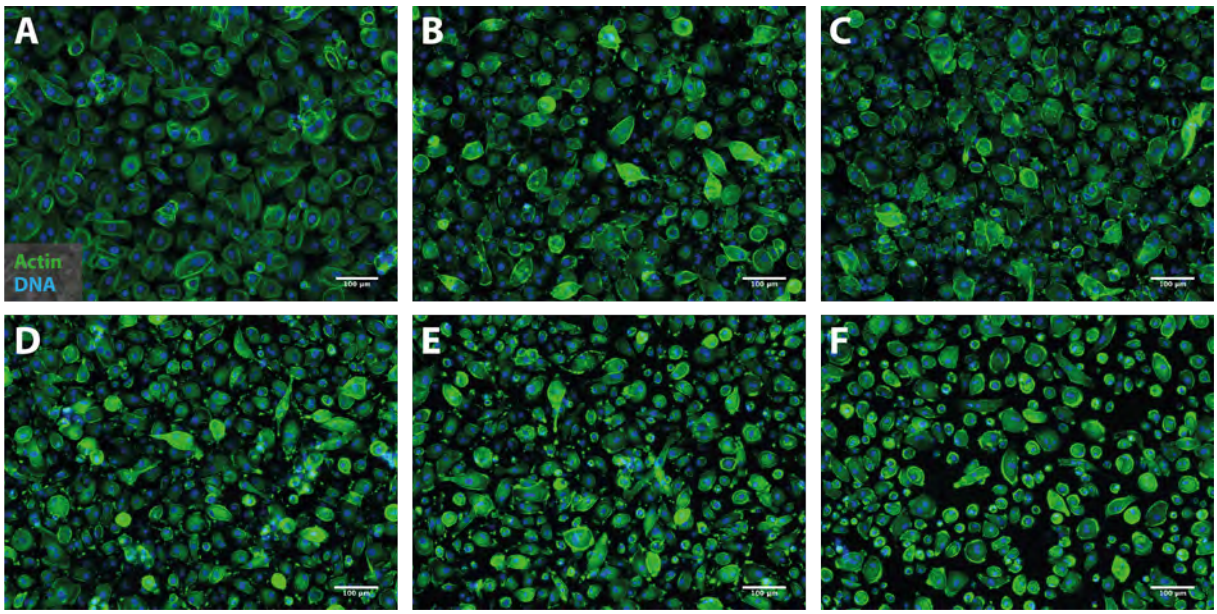


Fig. S6. Effect of increasing cell load of *P. putida* KT2440 on the morphology of bronchial epithelial cells. (A) Control cells. (B) 10 bacterial cells. (C) 50 bacterial cells. (D) 100 bacterial cells. (E) 500 bacterial cells. (F) 1000 bacterial cells. After 24h incubation, an increase in cell damage with increasing bacterial cell load was observed. As few as 10 bacterial cells have already a strong impact on cell morphology. Indeed, cells became rounder, and actin got more agglomerated, compared to the cells-only control. Culture medium volume was 200 µl/well. Scale bars = 100 µm.

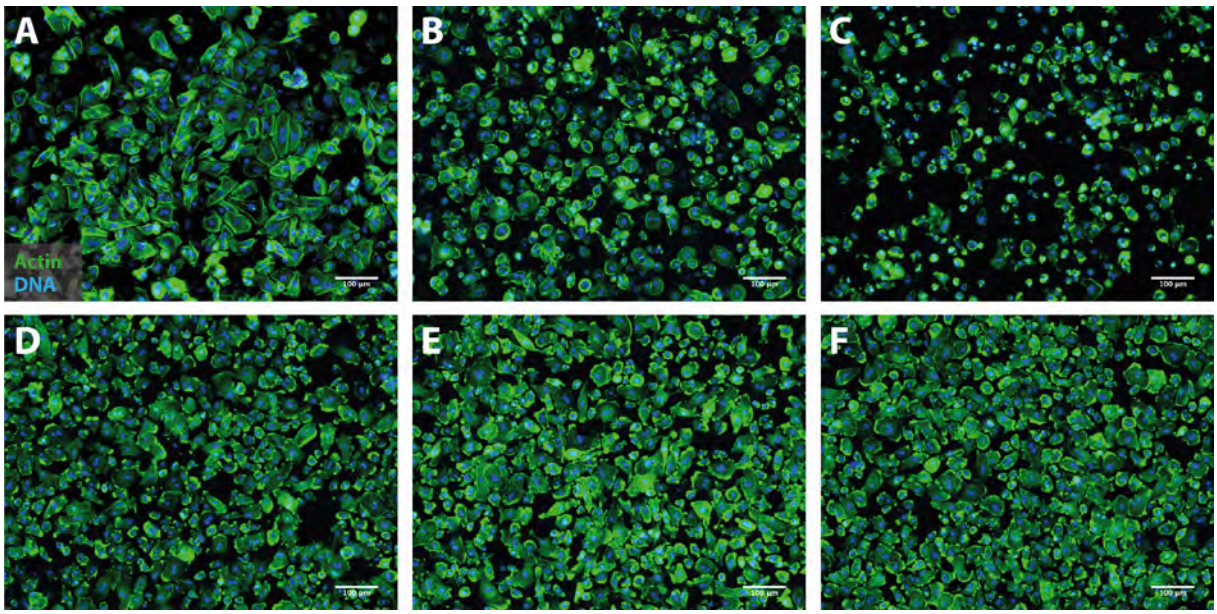


Fig. S7. Effect of co-culturing *C. oxalaticus* with *A. niger* on cell morphology in submerged undifferentiated bronchial epithelium. (A) Cell control. (B) 10 *A. niger* conidia. (C) 500 *A. niger* conidia. (D) 10 *C. oxalaticus* cells. (E) 10 *A. niger* conidia + 10 *C. oxalaticus* cells. (F) 500 *A. niger* conidia + 10 *C. oxalaticus* cells. After 72h incubation, the damage and cytopathic effect of *A. niger* conidia was clearly visible with as few as 10 conidia per well (200 µl) (B). The cells appear even more damaged with a conidial load of 500 (C). Ten *C. oxalaticus* cells also changed the morphology of the epithelial cells, but no cytopathic effect was observed (D). With the co-inoculation of as few as 10 *C. oxalaticus* cells (E and F), the morphology of bronchial cells infected with *A. niger* was similar the morphology of bacteria-only control. Culture medium volume was 200 µl/well. Scale bars = 100 µm.

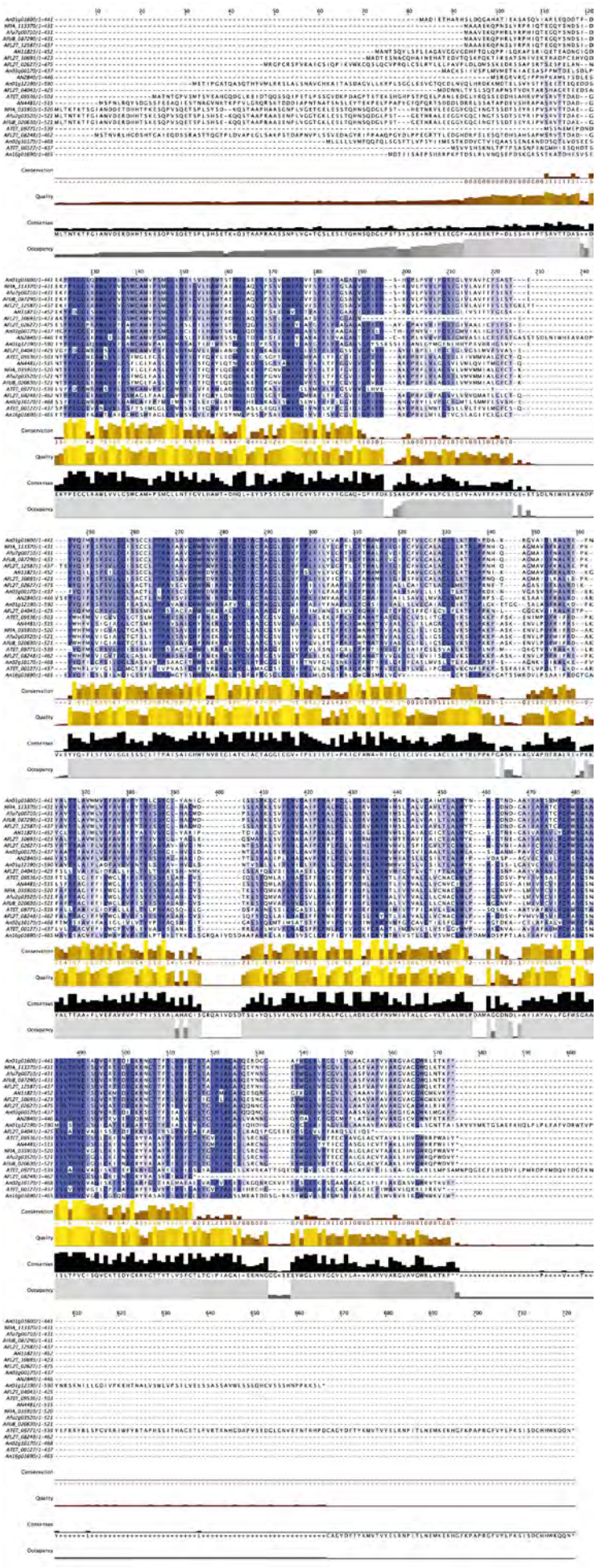
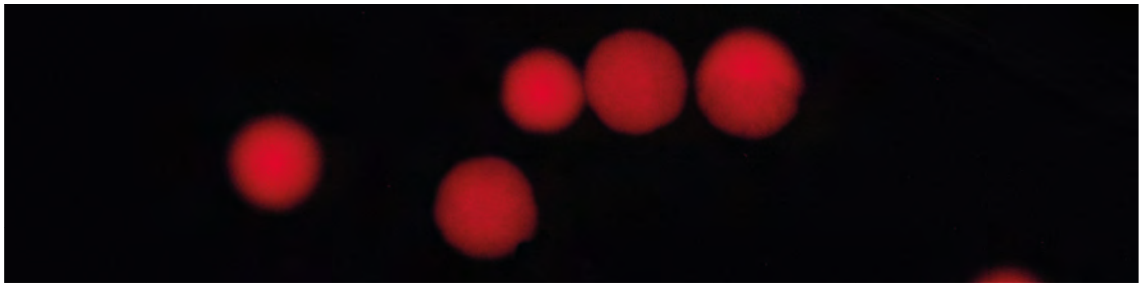


Fig. S8. Genomic screening of the oxalate/formate antiporter in other *Aspergillus* spp. Multiple sequence alignment of the protein sequences orthologous to the oxalate/formate antiporter of *A. niger* CBS 513.88 (GenBank accession number XP_001388599.2) revealed they were conserved to a lesser extent as compared to OAH, as indicated by an intense purple color of the amino acids. Multiple sequence alignments were performed using the MUSCLE protein alignment algorithm in Jalview (version 2.11.1.2). An = *A. niger* CBS 513.88, NFIA = *Neosartorya fisheri* NRRL 181 (formerly *A. fisheri*), Afu = *A. fumigatus* Af293, AFUB = *A. fumigatus* A1163, AFL2T = *A. flavus* NRRL 3357, AN = *A. nidulans* FGSC A4, ATET = *A. terreus* NIH2624.

CHAPTER 5



Genome analysis of *Cupriavidus oxalaticus* strain Ox1

Foreword

In this chapter, we present the analysis of the full genomes of *Cupriavidus oxalaticus* strain Ox1 and its isogenic mCherry-tagged mutant (*C. oxalaticus* Ox1 mCherry), and we investigated oxalotrophy in this model bacterial species. Moreover, we identified markers of oxalotrophy that could be used to evaluate this metabolism in the lung bacterial microbiota.

The key findings of this chapter are:

- The oxalyl-CoA decarboxylase (Oxc), formyl-CoA transferase (Frc), and the oxalate/formate antiporter (OxlT) were organized in an operon. Indeed, a promoter, as well as Rho-independent bacterial terminators, were predicted upstream of the oxc gene, and downstream from the frc and oxlT genes. Moreover, several transcriptional regulators were found in the vicinity of the oxc, frc and oxlT genes.
- The use of a mCherry-tagged isogenic strain of *C. oxalaticus* Ox1 permitted to confirm the ability of this soil bacterium to move along fungal hyphae in order to access calcium oxalate crystals as sole carbon source.

5. Genome analysis of Cupriavidus oxalaticus strain Ox1

Fabio Palmieri[1], Pauline Udriet[1], Shannon L. Johnson[2], Karen Davenport[2], Patrick S. G. Chain[2], Saskia Bindschedler[1], Pilar Junier[1].

[1] Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland. [2] Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA.

Abstract

Oxalic acid is the most common and ubiquitous low molecular weight organic acid in nature. In soils, it is produced predominantly by plants and fungi and it mostly occurs in the form of calcium oxalate (CaOx). Oxalate can be metabolized both aerobically and anaerobically by a very specialized group of bacteria called oxalotrophic bacteria. Oxalotrophy, the ability of these bacteria to degrade oxalate, involves the action of two main enzymes: the formyl-CoA transferase (Frc), and the oxalyl-CoA decarboxylase (Oxc). These enzymes participate in the decarboxylation of oxalate into formate and CO_2. The biochemistry of oxalotrophy has been extensively studied in the aerobic bacterial species *Cupriavidus oxalaticus* Ox1, formerly named *Pseudomonas oxalaticus*. In contrast, the genomic analysis of the genes encoding the key enzymes involved in this metabolism is still lacking. In this chapter, we analyzed the genome of *C. oxalaticus* strain Ox1 and more specifically the organization of the genes related to oxalate degradation. The genome analysis of *C. oxalaticus* Ox1 revealed that the genes for the enzymes related to oxalate degradation, i.e. *frc*, *oxc* and *oxlT* (a gene coding for an oxalate/formate antiporter), are organized in a putative transcriptional operon that also includes several transcriptional regulators. Further analysis of the genome revealed the existence of genes coding for virulence factors, such as for instance, those participating in the production of β-hemolysin or a macrophage infectivity potentiator-related protein, suggesting a pathogenic potential of this strain. This study provides valuable insights into the key genetic components required for designing a purely enzymatic oxalotrophic system to reduce the risk of using living bacteria in a future therapeutic application.

5.1. Introduction

Oxalic acid is the most common and ubiquitous low molecular weight organic acid (LMWOA) produced by fungi, bacteria, plant and animals. Accordingly, it occurs in various environments in which these organisms are common, including soils or even the gastrointestinal tract of many animals (1, 2). Since oxalic acid is a relatively strong organic acid (pKa_1 = 1.27 and pKa_2 = 4.27), in the environment, it is mostly found as its conjugated base, oxalate (3). Oxalate can form several salts with metal ions, such as Ca^{2+}, K^{+}, Mg^{2+}, Cu^{2+}, depending on their availability and the solubility products of the corresponding salts (1, 2). In soils, oxalic acid is predominantly produced by plants and fungi. It accumulates in the form of calcium oxalate (CaOx) crystals, which are the most commonly found metal oxalates in terrestrial ecosystems (4). Despite their low solubility (Ksp of calcium oxalate monohydrate = 2.32×10^{-9}) (5, 6), CaOx crystals rarely accumulate in the environment, something that has been suggested to be the result of their metabolization by oxalotrophic bacteria (7). In contrast to other LMWOAs (e.g. acetate, citrate or succinate), which are used by a large diversity of bacteria, the use of oxalate or oxalic acid as a carbon and energy source is exclusively found in oxalotrophic bacteria (2, 7). Oxalotrophy, the ability to degrade oxalic acid, is not a phylogenetic trait and has been shown to be both species and strain specific (2, 8). Oxalotrophic bacteria have been found among representatives of the phyla *Actinobacteria*, *Firmicutes* and *Proteobacteria* (2).

Oxalotrophic bacteria are highly abundant in soils. This is true especially in the vicinity of plant roots, as highlighted for instance by Sun *et al.* (9) in the case of the ectomycorrhizosphere of forest trees, or in association to oxalogenic fungi (2). Oxalotrophic bacteria are also highly abundant in the mammalian gut. For instance, in the case of humans, oxalate enters the body either through the consumption of oxalate-rich food, or it is produced endogenously in the liver. Humans do not have the capability of degrading oxalate. Instead, this function is carried out by gastrointestinal oxalotrophic bacteria such as *Oxalobacter formigenes* (1, 10). The gastrointestinal tract of the woodrat *Neotoma albigula*, a mammalian herbivore that feeds on oxalate-rich plants, also harbors a large diversity of oxalotrophic bacteria belonging to the genera *Lactobacillus*, *Enterococcus* and *Clostridium* (11).

Oxalate degradation via the decarboxylation of oxalate into formate and CO_2 can occur either aerobically or anaerobically and include two principal steps: (1) oxalate is first transformed into oxalyl-CoA by the transfer of CoA from formyl-CoA by the formyl-CoA transferase (Frc, EC 2.8.3.16). This step results also in the production of formate; and (2) oxalyl-CoA is then decarboxylated to formyl-CoA and CO_2 by the oxalyl-CoA decarboxylase (Oxc, EC 4.1.1.8) (12-15). Thiamine pyrophosphate (ThPP) is a cofactor of the Oxc (1, 10). In aerobic bacteria in order to produce energy, the formate dehydrogenase (Fdh, EC 1.17.1.9)

oxidizes formate into CO_2 and NADH. This is coupled to ATP generation by oxidative phosphorylation with O_2 as a terminal electron acceptor (16). In anaerobic bacteria, oxalate fermentation is linked to the excretion of formate outside the cell by the oxalate/formate antiporter OxlT (17). This oxalate-formate transport is coupled with the decarboxylation of oxalate, which consumes "protons" inside the cell and generates a proton pump gradient that is exploited by the cell for ATP production via an ATPase in the membrane (8). In either case, the energy yield of oxalotrophy is extremely low. Finally, in the anabolic reactions leading to biomass production, oxalyl-CoA is reduced in glyoxylate by the oxalyl-CoA reductase (Oxr, EC 1.2.1.17), which is then further transformed into cell constituents and integrated in cell biomass (18).

Both the *oxc* and *frc* genes, which encode the enzymes Oxc and the Frc, respectively, have been shown to co-occur in an oxalate degradation operon in a couple of bacterial species (1, 2). This is notably the case of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM (19, 20). Indeed, a putative promoter, as well as a high-energy rho-independent terminator have been predicted upstream of *oxc* and downstream of *frc*, respectively, indicating that this set of genes can be transcribed simultaneously (19). In contrast, no putative oxalate permease or oxalate/formate antiporter could be identified in this bacterial species (19). Transcriptional analyses showed that the expression of both genes involved in oxalate degradation in *L. acidophilus* NCFM seems to be pH dependent. Induction of these genes occurred in cells that were initially adapted to sub-inhibitory concentrations of oxalate, and then exposed to a pH of 5.5 (19). The *oxc* and *frc* genes were also shown to cluster together, and thus potentially constituting an operon, in *Bradyrhizobium japonicum* and *Streptomyces corchorusii* (21). However, previous analyses in *Oxalobacter formigenes* showed that the *oxc* and *frc* genes did not form an operon (21).

The understanding of the metabolic pathway of oxalotrophy in anaerobic bacteria, such as *O. formigenes*, has advanced simultaneously with the study of the organization of the genes involved in this metabolic process. In the case of aerobic bacteria, previous work from Quayle (12), Quayle (13), Blackmore *et al.* (14), and Dijkhuizen *et al.* (15) was seminal for the biochemical description of oxalotrophy in *C. oxalaticus* Ox1 (previously known as *Pseudomonas oxalaticus* Ox1, or *Ralstonia oxalatica*). This oxalotrophic bacterium was isolated from the intestinal tract of an Indian earthworm in 1953 (22, 23). Despite the fact that its metabolism has been extensively studied, the description of the genomic determinants of oxalotrophy in *C. oxalaticus* Ox1 is still lacking. Thus, the aim of this chapter was to analyze the genome of this soil bacterium, as well as the genomic organization of the genes involved in oxalotrophy.

5.2. Materials & Methods

5.2.1. Bacterial strains

Two *Cupriavidus oxalaticus* Ox1 strains – NEU 1047 (WT) and NEU 1287 (mCherry) – were obtained from the collection of Laboratory of Microbiology of the University of Neuchâtel (LAMUN). *C. oxalaticus* Ox1 strain NEU 1287 is a mCherry fluorescent isogenic mutant of *C. oxalaticus* Ox1 strain NEU 1047. The fluorescence label allows to track it visually. *C. oxalaticus* Ox1 strain NEU 1287 was tagged *in-house* using insertion with a Mini-Tn7-*mcherry* system contained in the delivery plasmid pME9407 (24). Both bacterial strains were routinely cultured on Nutrient Agar (NA, Carl Roth, Karlsruhe, Germany) medium.

5.2.2. Whole-genome sequencing

High molecular weight genomic DNA of both strains of *Cupriavidus oxalaticus* Ox1 was extracted using the QIAGEN Genomic-tip 20/G kit, following the manufacturer's instructions. All buffers required for bacterial genomic DNA extraction were prepared in-house according to the manufacturer's instruction. Prior to resuspension in Buffer B1, the bacterial pellets were washed with PIV solution (10mL 1M Tris-HCl pH 8 and 58.4g NaCl in 990mL MilliQ® water). Both bacterial genomes were sent for sequencing to the Lausanne Genomic Technologies Facility (University of Lausanne, https://www.unil.ch/gtf/en/home.html). Sequencing was performed using a PacBio SEQUEL system. De novo microbial assembly was performed on SMRT-Link (version 9.0.0.92188) using SMRT-Tools (version 9.0.0.92188). Default parameters were used, except for Genome Length, which was set at 8'500'000 instead of 5'000'000, respectively. Annotation was performed in the EDGE Bioinformatics platform (25).

5.2.3. Nucleotide sequence accession numbers

This whole-genome sequencing project has been deposited at GenBank under the Bioproject no. PRJNA695296. The genome assemblies of *C. oxalaticus* Ox1 and *C. oxalaticus* Ox1 mCherry are available under the accession no. GCA_016894385.1 and GCA_016894365.1, respectively.

5.2.4. Genome sequence analysis and comparative genomics

The annotated genome sequences of both *C. oxalaticus* Ox1 strains were analyzed using the RAST server (26) and the EDGAR 2.0 software platform (27). The additional whole genome sequences presented in Table 1 were selected based on the genomes presented in Abratt *et al.* (8), as well as strains that are present in the culture collection of the Laboratory of Microbiology of the University of Neuchâtel, and added to EDGAR in order to compare the genes related to oxalotrophy (i.e. *oxc*, *frc* and *oxlT*).

Escherichia coli K-12 and *Pseudomonas putida* KT2440 were used as negative strains for oxalotrophy for the comparative genomics. Bacterial sigma70 promoter and Rho-independent bacterial terminators were predicted using the web tools BPROM and FindTerm (http://www.softberry.com/), respectively (28).

Table 1. Strains used for comparative genomics

Strain	BioProject (NCBI)
Cupriavidus oxalaticus T2	PRJNA488920
Cupriavidus oxalaticus X2	PRJNA525272
Oxalobacter formigenes OXCC13	PRJNA353994
Lactobacillus acidophilus ATCC 4796	PRJNA31477
Bifidobacterium lactis DSM 10140	PRJNA32893
Providensia rettgeri DSM 1131	PRJNA29299
Burkholderia phytofirmans PSJn	PRJNA17463
Ammoniphilus oxalaticus RAOx-1	PRJNA336123
Cupriavidus necator H16	PRJNA531660
Escherichia coli K-12	PRJNA596906
Pseudomonas putida KT2440	PRJNA267

5.2.5. Metabolic tests

5.2.5.1. Hemolysis

Hemolysis was tested by streaking both *C. oxalaticus* Ox1 strains (WT and mCherry) on Columbia agar plates with 5% sheep blood (bioMérieux SA, France). The inocula were prepared from an overnight culture of both bacteria on NA. The plates were incubated for six days at 37°C. *Bacillus subtilis* (NEU1) and *Streptococcus agalactiae* (NEU1203) were used as positive controls for β-hemolysis, *Escherichia coli* (NEU1006) was used as positive control for α-hemolysis, and *Staphylococcus epidermidis* (NEU1229) was used as negative control (γ-hemolysis).

5.2.5.2. Production of siderophores

Siderophore production was assessed by streaking both *C. oxalaticus* Ox1 strains on CAS agar medium, which was prepared as described in Schwyn *et al.* (29). The inocula were prepared from an overnight culture of both bacteria on NA. *Escherichia coli* NEU1006 and *S. epidermidis* NEU1229 were used as positive and negative controls, respectively. Plates were incubated at 37°C for six days.

5.2.5.3. Antimicrobial resistance

An antimicrobial susceptibility test was performed by spreading homogeneously bacterial suspensions with an optical density equal to 0.5 MacFarland of both *C. oxalaticus* Ox1 strains using a sterile swab onto Mueller Hinton agar plates (38 g/L, Carl Roth, Karlsruhe, Germany). Then, ampicillin (10 µg), chloramphenicol

(30 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 units/disk, ROTI® Antibiotic Discs Penicillin G (P), Carl Roth, Karlsruhe, Germany) and vancomycin (30 µg) antimicrobial susceptibility disks (Thermo Scientific™ Oxoid™ Antimicrobial Susceptibility Disks, Fisher Scientific, Switzerland) were placed onto the streaked plates. A five µg ciprofloxacin disk was obtained by adding 30 µL of 0.166 mg/mL ciprofloxacin to a blank disk (Thermo Scientific™ Oxoid™ Blank Antimicrobial Susceptibility Disks, Fisher Scientific, Switzerland). A blank disk soaked in physiological water (9 g/L NaCl) was used as negative control. Plates were incubated at 37°C for 24 and 48 h and the inhibition halo diameter was recorded for each disk.

5.2.5.4. Motility test

Motility was assessed through the TTC motility test. TTC motility medium was composed of 10 g/L casein peptone, 5 g/L NaCl, 3 g/L yeast extract, 0.05 g/L TTC, and 3 g/L technical agar in 1L deionized water. Ten mL of TTC medium were distributed in test tubes prior to autoclaving. Bacterial strains were inoculated with an inoculation needle by stabbing down the center of the medium to approximately half of the height of the medium. *Enterococcus faecalis* NEU1012 and *P. putida* KT2440 NEU1264 were used as negative and positive controls, respectively. Tubes were incubated at 37°C for six days. A non-inoculated control was also kept and incubated at 37°C for the same time.

Additionally, the ecological advantage of flagellar motility of *C. oxalaticus* Ox1 was linked to its ability to access and degrade calcium oxalate (CaOx) alone or in association to dispersal along a mycelial network. To do so, five µL of a 10^6 *C. oxalaticus* Ox1 mCherry cells per µL suspension was inoculated as a spot onto bi-layered Angle medium containing 4 g/L CaOx, and a six days pre-grown *Trichoderma rossicum* mycelial network. The first layer (approx. 15 mL) of the Angle medium was prepared as explained in the Supplementary material of Chapter 3, but without glucose. The second layer (approx. five mL) contained Angle medium which was supplemented with 4 g/L CaOx as the sole carbon source. The plates were incubated at room temperature for 21 days and oxalate degradation was assessed visually. *C. oxalaticus* Ox1 mCherry growth and dispersal extent was assessed using a Nikon SMZ18 epifluorescence stereoscope, connected to a Nikon DS-Ri2 camera.

5.3. Results & Discussion

5.3.1. Genome assembly and annotation

PacBio sequencing yielded 2 contigs for both strains, with a total assembly size of 6’694’750 bp and 6’697’997 bp for *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry, respectively (Table 2). 5’944 and 5’948 coding sequences (CDS) were identified for *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry,

respectively. From those, 2'641 and 2'645 corresponded to hypothetical proteins, respectively (Table 2). The genome size of *C. oxalaticus* strain Ox1 is smaller than the two other complete whole genome sequences of *C. oxalaticus* (strains X32 and T2) available in NCBI. Those genomes are reported to contain 7'968'737 bp and 7'296'847 bp, respectively.

Table 2. Summary statistics of the assembly and annotation of the genomes of C. oxalaticus Ox1 WT and mCherry

	***C. oxalaticus* Ox1 WT**	***C. oxalaticus* Ox1 mCherry**
Assembly		
Number of contigs	2	2
N50	3'885'446 bp	3'888'701 bp
Max contig size	3'885'446 bp	3'888'701 bp
Min contig size	2'809'304 bp	2'809'296 bp
Total assembly size	6'694'750 bp	6'697'997 bp
Annotation		
CDS	5'944	5'948
Hypothetical proteins	2'641	2'645
rRNA	15	15
tRNA	73	73

An analysis of the core genome of *C. oxalaticus*, revealed that the core genome of the species corresponds to 4'558 CDS. Strain Ox1 shares more CDS with the strain T2 (5'312 shared CDS), as compared to the strain X32 (4'670 shared CDS) (Fig 1). This is corroborated by a phylogenetic analysis based on the whole genome of the three *C. oxalaticus* strains and *Cupriavidus necator* strain H16, with *P. putida* strain KT2440 used as an outgroup (Fig. 2).

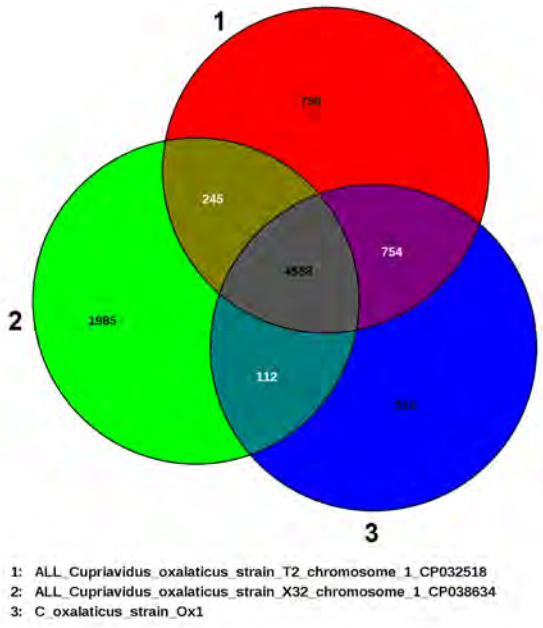


Fig. 1. Venn-diagram showing the overlap in CDS content between *C. oxalaticus* Ox1, *C. oxalaticus* X32 and *C. oxalaticus* T2. This Venn-diagram indicates that *C. oxalaticus* Ox1 shares a larger fraction of its genome with *C. oxalaticus* T2 than with *C. oxalaticus* X32, as they share 5'312 CDS, compared to 4'670 shared CDS with *C. oxalaticus* X32.

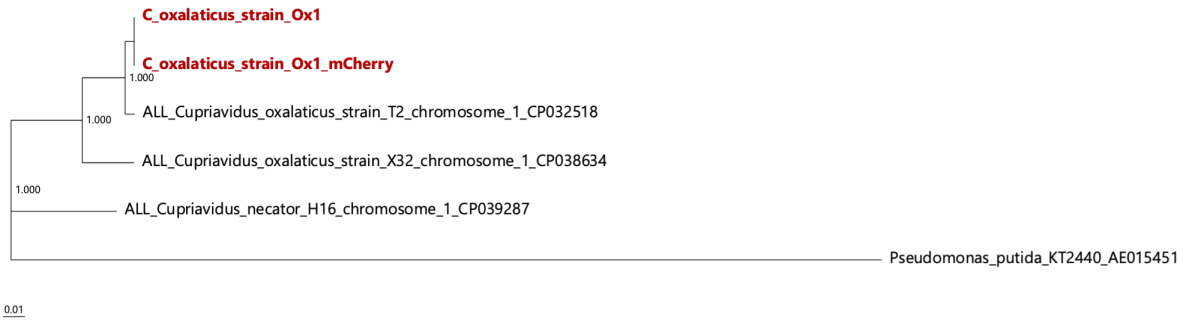


Fig. 2. Phylogenetic position of *C. oxalaticus* Ox1 WT and mCherry compared to other *C. oxalaticus* strains. Both strains *C. oxalaticus* Ox1 (in red) are more closely related to *C. oxalaticus* T2 than to *C. oxalaticus* X32. *P. putida* KT2440 was used as an outgroup.

Analysis of the genome with the RAST server produced an overview of putative functions. The number of predicted CDS differed from those predicted with the EDGE platform, but this is not unexpected as the number of annotated CDS can vary depending on the annotation tool used (30). The pie chart in Fig. 3 represents all annotated CDS that could be classified into 27 subsystems corresponding to specific metabolic or physiological pathways (52% of the CDS). The remaining 48% of the annotated CDS could not be classified in the listed subsystems. Among the CDS with a predicted function, we found genes related to the transport of fumarate, L-(D-)malate, succinate and citrate, indicating a potential use of these compounds as carbon source (Table S1) (7, 31). We also found genes involved in Type IV pili (T4P)-mediated twitching motility (Table S1). T4P have been shown to be a major virulence factor for epithelial cell adhesion and biofilm formation through twitching motility in *P. aeruginosa* (32). A number of phage-related genes coding for tail proteins, integrase, lysin or peptidoglycan binding protein for instance have been found in the genome of *C. oxalaticus* Ox1, indicating the presence of multiple prophages (Table S1). Genes coding for bacterioferritins, which are iron storing proteins (33), were also detected (Table S1). Two CDS have been annotated as immune-responsive protein 1 (Table S1). Finally, a macrophage infectivity potentiator-related protein has also been detected (Table S1). Macrophage infectivity potentiator protein (Mip) has been shown to be a major virulence factor in *Legionella pneumoniae,* which is required for an optimal growth of the bacterial pathogen inside alveolar macrophages (34). The presence of this gene indicates potential pathogenicity of the *C. oxalaticus* strain. Indeed, several *Cupriavidus* spp. strains are described to be pathogenic and to be involved in invasive bacteraemia and pneumonia (35-38).

The two genomes of *C. oxalaticus* Ox1 WT and mCherry were compared using a circular plot (Fig. 4). The core genome (red ring) includes 99.88% and 99.81% of the genomes of *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry, respectively (identical fraction). The two other rings represent the GC-content and GC-skew data. The mean GC-content for both *C. oxalaticus* strains amounts to 66.9%. The GC-skew data indicates if the guanine and cytosine nucleotides are over- or under-abundant in 1000 bp window. Based on this matrix, the genome can be divided into four regions with positive and negative GC skew,

in alternance. This indicated that the genome of *C. oxalaticus* Ox1 is most probably composed of two leading (largely positive GC skew) and two lagging (largely negative GC skew) strands (39).

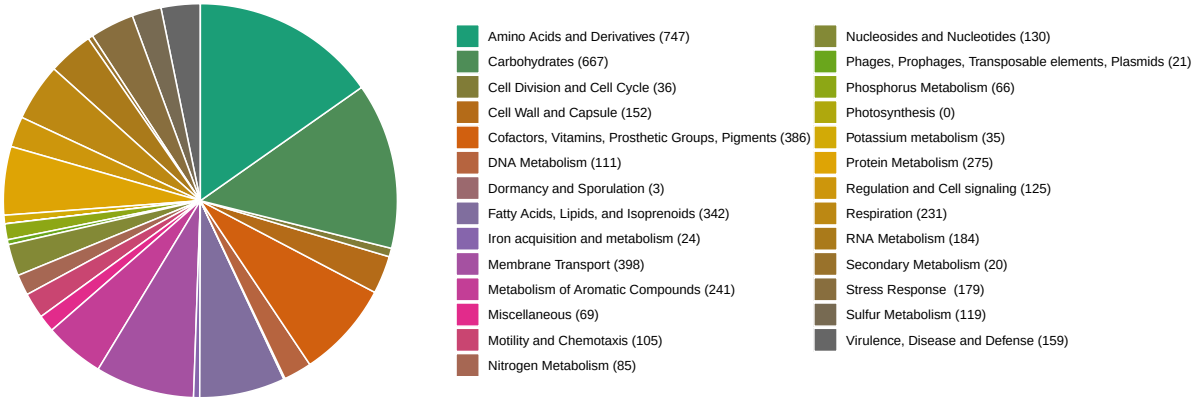


Fig. 3. Functional classification of annotated CDS using annotation in the RAST server. The annotated CDS are classified into 27 subsystems corresponding to specific functional categories. The total number of CDS identified in RAST are 6'103 and 6'111, of which 1418 and 1424 are hypothetical, for *C. oxalaticus* Ox1 WT and mCherry, respectively.

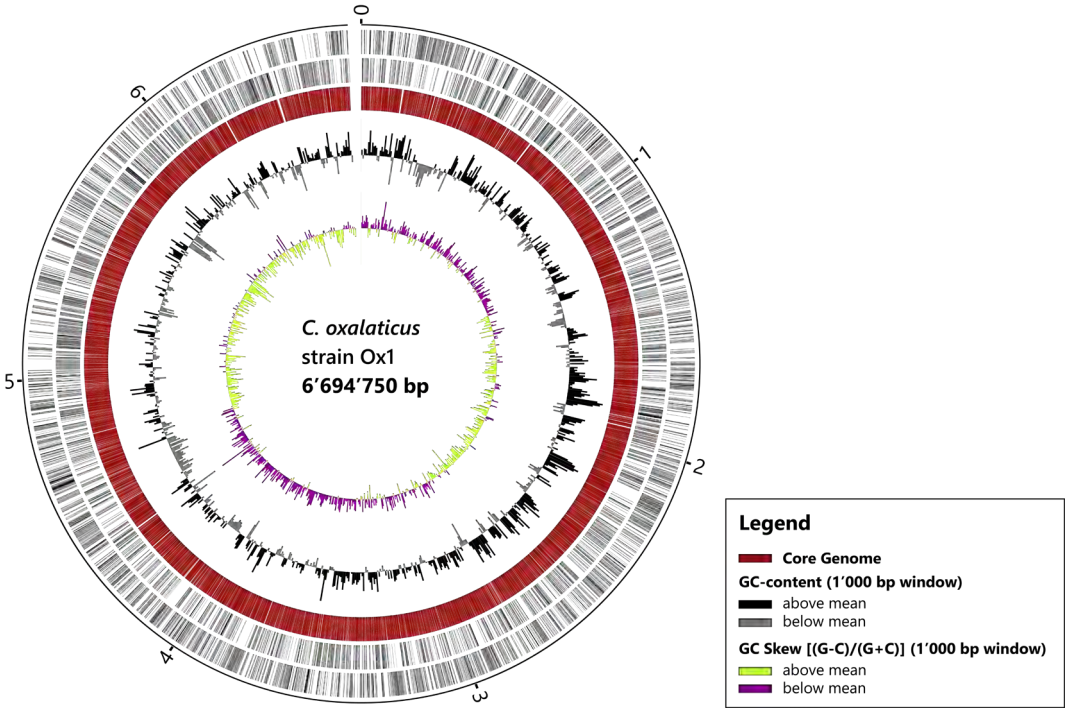


Fig. 4. Circular plot of the genomes of *Cupriavidus oxalaticus* Ox1 WT & mCherry. The two outer rings indicate coding sequences (CDS) on both "+" and "-" strands. The red ring indicates the core genome, i.e. the genes present in both genomes, the black/gray bars ring the GC-content, and the purple/green bars ring the GC skew. The almost entirety of the genome is identical among both strains (5926 shared genes, 99.88% and 99.81% of the genomes of *C. oxalaticus* Ox1 and *C. oxalaticus* Ox1 mCherry, respectively). The average GC-content of both strains is of 66.9%.

In order to identify genes only present in one specific genome, a Venn-diagram analysis was performed. The analysis showed that 7 and 11 CDS were unique to *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry, respectively (Fig. 5). These unique genes are listed in Table 3. Both strains have transposases, which are known to be associated with the transposition of antimicrobial resistance genes to plasmids

and their spread in the environment (40). The WT strain of *C. oxalaticus* Ox1 possesses two genes related to the transport of 4-hydroxybenzoate and sialic acid, as well as one gene related to purine biosynthesis. Moreover, a paralog of the DNA gyrase subunit B (*gyrB*), one of two subunits composing the tetrameric DNA gyrase enzyme, is present in the genome of *C. oxalaticus* Ox1 WT. Mutations in the DNA gyrase subunits *gyrA* and *gyrB* are known common resistance mechanisms to fluoroquinolones, especially in *Mycobacterium tuberculosis* (41). This could indicate a potential resistance mechanism to fluoroquinolones such as ciprofloxacin in this strain. The mCherry-tagged strain has genes related to the uptake of 4-hydroxybenzoate and the catabolism of L-arabinose. It also possesses a gene coding for the Type VI secretion protein IcmF-related protein, which is the intracellular multiplication factor (IcmF) of the Type VI secretion system (T6SS) related to eukaryotic cell invasion (42, 43). The three other CDS, only found in the mCherry-tagged strain, are the Lactose operon repressor *lacI*, the mCherry fluorescent protein and the Gentamicin 3-N-acetyltransferase. These three genes are all part of Mini-Tn7 transposon system used for the insertion of the mCherry fluorescent protein. Indeed, the pME9407 plasmid used contains a P_{tac} promoter that is repressible by the Lactose operon repressor (44, 45), which implies that the fluorescence can be repressed in the presence of lactose, something that has not yet been tested in our strains. Moreover, the insertion of genes with the Mini-Tn7 transposon system is targeted. Indeed, the target site of the Tn7 transposon is situated upstream of the *glmSU* genes (44, 46), something that was confirmed by the genome sequencing (Fig. 6). With the exception of the mCherry expression, both strains have been considered as "isogenic", as both derive from the same parental strain. After genome sequencing, we can say that this is globally true. Several unique genes in the WT strain are also present in the mCherry strain (especially the transposases and the 4-hydroxybenzoate transporter), confirming that the strains are largely "isogenic".

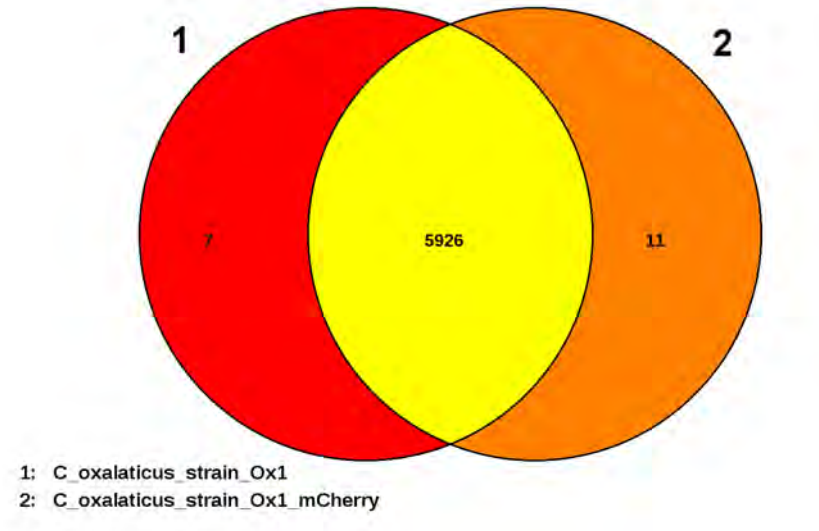


Fig. 5. Venn-diagramm comparing the genomes of *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry. Seven and 11 genes are unique to *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry, respectively.

Aside the presence of the mCherry tag, the gentamicin resistance gene and the lactose operon repressor, the other differences might be due to independent genetic drift in both strains.

Table 3. Genes unique to *C. oxalaticus* Ox1 WT and *C. oxalaticus* Ox1 mCherry

CDS ID	Annotation	Function
Genes only in *C. oxalaticus* Ox1 WT		
Fabio_yZM_00312	4-hydroxybenzoate transporter PcaK	Uptake of 4-hydroxybenzoate (4-HB)
Fabio_yZM_00313	Putative sialic acid transporter	Sialic acid transmembrane transporter activity
Fabio_yZM_00528	IS110 family transposase ISRta3	Gene transposition
Fabio_yZM_03825	IS110 family transposase ISRta3	Gene transposition
Fabio_yZM_05357	Phosphoribosylformylglycinamidine cyclo-ligase	Purine (inosine monophosphate) biosynthesis
Fabio_yZM_05490	IS4 family transposase ISAzo5	Gene transposition
Fabio_yZM_06033	DNA gyrase subunit B (gyrB)	Supercoiling of chromosomal DNA, resistance to fluoroquinolones
Genes only in *C. oxalaticus* Ox1 mCherry		
Fabio_M9m_00861	L-arabonate dehydratase	Degradation of L-arabinose
Fabio_M9m_01932	IS110 family transposase ISRta3	Gene transposition
Fabio_M9m_02147	4-hydroxybenzoate transporter PcaK	Uptake of 4-hydroxybenzoate (4-HB)
Fabio_M9m_02376	Type VI secretion protein IcmF-related protein	Intracellular multiplication factor of the type VI secretion system
Fabio_M9m_02929	IS4 family transposase ISAzo5	Gene transposition
Fabio_M9m_04369	Hypothetical protein	N.A.
Fabio_M9m_04594	IS110 family transposase ISRta3	Gene transposition
Fabio_M9m_05739	Acetone carboxylase beta subunit	Carboxylation of acetone to form acetoacetate
Fabio_M9m_05771	Lactose operon repressor (lacI)	Repressor of the lactose operon
Fabio_M9m_05772	mCherry fluorescent protein	Fluorescent tag
Fabio_M9m_05773	Gentamicin 3-N-acetyltransferase (aacC1)	Resistance to gentamicin

Fig. 6. Genomic organization of the inserted mCherry fluorescent protein. As predicted, the Mini-Tn7 insertion site lies upstream of the *glmSU* genes. *lacI* = Lactose operon repressor; *mcherry* = mCherry fluorescent protein; *aacC1* = Gentamicin 3-N-acetyltransferase; *glmS* = Glutamine--fructose-6-phosphate aminotransferase; *glmU* = Bifunctional protein GlmU.

5.3.2. Analysis and genomic organization of oxalotrophy genes in *C. oxalaticus* Ox1

While oxalate degradation in *C. oxalaticus* Ox1 has been extensively studied enzymatically, no confirmation of the presence of the genes encoding for the Oxc, Frc and OxlT in its genome has been made so far. Figure 7 shows the genomic organization of the *oxc-frc-oxlT* gene cluster. The *oxc* gene is followed by the *frc* gene. A sensor protein FixL lies just after it. The sensor protein FixL is a putative oxygen sensor that modulates the activity of FixJ, a transcriptional activator of the nitrogen fixation *fixK* gene (47). However, no nitrogen fixation-related genes were found in the genome. These genes are followed by a LysR-transcriptional regulator, as well as an HTH-type transcriptional activator and a regulator (CmpR and ArgP, respectively). Such LysR-transcriptional regulators have been found in the vicinity of the *oxc-frc* genes in the genomes of *Oxalobacter formigenes* and *Burkholderia japonicum* (8). Moreover, we also found orthologs of these transcriptional regulator genes in *C. oxalaticus* T2 and X32, as well as in *P. phytofirmans* PsJN (Table S2). The presence of these regulators could suggest that they play a role in the regulation of the expression of the oxalotrophy genes. Finally, an oxalate/formate antiporter (*oxlT*) gene lies at the end of the cluster. The same genomic organization of the oxalate degradation gene cluster was found for the two other *C. oxalaticus* strains, i.e. T2 and X32 (see orthologs in Table S2).

The existence of an *oxc-frc* operon has been proposed for *Lactobacillus acidophilus* NCFM (19). The presence of an operon implies the presence of promoters and Rho-independent bacterial terminators. Such promoters and transcriptional terminators have been found in the genome of *L. acidophilus* NCFM upstream of the *oxc* gene and downstream of the *frc* gene, respectively. We found a sigma70 promoter upstream of the *oxc* gene, as well as Rho-independent bacterial terminators downstream of the *frc* and *oxlT* genes. This indicates that the *oxc*, *frc*, and *oxlT* genes could be organized in an operon. However, the presence of a second terminator downstream of the *frc* suggests that *C. oxalaticus* Ox1 might use a hybrid pathway from those described in anaerobic and aerobic bacteria that might be differentially expressed between anabolism (i.e. oxalate used as carbon for biomass production), and catabolism (i.e. oxalate degradation and energy generation). Also, the co-occurrence of the *oxc* and *frc* genes in all Uniprot reference bacterial proteomes might confirm the existence of an oxalate-degradation operon.

Fig. 7. Genomic organization of the *oxc-frc-oxlT* genes in *C. oxalaticus* Ox1. The *oxc*, *frc* and *oxlT* genes occur in an operon. The oxc, frc and oxlT genes encode the proteins oxalyl-CoA decarboxylase (Oxc), Formyl-CoA transferase (Frc), and an oxalate/formate antiporter (OxlT). A promoter has been predicted upstream from the *oxc* gene, indicated by the red arrow. Two Rho-independent bacterial terminators have been predicted downstream from the *frc* and *oxlT* genes, represented by a red pinhole. Several transcriptional regulators, i.e. *lysR, cmpR* and *argP*, are found in the vicinity of the *oxc*, *frc* and *oxlT* genes.

5.3.3. Genomic determinants of oxalotrophy in other oxalotrophic bacteria

In order to get a better idea of the genomic determinants of oxalotrophy, we screened for the Frc, Oxc, and OxlT proteins in the proteomes of the species presented in Table 1. The occurrence of these three key proteins and known oxalotrophic activity for each species is presented in Table 4. First of all, we can see that the limiting factor for oxalotrophy appears to be the occurrence of Oxc. Indeed, this protein is present in a single copy in most of the genomes. As indicated in Fig. 7, the *oxc* gene occurs in an operon along with *frc*, which is usually situated downstream of *oxc*, and the oxalate/formate antiporter. Orthologs of these three different genes are found in all the genomes analyzed here (Table S2). The *frc* seems to be present in multiple copies, especially in the case of the *Cupriavidus* strains (Table 4). Energy production seems to be coupled both with formate oxidation, and with the transport of oxalate and formate in *C. oxalaticus*, given the presence of a formate dehydrogenase (data not shown) and the oxalate/formate antiporter (OxlT), respectively. However, no orthologs of OxlT have been identified in the genomes of *L. acidophilus* and *B. lactis*, but putative permease or transporter (red notes * & #) have been found in the vicinity of the *oxc* and *frc* genes.

Table 4. Occurrence of the Frc, Oxc and OxlT proteins in the selected whole genome sequences

Strain	Frc	Oxc	OxlT	Oxalotrophic
***C. oxalaticus* Ox1**	7	1	2	Yes
***C. oxalaticus* Ox1 mCherry**	8	1	2	Yes
***C. oxalaticus* T2**	7	1	2	Yes
***C. oxalaticus* X32**	6	2	2	Yes
***C. necator* H16**	5	2	2	Yes
***P. phytofirmans* PsJN**	3	2	1	Yes
***A. oxalaticus* RAOx**	2	1	1	Yes
***B. animalis* subsp. *lactis* DSM 10140**	1	2	1*	Yes
***L. acidophilus* ATCC 4796**	1	1	1#	Yes
***O. formigenes* OXCC13**	2	2	1	Yes
***P. rettgeri* DSM 1131**	1	2	0	No
***E. coli* K12**	1	1	0	No
***P. putida* KT2440**	1	0	0	No

*presence of a permease downstream of the *oxc* gene
ABC transporter, ATP-binding protein

In the case of *P. putida* KT2440, only one *frc* gene has been found, but no *oxc* or *oxlT* genes. This is not surprising as this species is not oxalotrophic. However, this is not the case of *E. coli* K12 and *P. rettgeri*. Indeed, for both species the *oxc* and *frc* genes are present in their genomes. *E. coli* has not been previously reported as being oxalotrophic. However, certain freshly isolated *E. coli* strains were able to grow on an oxalate-containing medium, but this ability was lost with sub-culturing (Abratt 2010). The same was observed for an oxalate-degrading strain of *P. rettgeri*. This might suggest that gastrointestinal

bacteria may have the ability to degrade oxalate *in-vivo* but that this poorly energetic phenotype is lost *in-vitro*. Further studies in *in-vivo*-like conditions could unravel the exact conditions that promote oxalate degradation in these bacterial species.

One other key determinant of the capability to degrade oxalate might be the presence of transcriptional activators or regulators such as those found in the case of *C. oxalaticus* Ox1 (Fig. 7). Indeed, those were only found in the genomes of *Cupriavidus* strains, as well as in *P. phytofirmans* PsJN (Table S2). Targeted knockouts of these transcriptional regulators might be needed to determine the role of those regulators in the transcription of oxalate-degradation-related genes. Moreover, a more extensive analysis of the presence of promoters and transcriptional terminators upstream and downstream of the genes related to oxalotrophy might be needed for the identification of an oxalate operon in other species.

5.3.4. Screening of virulence-related genes and other functions

Besides confirming the presence of oxalotrophy-related genes, the genomic screening of *C. oxalaticus* Ox1 also revealed the presence of genes related to virulence, such as the production of β-hemolysin, or antimicrobial resistance to vancomycin, as well as all the machinery for flagellar motility and chemotaxis (Table S3). While genes for siderophore production were not found in the genome of *C. oxalaticus* Ox1, genes related to siderophore uptake were detected (Table S3). All these findings were confirmed by culture-based assays. Indeed, β-hemolysin production by both *C. oxalaticus* Ox1 strains was confirmed on Columbia blood agar + 5% sheep blood (Fig. 8). This is consistent with a previous study that showed that *Cupriavidus* spp. strains isolated from a cave were also capable of hemolysis (48). However, a novel *Cupriavidus* species isolated from infected airways of patients with cystic fibrosis showed no hemolysis activity, which could suggest that the capacity of hemolysis is not crucial in pathogenesis (Kalka-Moll 2009). The absence of siderophore production was also confirmed on CAS agar (Fig. 9).

Both strains were shown to be resistant to vancomycin (Fig. 10), which is in accordance with another study that showed that a nickel-resistant strain of *Cupriavidus* spp. was also resistant to vancomycin, as well as to other antimicrobial compounds such as chloramphenicol, ampicillin, gentamicin, and kanamycin (49). As expected by the insertion of the mCherry tag, *C. oxalaticus* Ox1 mCherry is resistant to gentamicin (Fig. 10). Despite the resistance-related genes found in their genomes (Table S3), our two strains did not show resistance to any other antimicrobial agents tested, i.e. kanamycin (an aminoglycoside); ampicillin and penicillin (β-lactams); and ciprofloxacin (fluoroquinolone). Moreover, we observed that the mCherry-tagged strain is globally more susceptible to all the other antimicrobial compounds tested (Fig. 10). This could be explained by the fact that the gentamicin resistance gene, as well as the mCherry

fluorescent protein gene, are under the control of a constitutive promoter (P_{tac}) (44, 45), resulting maybe in a decreased expression of other antimicrobial-resistance genes.

Finally, we were able to confirm the motility of both our strains using a TTC motility test (Fig. 11A-E). Moreover, we could show the ecological advantage of flagellar motility in a fungal network for dispersal and accessibility of resources. Indeed, thanks to the mCherry tag of the *C. oxalaticus* Ox1 mCherry strain, we could visualize the interaction of this bacterium with the mycelial network of the soil fungal strain *T. rossicum*, which permitted the bacterium to disperse on its hyphae and increased its access of CaOx in Angle medium supplemented with 4g/L CaOx. This was visible by the larger dissolution zone in the plate where *C. oxalaticus* Ox1 mCherry was inoculated on *T. rossicum*, as compared to the plate where the bacterium was inoculated alone (Fig. 11H-I *versus* F-G).

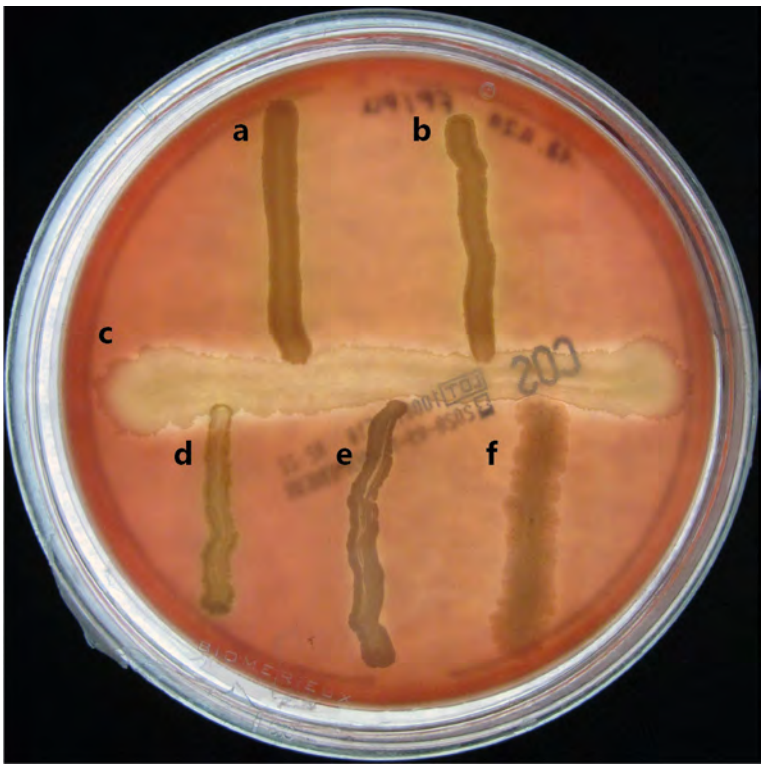


Fig. 8. Hemolysis test on Columbia blood agar + 5% sheep blood. This hemolysis test revealed that *C. oxalaticus* Ox1 (a) and *C. oxalaticus* Ox1 mCherry (b) are positive for β-hemolysis (complete hemolysis, presence of a degradation halo around the inoculum), confirming the presence of genes related to β-hemolysin in their genome. *Bacillus subtilis* NEU1 (c) and *Streptococcus agalactiae* NEU1203 (d) were used as positive controls for β-hemolysis, *Staphylococcus epidermidis* NEU1229 (e) was used as negative control (γ-hemolysis, no hemolysis), and *Escherichia coli* NEU1006 (f) was used as positive control for α-hemolysis (partial hemolysis, appears blackish).

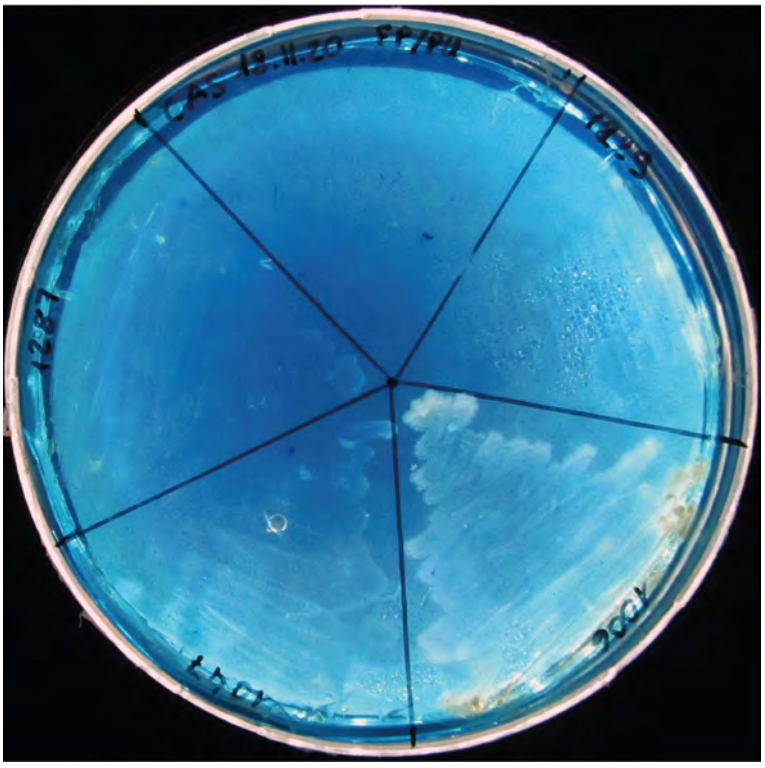


Fig. 9. Siderophores production assay on CAS agar. Both *C. oxalaticus* Ox1 strain were not able to produce siderophores. A color change from blue to yellow, due to the chelation of iron by siderophores, means the reaction is positive. Clockwise from the top: non-inoculated control; 1229: *Staphylococcus epidermidis*, negative control; 1006: *Escherichia coli*, positive control; 1047: *C. oxalaticus* Ox1; 1287: *C. oxalaticus* Ox1 mCherry.

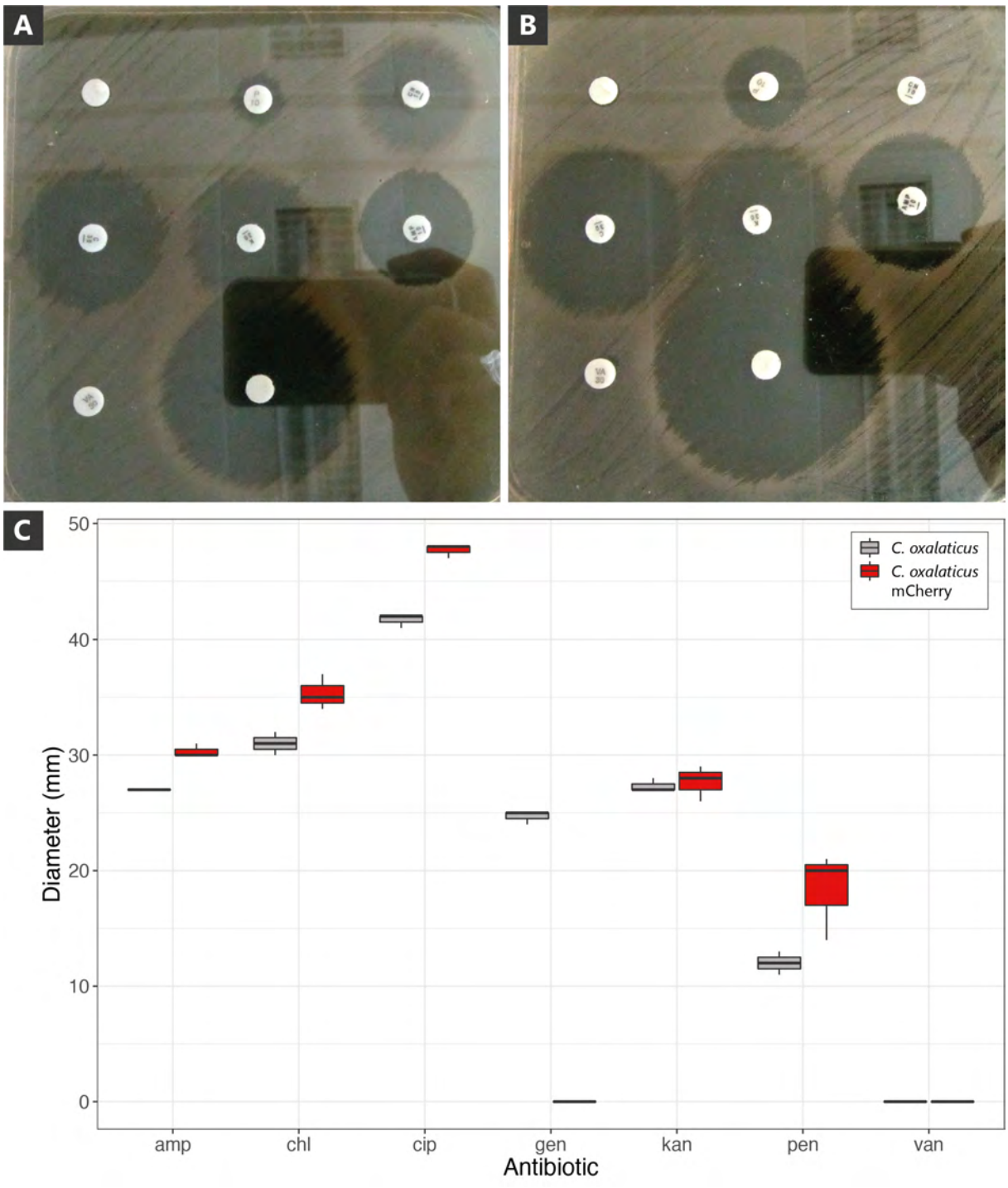


Fig. 10. Antimicrobial susceptibility test. (A) Antibiogram for *C. oxalaticus* Ox1. (B) Antibiogram for *C. oxalaticus* Ox1 mCherry. From left to right, and up to bottom: blank, penicillin, gentamicin, chloramphenicol, kanamycin, ampicillin, vancomycin, and ciprofloxacin. Both strains are resistant to vancomycin. *C. oxalaticus* Ox1 mCherry is confirmed to be resistant to gentamicin, due to the insertion of the mCherry fluorescent protein, as revealed by the genomic screening. (C) Boxplot of the diameter of the inhibition area around antimicrobial disks after 24h. Amp = ampicillin; Chl = chloramphenicol; Cip = ciprofloxacin; Gen = gentamicin; Kan = kanamycin; Pen = penicillin; Van = vancomycin.

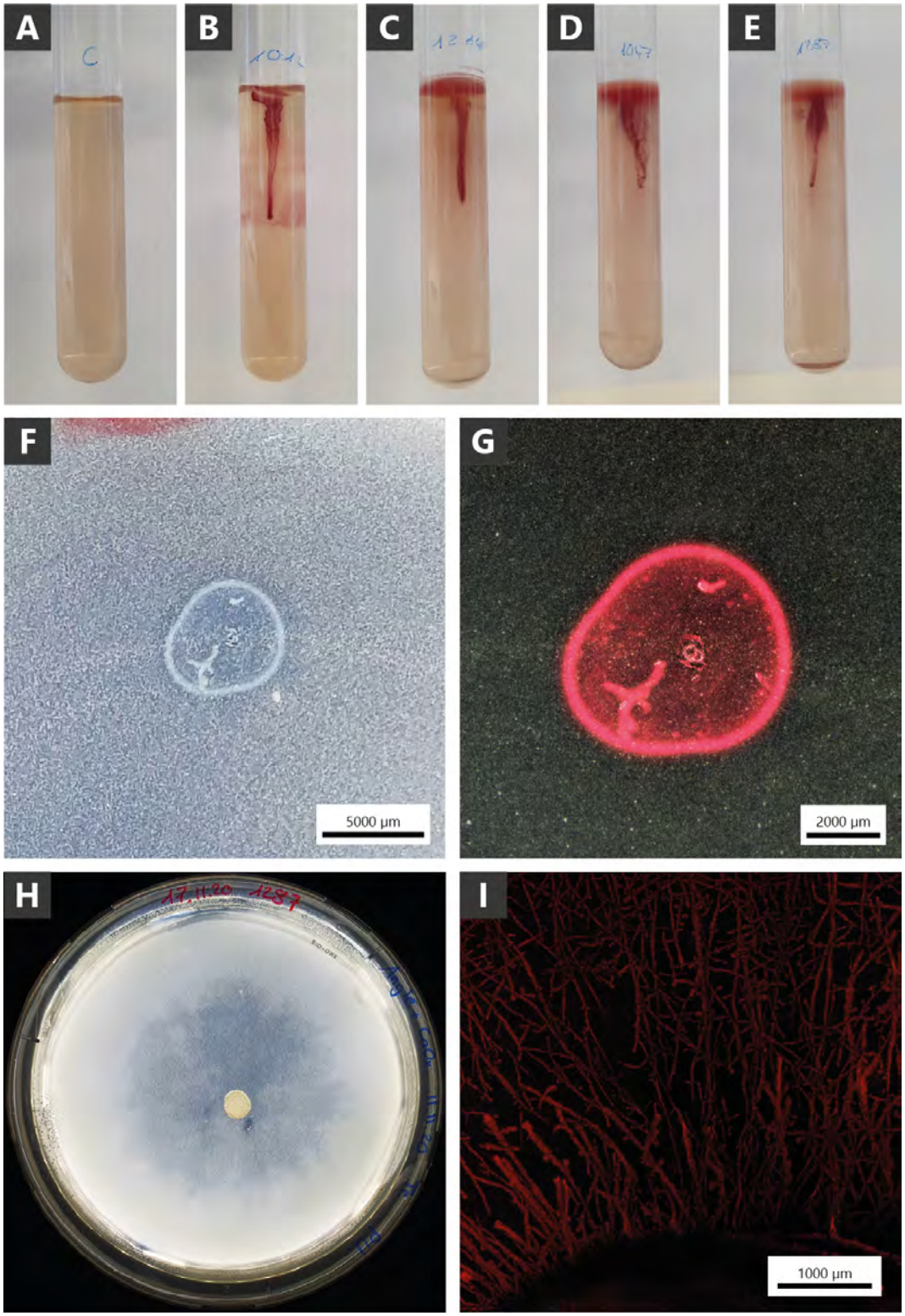


Fig. 11. TTC Motility test & oxalate-degradation extent with and without dispersal on fungal hyphae. (A-E) TTC Motility test. A strain positive to this test will show a diffuse red color, due to the reduction of TTC to formazan, in the growth area from the stab line of inoculation. (A) Non-inoculated control TTC motility medium. (B) Negative control, *Enterococcus faecalis* NEU1012. (C) Positive control, *Pseudomonas putida* KT2440 NEU1264. (D) *C. oxalaticus* Ox1 NEU1047. (E) *C. oxalaticus* Ox1 mCherry NEU1287. (F) Extent of the degradation of calcium oxalate by *C. oxalaticus* Ox1 mCherry alone, which is visible by a transparent halo under the colony. (G) Growth extent of (F) observed under epifluorescence stereoscopy (H) Extent of the degradation of calcium oxalate by *C. oxalaticus* Ox1 mCherry when inoculated on the mycelial network of *Trichoderma rossicum*. *C. oxalaticus* Ox1 mCherry benefit from the interaction with *T. rossicum* through fungal highways by being able to access and degrade more CaOx. (I) Fungal highways of (H) observed under epifluorescence stereoscopy.

5.4. Conclusions

To conclude, the genomic screening of *C. oxalaticus* Ox1 revealed that the genes related to oxalotrophy, i.e., *oxc*, *frc* and *oxlT*, are most probably organized in an operon, which is under the regulation of a promoter, transcriptional terminators, as well as several transcriptional regulators which are found in the vicinity of the oxalate-degradation genes. However, a more extensive investigation of the specific role of the transcriptional regulators found in the vicinity of the genes related to oxalate-degradation will be needed in order to better understand oxalotrophy in this model oxalotrophic bacterial species, and to engineer a viable cell-free oxalate-degrading pathway.

Our biocontrol study to inhibit the growth of *Aspergillus niger* in 3D-lung cell cultures in lung-on-a-chip systems (Chapter 4) showed that *C. oxalaticus* Ox1 causes cellular damage. This is not surprising as the *Cupriavidus* genus is known to contain several pathogenic species infecting cystic fibrosis patients. However, the analysis performed here provided further support to this as we found virulence factors such as the production of β-hemolysin or resistance to vancomycin. This confirms that this bacterium cannot be used as a live biotherapeutic strain for the fight against aspergillosis.

Genes related to flagellar motility and chemotaxis were also found, which is not surprising given the soil origin of this bacterium and its known interaction with filamentous fungi. Additionally, we could also highlight a fungal highway like interaction between *C. oxalaticus* Ox1 and the fungus *T. rossicum* and its ecological advantage in the accessibility of nutrients, such as calcium oxalate, in a heterogenous unsaturated habitat such as soil.

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References

1. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. In: Gadd GM, Sariaslani S, editors. 106: Academic Press; 2019. p. 49-77.

2. Hervé V, Junier T, Bindschedler S, Verrecchia E, Junier P. Diversity and ecology of oxalotrophic bacteria. World Journal of Microbiology and Biotechnology. 2016;32(2):1-7.

3. Strobel BW. Influence of vegetation on low-molecular-weight carboxylic acids in soil solution—a review. Geoderma. 2001;99(3-4):169-98.

4. Gadd GM. Fungal Production of Citric and Oxalic Acid: Importance in Metal Speciation, Physiology and Biogeochemical Processes. Advances in Microbial Physiology. 1999;41:47-92.

5. Certini G, Corti G, Ugolini FC. Vertical trends of oxalate concentration in two soils under Abies alba from Tuscany (Italy). Journal of Plant Nutrition and Soil Science. 2000;163(2):173-7.

6. Hofmann BA, Bernasconi SM. Review of occurrences and carbon isotope geochemistry of oxalate minerals: implications for the origin and fate of oxalate in diagenetic and hydrothermal fluids. Chemical Geology. 1998;149(1-2):127-46.

7. Braissant O, Verrecchia EP, Aragno M. Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? Naturwissenschaften. 2002;89(8):366-70.

8. Abratt VR, Reid SJ. Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease. 72: Academic Press; 2010. p. 63-87.

9. Sun Q, Li J, Finlay RD, Lian B. Oxalotrophic bacterial assemblages in the ectomycorrhizosphere of forest trees and their effects on oxalate degradation and carbon fixation potential. Chemical Geology. 2019;514:54-64.

10. Stewart CS, Duncan SH, Cave DR. Oxalobacter formigenes and its role in oxalate metabolism in the human gut. FEMS Microbiology Letters. 2006;230(1):1-7.

11. Miller AW, Kohl KD, Dearing MD. The gastrointestinal tract of the white-throated Woodrat (Neotoma albigula) harbors distinct consortia of oxalate-degrading bacteria. Appl Environ Microbiol. 2014;80(5):1595-601.

12. Quayle JR. Carbon assimilation by Pseudomonas oxalaticus (OX 1). 6. Reactions of oxalyl-coenzyme A. Biochemical Journal. 1963;87(2):368-73.

13. Quayle JR. Carbon Assimilation by Pseudomonas Oxalaticus (Ox1). 7. Decarboxylation of Oxalyl-Coenzyme a to Formyl-Coenzyme A. Biochem J. 1963;89:492-503.

14. Blackmore MA, Quayle JR. Microbial growth on oxalate by a route not involving glyoxylate carboligase. The Biochemical journal. 1970;118(1):53-9.

15. Dijkhuizen L, Wiersma M, Harder W. Energy production and growth of Pseudomonas oxalaticus OX1 on oxalate and formate. Arch Microbiol. 1977;115(2):229-36.

16. Kuhner CH, Hartman PA, Allison MJ. Generation of a proton motive force by the anaerobic oxalate-degrading bacterium Oxalobacter formigenes. Applied and environmental microbiology. 1996;62(7):2494-500.

17. Anantharam V, Allison MJ, Maloney PC. Oxalate:formate exchange. The basis for energy coupling in Oxalobacter. The Journal of biological chemistry. 1989;264(13):7244-50.

18. Sahin N. Oxalotrophic bacteria. Research in Microbiology. 2003;154(6):399-407.

19. Azcarate-Peril MA, Bruno-Bárcena JM, Hassan HM, Klaenhammer TR. Transcriptional and Functional Analysis of Oxalyl-Coenzyme A (CoA) Decarboxylase and Formyl-CoA Transferase Genes from <em>Lactobacillus acidophilus</em&gt. Applied and Environmental Microbiology. 2006;72(3):1891 LP-9.

20. Turroni S, Vitali B, Bendazzoli C, Candela M, Gotti R, Federici F, et al. Oxalate consumption by lactobacilli: Evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in Lactobacillus acidophilus. Journal of Applied Microbiology. 2007;103(5):1600-9.

21. Jiang T, Chen W, Cao L, He Y, Zhou H, Mao H. Abundance, Functional, and Evolutionary Analysis of Oxalyl-Coenzyme A Decarboxylase in Human Microbiota. Front Microbiol. 2020;11:672.

22. Khambata SR, Bhat JV. Studies on a new oxalate-decomposing bacterium, Pseudomonas oxalaticus. J Bacteriol. 1953;66(5):505-7.

23. Şahin N, Işik K, Tamer AÜ, Goodfellow M. Taxonomic position of 'Pseudomonas oxalaticus' strain Ox1(T) (DSM 110(T)) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia oxalatica comb, nov. Systematic and Applied Microbiology. 2000;23(2):206-9.

24. Rochat L, Pechy-Tarr M, Baehler E, Maurhofer M, Keel C. Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow cytometry. Mol Plant Microbe Interact. 2010;23(7):949-61.

25. Li PE, Lo CC, Anderson JJ, Davenport KW, Bishop-Lilly KA, Xu Y, et al. Enabling the democratization of the genomics revolution with a fully integrated web-based bioinformatics platform. Nucleic Acids Research. 2017;45(1):67-80.

26. Aziz RK, Bartels D, Best A, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: Rapid annotations using subsystems technology. BMC Genomics. 2008;9:15-.

27. Blom J, Kreis J, Spänig S, Juhre T, Bertelli C, Ernst C, et al. EDGAR 2.0: an enhanced software platform for comparative gene content analyses. Nucleic acids research. 2016;44(W1):W22-W8.

28. Solovyev V. V. Solovyev, A Salamov (2011) Automatic Annotation of Microbial Genomes and Metagenomic Sequences. In Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies (Ed. R.W. Li), Nova Science Publishers, p.61-78. 2011. p. 61-78.

29. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry. 1987;160(1):47-56.

30. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30(14):2068-9.

31. Janausch IG, Zientz E, Tran QH, Kröger A, Unden G. C4-dicarboxylate carriers and sensors in bacteria. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2002;1553(1-2):39-56.

32. Nolan LM, Whitchurch CB, Barquist L, Katrib M, Boinett CJ, Mayho M, et al. A global genomic approach uncovers novel components for twitching motility-mediated biofilm expansion in Pseudomonas aeruginosa. Microb Genom. 2018;4(11).

33. Rivera M. Bacterioferritin: Structure, Dynamics, and Protein–Protein Interactions at Play in Iron Storage and Mobilization. Accounts of Chemical Research. 2017;50(2):331-40.

34. Riboldi-Tunnicliffe A, Konig B, Jessen S, Weiss MS, Rahfeld J, Hacker J, et al. Crystal structure of Mip, a prolylisomerase from Legionella pneumophila. Nat Struct Biol. 2001;8(9):779-83.

35. Kalka-Moll WM, LiPuma JJ, Accurso FJ, Plum G, van Koningsbruggen S, Vandamme P. Airway infection with a novel Cupriavidus species in persons with cystic fibrosis. J Clin Microbiol. 2009;47(9):3026-8.

36. Zhu C, Guo R, Zhou M, Yu Y. Bloodstream infection caused by Cupriavidus gilardii in a systemic lupus erythematosus patient with invasive pulmonary aspergillosis. Journal of Emergency and Critical Care Medicine. 2018;2:46-.

37. Ruiz C, McCarley A, Espejo ML, Cooper KK, Harmon DE. Comparative Genomics Reveals a Well-Conserved Intrinsic Resistome in the Emerging Multidrug-Resistant Pathogen Cupriavidus gilardii. mSphere. 2019;4(5).

38. Massip C, Coullaud-Gamel M, Gaudru C, Amoureux L, Doleans-Jordheim A, Hery-Arnaud G, et al. In vitro activity of 20 antibiotics against Cupriavidus clinical strains. J Antimicrob Chemother. 2020;75(6):1654-8.

39. Lu J, Salzberg SL. SkewIT: The Skew Index Test for large-scale GC Skew analysis of bacterial genomes. PLoS Comput Biol. 2020;16(12):e1008439.

40. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev. 2018;31(4).

41. Chien JY, Chiu WY, Chien ST, Chiang CJ, Yu CJ, Hsueh PR. Mutations in gyrA and gyrB among Fluoroquinolone- and Multidrug-Resistant Mycobacterium tuberculosis Isolates. Antimicrob Agents Chemother. 2016;60(4):2090-6.

42. de Pace F, Boldrin de Paiva J, Nakazato G, Lancellotti M, Sircili MP, Guedes Stehling E, et al. Characterization of IcmF of the type VI secretion system in an avian pathogenic Escherichia coli (APEC) strain. Microbiology (Reading). 2011;157(Pt 10):2954-62.

43. Navarro-Garcia F, Ruiz-Perez F, Cataldi A, Larzabal M. Type VI Secretion System in Pathogenic Escherichia coli: Structure, Role in Virulence, and Acquisition. Front Microbiol. 2019;10:1965.

44. Remus-Emsermann MN, Gisler P, Drissner D. MiniTn7-transposon delivery vectors for inducible or constitutive fluorescent protein expression in Enterobacteriaceae. FEMS Microbiol Lett. 2016;363(16).

45. de Boer HA, Comstock LJ, Vasser M. The tac promoter: a functional hybrid derived from the trp and lac promoters. Proc Natl Acad Sci U S A. 1983;80(1):21-5.

46. DeBoy RT, Craig NL. Target site selection by Tn7: attTn7 transcription and target activity. J Bacteriol. 2000;182(11):3310-3.

47. de Philip P, Batut J, Boistard P. Rhizobium meliloti Fix L is an oxygen sensor and regulates R. meliloti nifA and fixK genes differently in Escherichia coli. J Bacteriol. 1990;172(8):4255-62.

48. Chalia S, Baskar S, Minakshi P, Baskar R, Ranjan K. Biomineralization Abilities of Cupriavidus Strain and Bacillus subtilis Strains In Vitro Isolated from Speleothems, Rani Cave, Chhattisgarh, India. Geomicrobiology Journal. 2017;34(9):737-52.

49. Lee S, Khanal A, Cho AH, Lee H, Kang MS, Unno T, et al. Cupriavidus sp. strain Ni-2 resistant to high concentration of nickel and its genes responsible for the tolerance by genome comparison. Arch Microbiol. 2019;201(10):1323-31.

Supplementary Material

Table S1. Selected genes with potential interesting functions in *C. oxalaticus* Ox1

Gene ID	Function
Transport of C-sources	
fig|6666666.679904.peg.290	Aerobic C4-dicarboxylate transporter for fumarate, L-malate, D-malate, succinate
fig|6666666.679904.peg.1977	citrate transporter, CitM family
Twitching motility	
fig|6666666.679904.peg.3204	twitching motility protein PilG
fig|6666666.679904.peg.3205	twitching motility protein PilH
fig|6666666.679904.peg.3206	type IV pili signal transduction protein PilI
fig|6666666.679904.peg.3207	twitching motility protein PilJ
fig|6666666.679904.peg.3930	Type IV pilus biogenesis protein PilZ
fig|6666666.679904.peg.4964	Tfp pilus assembly protein FimV
Phage	
fig|6666666.679904.peg.1114	Phage-related integrase
fig|6666666.679904.peg.1899	Phage tail sheath protein FI
fig|6666666.679904.peg.1901	Phage tail sheath protein FI
fig|6666666.679904.peg.1905	Phage protein D
fig|6666666.679904.peg.3693	Phage-related protein
fig|6666666.679904.peg.3698	Phage related protein
fig|6666666.679904.peg.3713	Phage protein
fig|6666666.679904.peg.3717	Phage protein
fig|6666666.679904.peg.3730	elements of external origin; phage-related functions and prophages
fig|6666666.679904.peg.3744	Phage lysin (EC 3.2.1.17) # Phage lysozyme or muramidase (EC 3.2.1.17)
fig|6666666.679904.peg.4843	Phage tail X
fig|6666666.679904.peg.4844	phage-related protein
fig|6666666.679904.peg.4845	elements of external origin; phage-related functions and prophages
fig|6666666.679904.peg.4846	Putative phage-encoded peptidoglycan binding protein
fig|6666666.679904.peg.4851	Phage baseplate assembly protein
fig|6666666.679904.peg.4865	Phage Rha protein
fig|6666666.679904.peg.4866	Phage-related protein
fig|6666666.679904.peg.5354	Phage protein
Bacterioferritin	
fig|6666666.679904.peg.2707	Bacterioferritin
fig|6666666.679904.peg.2763	Bacterioferritin-associated ferredoxin
fig|6666666.679904.peg.2764	Bacterioferritin
fig|6666666.679904.peg.4603	Bacterioferritin
Virulence factor in macrophages	
fig|6666666.679904.peg.4363	Macrophage infectivity potentiator-related protein

Table S2. Orthologous genes of the genes present in the oxalate operon in *C. oxalaticus* Ox1

Fabio_yZM_01645 – Oxalate/formate antiporter

Organism	Gene	Description	Start	Stop	Strand
Ammoniphilus_oxalaticus_strain_RAOx_1_MCHY00000000	BEP19_14235	oxalate/formate MFS antiporter	3027853	3026579	-
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03255	oxalate/formate MFS antiporter	760386	759067	-
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02455	oxalate/formate MFS antiporter	576557	575238	-
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00814	Oxalate:formate antiporter	915108	916427	+
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27780	oxalate/formate MFS antiporter	1936344	1935025	-
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6027	major facilitator superfamily MFS_1	2258982	2257639	-
Oxalobacter_formigenes_OXCC13_CP019430	BRW84_01465	oxalate/formate MFS antiporter	298386	299669	+

Fabio_yZM_01646 - Hypothetical

Organism	Gene	Description	Start	Stop	Strand
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03260	hypothetical protein	761263	761045	-
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02460	hypothetical protein	577473	577255	-
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00813	hypothetical protein	914232	914450	+
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27785	hypothetical protein	1937242	1937024	-

Fabio_yZM_01648 - HTH-type transcriptional regulator ArgP

Organism	Gene	Description	Start	Stop	Strand
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03265	LysR family transcriptional regulator	762327	761404	-
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02465	LysR family transcriptional regulator	578544	577612	-
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00811	HTH-type transcriptional regulator ArgP	911833	912756	+
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27790	LysR family transcriptional regulator	1938314	1937391	-
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6026	transcriptional regulator, LysR family	2257609	2256638	-

Fabio_yZM_01649 - HTH-type transcriptional activator CmpR

Organism	Gene	Description	Start	Stop	Strand
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03270	LysR family transcriptional regulator	762618	763559	+
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02475	LysR family transcriptional regulator	579452	580393	+
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00810	HTH-type transcriptional activator CmpR	911542	910601	-
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27795	LysR family transcriptional regulator	1938633	1939574	+
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6028	transcriptional regulator, LysR family	2259392	2260339	+

Fabio_yZM_01650 – LysR family transcriptional regulator

Organism	Gene	Description	Start	Stop	Strand
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03275	LysR family transcriptional regulator	763683	763988	+
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02480	LysR family transcriptional regulator	580513	580818	+
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00809	hypothetical protein	910477	910172	-
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27800	LysR family transcriptional regulator	1939679	1939984	+
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6738	conserved hypothetical protein	3064206	3063817	-

Fabio_yZM_01651 – Hypothetical

Organism	Gene	Description	Start	Stop	Strand
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03280	hypothetical protein	764410	764871	+
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02495	hypothetical protein	581782	582243	+
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00808	hypothetical protein	909807	909289	-

Fabio_yZM_01652 – Sensor protein FixL

Organism	Gene	Description	Start	Stop	Strand
Providencia_rettgeri_DSM_1131_GG705262	PROVRETT_09477	PAS domain S-box protein	607366	607776	+
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03285	PAS domain S-box protein	765523	765086	-
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02500	PAS domain S-box protein	582852	582415	-
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00807	Sensor protein FixL	908637	909074	+
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27825	PAS sensor domain-containing protein	1943816	1943379	-
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6742	putative PAS/PAC sensor protein	3069187	3069618	+

Fabio_yZM_01653 - Formyl-CoA:oxalate CoA-transferase

Organism	Gene	Description	Start	Stop	Strand
Escherichia_coli_K_12_CP047127	GRF62_15545	formyl-CoA transferase	3253316	3254566	+
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03290	formyl-CoA transferase	766924	765674	-
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00806	Formyl-CoA:oxalate CoA-transferase	907236	908486	+
Bifidobacterium_animalis_subsp_lactis_DSM_10140_CP001606	Balat_1449	formyl-coenzyme A transferase	1696307	1697638	+
Ammoniphilus_oxalaticus_strain_RAOx_1_MCHY00000000	BEP19_11385	formyl-CoA transferase	2439893	2438601	-
Lactobacillus_acidophilus_ATCC_4796_GG669566	HMPREF0492_0758	formyl-CoA transferase	747359	746022	-
Providencia_rettgeri_DSM_1131_GG705262	PROVRETT_09965	formyl-CoA transferase	118462	117212	-
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02505	formyl-CoA transferase	584253	583003	-
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27830	formyl-CoA transferase	1945222	1943972	-
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6741	formyl-CoA transferase	3067831	3069081	+
Oxalobacter_formigenes_OXCC13_CP019430	BRW84_05210	formyl-CoA--oxalate CoA-transferase	1113567	1112281	-

Fabio_yZM_01654 - Oxalyl-CoA decarboxylase

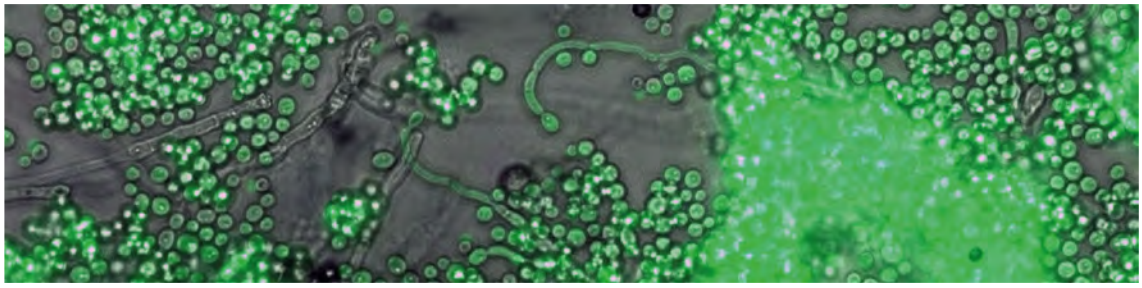
Organism	Gene	Description	Start	Stop	Strand
Escherichia_coli_K_12_CP047127	GRF62_15550	oxalyl-CoA decarboxylase	3254620	3256314	+
Cupriavidus_oxalaticus_strain_T2_chromosome_1_CP032518	D2917_03295	oxalyl-CoA decarboxylase	768706	766967	-
C_oxalaticus_strain_Ox1_mCherry	Fabio_M9m_00805	Oxalyl-CoA decarboxylase	905454	907193	+
Bifidobacterium_animalis_subsp_lactis_DSM_10140_CP001606	Balat_1453	putative oxalyl-CoA decarboxylase	1702832	1701051	-
Ammoniphilus_oxalaticus_strain_RAOx_1_MCHY00000000	BEP19_14240	oxalyl-CoA decarboxylase	3030011	3028323	-
Lactobacillus_acidophilus_ATCC_4796_GG669566	HMPREF0492_0759	oxalyl-CoA decarboxylase	749125	747359	-
Providencia_rettgeri_DSM_1131_GG705262	PROVRETT_09964	oxalyl-CoA decarboxylase	117147	115444	-
Cupriavidus_oxalaticus_strain_X32_chromosome_1_CP038634	E0W60_02510	oxalyl-CoA decarboxylase	586033	584294	-
Cupriavidus_necator_H16_chromosome_2_CP039288	E6A55_27835	oxalyl-CoA decarboxylase	1947004	1945265	-
Paraburkholderia_phytofirmans_PsJN_chromosome_2_CP001053	Bphyt_6740	oxalyl-CoA decarboxylase	3066064	3067806	+
Oxalobacter_formigenes_OXCC13_CP019430	BRW84_02545	oxalyl-CoA decarboxylase	535702	537408	+

Table S3. Virulence-related genes and other functions in *C. oxalaticus* Ox1

Gene ID	Function
Hemolysis	
fig|6666666.679904.peg.1136	hemolysin B
fig|6666666.679904.peg.4787	Putative hemolysin
fig|6666666.679904.peg.5917	21 kDa hemolysin precursor
Siderophores	
fig|6666666.679904.peg.1640	TonB-dependent siderophore receptor
fig|6666666.679904.peg.1642	N6-hydroxylysine O-acetyltransferase (EC 2.3.1.102), aerobactin biosynthesis protein IucB @ Siderophore synthetase small component, acetyltransferase
fig|6666666.679904.peg.1653	Siderophore biosynthesis diaminobutyrate--2-oxoglutarate aminotransferase (EC 2.6.1.76)
fig|6666666.679904.peg.2205	Pyoverdine chromophore precursor synthetase PvdL @ Siderophore biosynthesis non-ribosomal peptide synthetase modules
fig|6666666.679904.peg.3086	Ferric siderophore transport system, biopolymer transport protein ExbB
fig|6666666.679904.peg.4884	Ferric siderophore transport system, periplasmic binding protein TonB
fig|6666666.679904.peg.5515	Ferric siderophore transport system, periplasmic binding protein TonB
Antimicrobial resistance	
Beta-lactams	
fig|6666666.679904.peg.52	metallo-beta-lactamase superfamily protein
fig|6666666.679904.peg.341	Metallo-beta-lactamase family protein, RNA-specific
fig|6666666.679904.peg.799	Beta-lactamase class C and other penicillin binding proteins
fig|6666666.679904.peg.983	Metal-dependent hydrolases of the beta-lactamase superfamily III
fig|6666666.679904.peg.1033	Metallo-beta-lactamase
fig|6666666.679904.peg.1128	Beta-lactamase (EC 3.5.2.6)
fig|6666666.679904.peg.1255	Beta-lactamase class C and other penicillin binding proteins
fig|6666666.679904.peg.2631	Beta-lactamase-like
fig|6666666.679904.peg.3598	Metal-dependent hydrolases of the beta-lactamase superfamily I
fig|6666666.679904.peg.4190	Metallo-beta-lactamase family protein
fig|6666666.679904.peg.4516	Beta-lactamase (EC 3.5.2.6)
fig|6666666.679904.peg.4517	beta-lactamase domain protein
fig|6666666.679904.peg.5962	Beta-lactamase-like
Fluoroquinolones	
fig|6666666.679904.peg.2507	DNA gyrase subunit B (EC 5.99.1.3)
fig|6666666.679904.peg.3256	DNA gyrase subunit A (EC 5.99.1.3)
fig|6666666.679904.peg.5009	Topoisomerase IV subunit A (EC 5.99.1.-)
fig|6666666.679904.peg.5011	Topoisomerase IV subunit B (EC 5.99.1.-)
Aminoglycosides	
fig|6666666.679904.peg.3490	Predicted aminoglycoside phosphotransferase
Flagellar motility	
fig|6666666.679904.peg.218	Flagellar transcriptional activator FlhD
fig|6666666.679904.peg.219	Flagellar transcriptional activator FlhC
fig|6666666.679904.peg.220	Flagellar motor rotation protein MotA
fig|6666666.679904.peg.221	Flagellar motor rotation protein MotB
fig|6666666.679904.peg.232	Flagellar biosynthesis protein FlhB
fig|6666666.679904.peg.233	Flagellar biosynthesis protein FlhA
fig|6666666.679904.peg.234	Flagellar biosynthesis protein FlhF
fig|6666666.679904.peg.235	Flagellar synthesis regulator FleN
fig|6666666.679904.peg.236	RNA polymerase sigma factor for flagellar operon
fig|6666666.679904.peg.239	Flagellar biosynthesis protein FlgN
fig|6666666.679904.peg.240	Negative regulator of flagellin synthesis
fig|6666666.679904.peg.241	Flagellar basal-body P-ring formation protein FlgA
fig|6666666.679904.peg.242	Flagellar basal-body rod protein FlgB
fig|6666666.679904.peg.243	Flagellar basal-body rod protein FlgC
fig|6666666.679904.peg.244	Flagellar basal-body rod modification protein FlgD
fig|6666666.679904.peg.245	Flagellar hook protein FlgE
fig|6666666.679904.peg.246	Flagellar basal-body rod protein FlgF
fig|6666666.679904.peg.247	Flagellar basal-body rod protein FlgG
fig|6666666.679904.peg.248	Flagellar L-ring protein FlgH
fig|6666666.679904.peg.249	Flagellar P-ring protein FlgI
fig|6666666.679904.peg.250	Flagellar protein FlgJ [peptidoglycan hydrolase] (EC 3.2.1.-)
fig|6666666.679904.peg.251	Flagellar hook-associated protein FlgK
fig|6666666.679904.peg.252	Flagellar hook-associated protein FlgL
fig|6666666.679904.peg.434	Flagellar biosynthesis protein FliR
fig|6666666.679904.peg.435	Flagellar biosynthesis protein FliQ
fig|6666666.679904.peg.436	Flagellar biosynthesis protein FliP
fig|6666666.679904.peg.437	Flagellar biosynthesis protein FliQ
fig|6666666.679904.peg.438	Flagellar motor switch protein FliN
fig|6666666.679904.peg.439	Flagellar motor switch protein FliM
fig|6666666.679904.peg.440	Flagellar biosynthesis protein FliL
fig|6666666.679904.peg.823	Flagellar transcriptional activator FlhC
fig|6666666.679904.peg.1533	Flagellar transcriptional activator FlhD

fig|6666666.679904.peg.2072	RNA polymerase sigma-54 factor RpoN
fig|6666666.679904.peg.2329	Flagellar biosynthesis protein FliC
fig|6666666.679904.peg.2331	Flagellar hook-associated protein FliD
fig|6666666.679904.peg.2332	Flagellar biosynthesis protein FliS
fig|6666666.679904.peg.2333	Flagellar biosynthesis protein FliT
fig|6666666.679904.peg.2335	Flagellar biosynthesis protein FlhB
fig|6666666.679904.peg.2336	Flagellar hook-basal body complex protein FliE
fig|6666666.679904.peg.2338	Flagellar M-ring protein FliF
fig|6666666.679904.peg.2339	Flagellar motor switch protein FliG
fig|6666666.679904.peg.2340	Flagellar assembly protein FliH
fig|6666666.679904.peg.2341	Flagellum-specific ATP synthase FliI
fig|6666666.679904.peg.2342	Flagellar protein FliJ
fig|6666666.679904.peg.2343	Flagellar hook-length control protein FliK
Chemotaxis	
fig|6666666.679904.peg.213	Chemotaxis regulator - transmits chemoreceptor signals to flagelllar motor components CheY
fig|6666666.679904.peg.214	Positive regulator of CheA protein activity (CheW)
fig|6666666.679904.peg.215	Aerotaxis sensor receptor protein
fig|6666666.679904.peg.216	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
fig|6666666.679904.peg.222	Signal transduction histidine kinase CheA (EC 2.7.3.-)
fig|6666666.679904.peg.223	Positive regulator of CheA protein activity (CheW)
fig|6666666.679904.peg.224	Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)
fig|6666666.679904.peg.225	Chemotaxis protein CheD
fig|6666666.679904.peg.226	Chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61)
fig|6666666.679904.peg.227	Chemotaxis regulator - transmits chemoreceptor signals to flagelllar motor components CheY
fig|6666666.679904.peg.228	Chemotaxis response - phosphatase CheZ
fig|6666666.679904.peg.280	Dipeptide-binding ABC transporter, periplasmic substrate-binding component (TC 3.A.1.5.2)
fig|6666666.679904.peg.425	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
fig|6666666.679904.peg.438	Flagellar motor switch protein FliN
fig|6666666.679904.peg.439	Flagellar motor switch protein FliM
fig|6666666.679904.peg.1341	Dipeptide-binding ABC transporter, periplasmic substrate-binding component (TC 3.A.1.5.2)
fig|6666666.679904.peg.1882	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
fig|6666666.679904.peg.2106	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
fig|6666666.679904.peg.2177	Chemotaxis protein CheV (EC 2.7.3.-)
fig|6666666.679904.peg.2230	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
fig|6666666.679904.peg.2296	Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)
fig|6666666.679904.peg.2323	Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)
fig|6666666.679904.peg.2327	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)
fig|6666666.679904.peg.2339	Flagellar motor switch protein FliG
fig|6666666.679904.peg.2421	Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)

CHAPTER 6



Tripartite interaction between *Candida albicans*, *Aspergillus niger* & *Cupriavidus oxalaticus*

Foreword

In this chapter, we investigate for the first time Fungal-Fungal-Bacterial interactions between *A. niger*, *C. albicans* and *C. oxalaticus* in the case of fungal co-infection and the role of oxalate in this interaction.

The key findings of this chapter were:

- *C. albicans* was found to produce oxalic, formic and acetic acid depending on the trophic conditions and growth temperatures tested, with acetic acid being the most abundant.
- The interaction between *C. albicans* and *A. niger* is dependent on the inoculation method: when co-inoculated with *C. albicans*, *A. niger* conidia germination was completely inhibited, which is concomitant with the absence of a drop in pH and free calcium. On the contrary, when *A. niger* conidia are let to germinate for 24h, and then *C. albicans* is added to the system, *A. niger* grew but was unable to acidify the culture medium, also associated with the absence of pH and free calcium concentration drop.
- *C. albicans* seems to grow partly in hyphal form when grown alone. Moreover, the presence of *A. niger* does not seem to trigger the shift yeast-to-hyphal growth in *C. albicans*.

6. Tripartite interaction between *Candida albicans*, *Aspergillus niger* & *Cupriavidus oxalaticus*

Fabio Palmieri[1], Pauline Udriet[1], Saskia Bindschedler[1], Pilar Junier[1]

[1]Laboratory of Microbiology, University of Neuchâtel, Switzerland

Abstract

Candida spp. and *Aspergillus* spp. are the most prevalent fungal pathogens infecting immunocompromised and cystic fibrosis patients, and often co-occur in mixed pulmonary fungal infections in at-risk individuals. One key virulence factor in *C. albicans* is notably its ability to shift from yeast to hyphal growth, which is induced by several environmental factors, such as high pCO_2, neutral or alkaline pH, or the presence of serum, among others. Oxalic acid has also been shown to induce this shift in the growth morphotype. Thus, co-occurrence with *Aspergillus* spp., which are notorious oxalic acid producers, may trigger hyphal growth in *C. albicans*. We have previously shown that the oxalotrophic bacterium *Cupriavidus oxalaticus* was able to control the growth of the oxalate-producing *A. niger*, as well as to prevent the formation of calcium oxalate crystals through growth inhibition. We thus hypothesized that the co-culture of *C. albicans* with *A. niger* will induce the shift from yeast to hyphal growth in the former, while the addition of *C. oxalaticus* will block oxalic acid production of *A. niger* and result in the growth *C. albicans* as a yeast. To test this hypothesis, we performed co-cultures with two inoculation schemes (simultaneous and sequential) in a medium used to grow lung cells. In addition to growth monitoring, the following parameters were also assessed: pH, oxalic acid and calcium concentrations. We showed that when co-cultured simultaneously, *C. albicans* inhibits the germination of *A. niger* conidia. Therefore, *A. niger* could neither acidify the growth medium, nor precipitate calcium as calcium oxalate crystals, which was the case when *A. niger* was inoculated alone. When *C. albicans* was cultured alone, it exhibited yeast growth, and the pH and calcium concentration remained at the same level as the uninoculated controls. When *C. albicans* was inoculated after *A. niger* conidia had germinated (sequential inoculation), *C. albicans* did not shift into hyphal growth. In these conditions, the growth of *A. niger* was not impaired, however it appears that *C. albicans* is able to remobilize calcium potentially chelated as calcium oxalate. The oxalotrophic bacterium *C. oxalaticus* also inhibited the germination of *A. niger* conidia, and thus impede acidification and calcium oxalate precipitation. However, *C. oxalaticus* did not appear to have any impact on *C. albicans* growth. Finally, we also investigated the production of organic acids by *C. albicans* in various culture media by UHPLC. *C. albicans* produce mainly acetic acid, but also formic and oxalic acid (albeit at lower concentrations), in different culture media. This represents a relevant fungal-fungal interaction to considered within the lung microbiome and thus in the context of human health and fungal co-infection.

6.1. Introduction

Candida albicans is the most common commensal inhabitant of the mammalian mycobiome. This fungus colonizes the skin and mucosal surfaces of the mouth, female reproductive tract and gastrointestinal tract of most healthy individuals, without causing any harm (1, 2). *C. albicans* is also the most prevalent human opportunistic fungal pathogen, and affects immunologically weak or immunocompromised individuals (3, 4). Diseases caused by *C. albicans* range from infections of mucosal surfaces, to life-threatening invasive disseminated infections (4). Invasive candidiasis, the most lethal one, affects around 750'000 individuals annually (5).

The virulence of *C. albicans* is partly promoted by its ability to change growth morphotypes during its interaction with its host. Indeed, *C. albicans* is polymorphic and can shift between a wide range of cellular growth forms (6). The main morphologies are yeast, pseudohyphae and hyphae (3, 4, 7). While the yeast and hyphae morphotypes are the best characterized, this is not true in the case of pseudohyphae. Up to date, there are no known *in-vitro* conditions to induce a pure, stable population of pseudohyphae (3). The filamentous forms of *C. albicans* – i.e. hyphae and pseudohyphae – were traditionally viewed as pathogenic, whereas the yeast form was viewed as commensal. Unlike yeast, which disseminate through the bloodstream, hyphae can actively penetrate the host tissue, and have been shown to express a number of virulence factors such as adhesins, tissue-degrading enzymes, or antioxidant defence proteins, among others (3). However, all three cell types have been shown to co-exist in animals and human patients with disseminated candidiasis (8-10), and to be required for biofilm formation, which is also important in pathogenesis (3). Together, the yeast-hypha-pseudohypha morphogenesis plays a central role in *C. albicans*-host interaction (3).

The switch between yeast to hyphal growth is known to be induced by a variety of environmental triggers such as the presence of serum, neutral or alkaline pH, incubation at 37°C, high CO_2 concentration, growth in embedded conditions, quorum-sensing, carbon and nitrogen starvation, or even by contact with peptidoglycan (4, 6, 11). Additionally, *C. albicans* has been shown to auto-induce its switch to hyphal form by altering the pH of its environment through the production and release of ammonium ions (12). This process is regulated by the plasma membrane H^+-ATPase Pma1p (11, 13). Moreover, *C. albicans* can also acidify its environment, notably through acetate production in sucrose- or glucose-containing media (11, 14, 15). Organic acids such as acetate, formate, pyruvate and propionate secreted by *C. albicans* have been also suggested to have a role in pathogenesis (15).

Another organic acid for which a role in pathogenesis has been suggested is oxalic acid. For instance in the case of the phytopathogenic fungus *Sclerotinia sclerotiorum*, oxalic acid has been shown to facilitate

infection through acidification and sequestration of calcium ions in the middle lamella, which weakens the cell wall structure. Sequestration of calcium also inhibits plant defenses and induce apoptosis (16, 17). *Candida* spp. is reported as a fungal source of oxalate on the Human Metabolome Database (HMDB) page of oxalic acid (HMDB ID HMDB0002329). However, the production of oxalic acid by *Candida* spp. has not been shown experimentally. To the best of our knowledge, there are only two studies relating the presence of *Candida* spp. with oxalate: the first one describes the presence of *Candida*-like yeast cells in a bladder stone (18), and the second one reports the presence of *C. albicans* associated to a case of pulmonary aspergillosis in alpaca in which oxalate was detected (19). The search for genes involved in the oxalate biosynthetic pathway in the *Candida* Genome Database (CGD) yielded no genes associated to oxalate production in any of the *Candida* spp. represented in this database (*C. albicans*, *C. auris*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*). However, genes related to oxalate transport and oxalate catabolism are present in *C. albicans* and *Candida glabrata*, respectively, suggesting that some *Candida* spp. could use oxalate as a carbon source.

Another prevalent opportunistic fungal pathogen affecting immunocompromised and cystic fibrosis patients is *Aspergillus*. *Aspergillus* fungi can cause a variety of diseases ranging from mild allergies to life-threatening invasive infections (20). In contrast to *Candida* spp., *Aspergillus* spp. are known to directly produce and excrete oxalic acid (21-23). *Candida* spp. are often found in co-infection with *Aspergillus* species in immunocompromised patients (24), as well as patients with cystic fibrosis (25). A paper from Bonhomme *et al.* (26) reported that the addition of 10 mM oxalate resulted in the shift in the growth of *C. albicans* from yeast to hyphal. Thus, the presence of *Aspergillus* spp. and production of oxalic acid may be important in the co-existence with *C. albicans,* as well as the induction of a morphological shift in the latter. This offers the possibility of an indirect control of *C. albicans* growth through the control of *Aspergillus* spp. We thus hypothesize that **the co-culture of *A. niger* and *C. albicans* will induce the shift from yeast to hyphal growth in the latter** and that **the addition of oxalotrophic bacteria will block oxalic acid production of *A. niger* and thus suppress the morphological shift of *C. albicans***.

6.2. Materials & Methods

6.2.1. Bacterial and Fungal cultures

All fungal and bacterial strains used in this study are summarized in Table 1. *C. albicans* DSY4717 was kindly provided by Prof. Dominique Sanglard (Lausanne University Hospital). *A. niger* NEUM8 and *Cupriavidus oxalaticus* Ox1 were obtained from the collection of the Laboratory of Microbiology of the University of Neuchâtel (LAMUN). *C. oxalaticus* Ox1 was tagged in-house using insertion with a MiniTn7

system. *C. albicans* was routinely cultured on Yeast Extract-Peptone-Dextrose Agar (YEPDA). YEPDA medium was composed of 10 g/L yeast extract (Lab Logistics Group GmbH, Meckenheim, Deutschland), 20 g/L peptone from casein (tryptone, Biolife Italiana, Milano, Italy), 20 g/L dextrose (Carl Roth, Karlsruhe, Germany), and 20 g/L agar (Biolife Italiana, Milano, Italy), and the pH was adjusted to 5.6. *A. niger* was routinely cultured on Malt Agar (MA) medium, whereas Potato Dextrose Agar (PDA, Carl Roth, Karlsruhe, Germany) was used for *A. niger* conidia production. MA medium was composed of 12 g/L malt extract (Sios Homebrewing GmbH, Wald, Switzerland) and 15 g/L agar (Biolife Italiana, Milano, Italy). *C. oxalaticus* Ox1 was routinely cultured on Nutrient Agar (NA, Carl Roth, Karlsruhe, Germany) medium.

Table 1. Fungal and bacterial strains used

Strain #	Code	Species name	Fluorescent tag	References
DSY4717	**Ca**	*Candida albicans*	GFP	Lausanne University Hospital
NEUM8	**An**	*Aspergillus niger*	/	(27)
NEU1287	**Co**	*Cupriavidus oxalaticus* Ox1	mCherry	(28)

6.2.2. Low Molecular Weight Organic Acids (LMWOA) detection by Ultra-High Performance Liquid Chromatography (UHPLC)

A suspension of *C. albicans* was prepared by taking 5 colonies of *C. albicans* and resuspensding it into 6 mL of physiological water (9 g/L NaCl). A hundred µl of this suspension was inoculated in two sets of 6-well plates containing 5 mL of malt, Reasoner's 2, Potato Dextrose Broth (PDB), Angle, Yeast Extract Potato Dextrose (YEPD), and Air-Liquid Interface (ALI) liquid media, in triplicate. One set of plates was incubated at room temperature for 5 days, and the second set was incubated at 37°C for 5 days.

Table 2. List of media used for the assessement of LMWOA production by *C. albicans*

Medium	Composition	References
ALI	1:1 of DMEM/F12 (Cat.# 11320033, Thermo Fisher Scientific) and LHC Basal Medium (Cat.# 12677019, Thermo Fisher Scientific)	(29)
Angle (modified)	Composition in **Supp. Mat. of Chapter 3**	(30)
Malt	12 g of malt extract (Sios Homebrewing GmbH, Wald, Switzerland) per liter of deionized (DI) water	
PDB	4 g potato infusion powder, 20 g dextrose per liter of DI water	
Reasoner's 2	0.5 g yeast extract, 0.5 g Bacto Peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g Na-pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \times 7H_2O$ per liter of Milli-Q® water	(31)

Medium	Composition	References
YEPD	10 g yeast extract, 20 g peptone from casein (tryptone), 20 g dextrose per liter of DI water	

For the UHPLC analysis, 500 µl of 30 mM H_2SO_4 were added to 1 mL of a five-day liquid culture in malt, Reasoner's 2, PDB, Angle, YEPD and ALI liquid media in triplicate, to obtain 20 mM H_2SO_4 final concentration. The samples were incubated at 60°C for two hours in order to dissolve precipitated metal oxalate crystals, and then centrifuged at 3000 g for 10 min. All the samples were filtered at 0.22 µm (13mm syringe filters, PTFE, hydrophilic) and 200 µl were added into HPLC vials with 250 µl conical inserts. UHPLC (Ultimate 3000 RS-Dionex, Thermo Fisher Scientific, MA, USA) was coupled with DAD detector set at 210 ± 2 nm. A 5 µL of sample was injected onto a Prevail™ organic acid column (5 µm particle size, 150 x 4.6 mm, Grace Davison Discovery Sciences, Deerfield, IL, USA) with the temperature kept at 40°C. The mobile phase consisted of 50 mM phosphate buffer adjusted to pH 2.5 with phosphoric acid with a flow rate of 1 mL/min. Pure oxalic acid, malic acid, acetic acid, citric acid, succinic acid, and formic acid (Merck KGaA, Darmstadt, Germany) were identified by the retention time and were quantified by an external standard curve, linear regression from five calibration points (0.2 to 5 mg/mL).

6.2.3. *Candida-Aspergillus-Cupriavidus* tripartite interaction – simultaneous inoculation

The interaction between *C. albicans*, *A. niger* and *C. oxalaticus* was assessed in Air-Liquid Interface (ALI) medium. ALI medium was composed of DMEM/F12 (Cat.# 11320033, Thermo Fisher Scientific) and LHC Basal Medium (Cat.# 12677019, Thermo Fisher Scientific) at a 1:1 ratio (29). To produce fungal conidia, *A. niger* was cultured on PDA for 7 days at room temperature. Conidia were then harvested by using Dulbecco's Phosphate Buffer Saline (DPBS) supplemented with 0.16% (v/v) Tween 80. Harvested conidia were washed three times with DPBS following centrifugation at 2000xg for 5 min at room temperature. Finally, conidia were resuspended in 1 mL DPBS and quantified with an Improved Neubauer counting chamber. Ten µL of a 1.2×10^6-conidia, *Candida* cells, and/or bacterial cells suspension were then inoculated in 1 mL ALI medium in order to obtain a final cell density of 120 conidia, *Candida* cells or bacterial cells per well in a 12-well plate, in triplicate. The plates were incubated at 37°C for 72h. Images were taken with an EVOS® FL inverted microscope (Thermo Fisher Scientific, CA, USA). pH was measured using pH 1-14 universal indicator paper. Free calcium (Calcium Colorimetric Assay, Sigma-Aldrich, Darmstadt, Germany, Cat.# MAK022) and free oxalic acid (Oxalic Acid Colorimetric Assay Kit, Sigma-Aldrich, Darmstadt, Germany, Cat.# MAK179) were quantified in the culture medium using colorimetric assay kits following the manufacturer instructions.

6.2.4. Cross-inoculation of *C. albicans* and *A. niger* in spent medium of the other partner

Spent ALI medium in which *C. albicans* and *A. niger* were inoculated as conidia and mycelial microcolonies was used in order to check the influence of the compounds excreted by each partner on the growth of the other. For that, 1200 cells of *C. albicans* and 1200 *A. niger* conidia were inoculated in 10 mL ALI medium and incubated for 72 h at 37°C. Microcolonies of *A. niger* were obtained by letting 120 conidia germinate in 1 mL ALI medium. After incubation, the culture was harvested by centrifugation at 3000xg for 10 min in order to recover the spent medium. Afterwards, 120 *C. albicans* cells were inoculated in 1 mL of *A. niger* spent media, and 120 *A. niger* conidia or microcolonies were inoculated in the same amount of *C. albicans* spent medium and incubated for 72h. Imaging was performed using an EVOS® FL inverted microscope (Thermo Fisher Scientific, CA, USA).

6.2.5. *Candida-Aspergillus-Cupriavidus* tripartite interaction – sequential inoculation

Ten µL of a 1.2×10^6 of *A. niger* conidia were inoculated in 1mL ALI medium in triplicate. Conidia were incubated at 37°C for 24h to let them germinate. Ten µL of a 1.2×10^6 of *Candida* and/or bacterial cells were then added in the appropriate wells and the plates were incubated 72 h at 37°C. Each condition was done in triplicate. Images were taken with an EVOS® FL inverted microscope (Thermo Fisher Scientific, CA, USA). pH was measured using pH 1-14 universal indicator paper. Free calcium (Calcium Colorimetric Assay, Sigma-Aldrich, Darmstadt, Germany, Cat.# MAK022) and free oxalic acid (Oxalic Acid Colorimetric Assay Kit, Sigma-Aldrich, Darmstadt, Germany, Cat.# MAK179) were quantified in the culture medium using colorimetric assay kits following the manufacturer instructions.

6.2.6. Statistical analyses

All statistical analyses were performed using Prism 9 (Version 9.0.2). One-way ANOVAs with multiple comparisons (Tukey's multiple comparisons tests) were used to test the statistical significance of the calcium and oxalic acid quantification data for the simultaneous and sequential inoculation experiments. The statistical significance threshold was set to 5%.

6.3. Results & Discussion

6.3.1. LMWOAs production by *C. albicans* in different nutritional conditions

We first assessed the production of oxalic acid and other LMWOAs by *C. albicans* in different culture media at room temperature (RT) and 37°C. From the six organic acids tested (oxalic, malic, acetic, citric, succinic, and formic acids), only oxalic, formic and acetic acid were detected in the different conditions

tested (Fig. 1). Very low concentrations of oxalic acid (5-7 µM) were detected in PDB (RT and 37°C) and malt at 37°C. In contrast, 35-55 µM of formic acid were detected in PDB and Angle at 37°C. Finally, acetic acid was detected at concentrations ranging from 165 to 175 µM in Angle medium when incubated at 37°C, as well as in ALI medium at both RT and 37°C. This result confirms the report from Samaranayake *et al.* (15) that showed that acetate is the main organic acid excreted by *C. albicans*. Apart from this early report, which also showed differential production of formate, pyruvate and propionate in glucose- and sucrose-supplemented medium, no other study has previously investigated the production of LMWOA in *C. albicans*. LMWOAs such as oxalic acid are known to be differentially produced depending on the trophic conditions of the growth culture medium, which include, among others, the carbon and nitrogen sources and their availability, as well as the environmental pH (32, 33). This has been demonstrated to be the case for the filamentous fungi *Sclerotium rolfsii* (34) and *A. niger* (22). This study provides an exploratory overview into LMWOAs production in *C. albicans* in a limited range of conditions. A more extensive study with the use of a basal mineral medium and specific additions of different carbon and nitrogen sources will be needed in order to decipher the influence of medium composition on the production of organic acids in *C. albicans*.

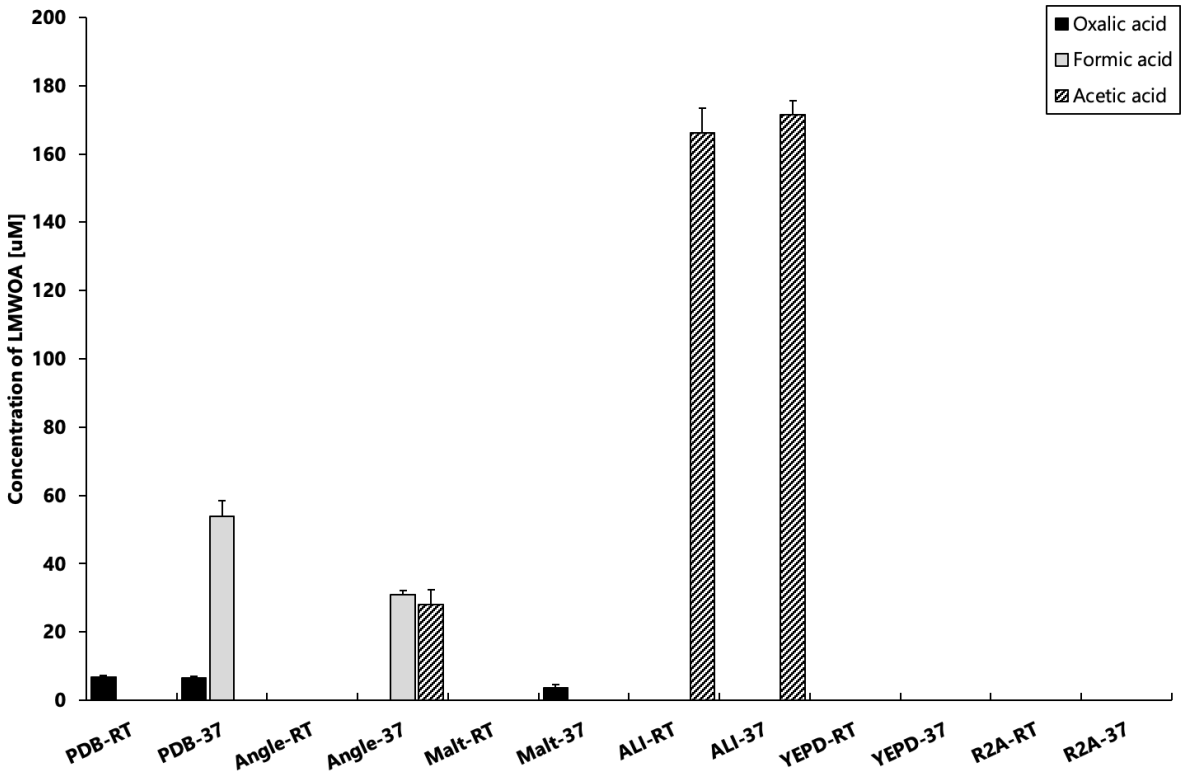


Fig. 1. Production of LMWOAs by *Candida albicans* in different culture media. Oxalic, formic and acetic acids were detected in some of the media tested, with acetic acid being the most abundantly produced in Angle medium at 37°C and in ALI medium at both room temperature and 37°C. The bars represent the mean concentration of three independent replicates + sd.

As indicated previously, despite the absence of genes related to oxalate biosynthesis in the candida genomes present in the *Candida* Genome Database (CGD), we measured a small amount of oxalic acid in some of the media tested (PDB and malt).

6.3.2. *Candida-Aspergillus-Cupriavidus* tripartite interaction – simultaneous inoculation

Next, we performed an interaction assay between *C. albicans*, *A. niger* and *C. oxalaticus* by inoculating them simultaneously in ALI medium. After 72h of incubation, the pH of the medium of the wells inoculated with *A. niger* shifted to an acidic pH as compared to the control (Fig. 2). This is indicated by the change of the color of the phenol red pH indicator from pink to yellow. This is correlated to the development of *A. niger* (Fig. 3A), and corroborated by the measured pH drop from 9 to 6 (Fig. 4A). This pH drop was accompanied by a drop in free calcium and free oxalic acid concentrations (Fig. 4B & C), which resulted from the formation of calcium oxalate crystals (Fig. 3B).

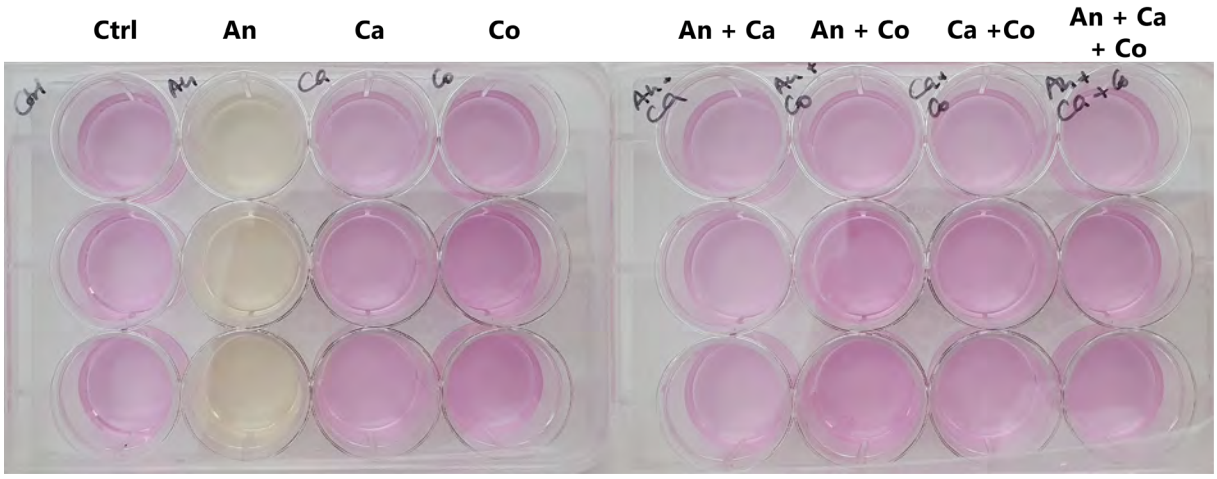


Fig. 2. Image of the plates in the simultaneous interaction after 72h. A yellow color indicates a pH < 7, and a pink color a pH > 7. Ctrl = control; An = *A. niger*; Ca = *C. albicans*; Co = *C. oxalaticus*; An + Ca = *A. niger* + *C. albicans*; An + Co = *A. niger* + *C. oxalaticus*; Ca + Co = *C. albicans* + *C. oxalaticus*; An + Ca + Co = *A. niger* + *C. albicans* + *C. oxalaticus*.

C. albicans seems to grow partly in hyphal form when grown alone (Fig. 3C). Moreover, *C. albicans* growth does not seem to have any impact on the pH nor on the free calcium concentrations, which both stay at the level of the control (Fig. 4A & B). However, *C. albicans* seems to produce around 0.45 mM of oxalic acid (Fig. 4C). *C. oxalaticus* grown alone (Fig. 3D) does not seem to have any impact either on the pH of the culture medium, or on the free calcium concentration (Fig. 4A & B).

When co-cultured with *C. albicans*, *A. niger* seems to be completely inhibited and outcompeted by the former (Fig. 3E). This is confirmed by the absence of a drop in pH or free calcium concentration (Fig. 4A & 4B). *In-vitro* inhibition of *A. fumigatus* by *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei* and *C. tropicalis* has already been reported by Randhawa (35). By performing inhibition assays in peptone glucose agar plates that were either unsealed or sealed with parafilm, they suggested that the predominant inhibitory molecule was a volatile molecule, as the inhibition was more important in sealed agar plates than in unsealed ones. However, in this particular case, our results suggest the inhibition of *A. niger* conidia germination by *C. albicans* is due to a soluble metabolite secreted in the culture medium, as the confrontation experiments have been done in liquid culture media. Further confrontation experiments would have to be performed in order to assess if inhibition is also partly due to a volatile metabolite. *A. niger* conidia germination was also inhibited by *C. oxalaticus* when co-cultured together, as ungerminated conidia were visible (star in the insert of Fig. 3F). Since C. *oxalatixus* completely inhibited the germination of *A. niger* conidia, no drop in pH and free calcium concentration were observed (Fig. 4A & B). However, *C. oxalaticus* does not seem to have a significant impact on the growth of *C. albicans* (Fig. 3G). In the tripartite interaction (*A. niger*, *C. albicans* and *C. oxalaticus*), *A. niger* was not able to grow (Fig. 3H), and thus neither the pH, nor the free calcium concentration were impacted (Fig. 4A & B). Concerning the free oxalic acid concentrations, *C. albicans* seem to produce oxalic acid at a concentration of 0.45 mM (Fig. 4C). Moreover, a small concentration of oxalic acid was detected in the case of *C. oxalaticus* (Fig. 4C). Because of the inhibition of *A. niger* by *C. albicans*, the potential triggering effect of biogenic oxalic acid on the yeast-to-hyphal growth switch in *C. albicans* could not be tested in simultaneous inoculation.

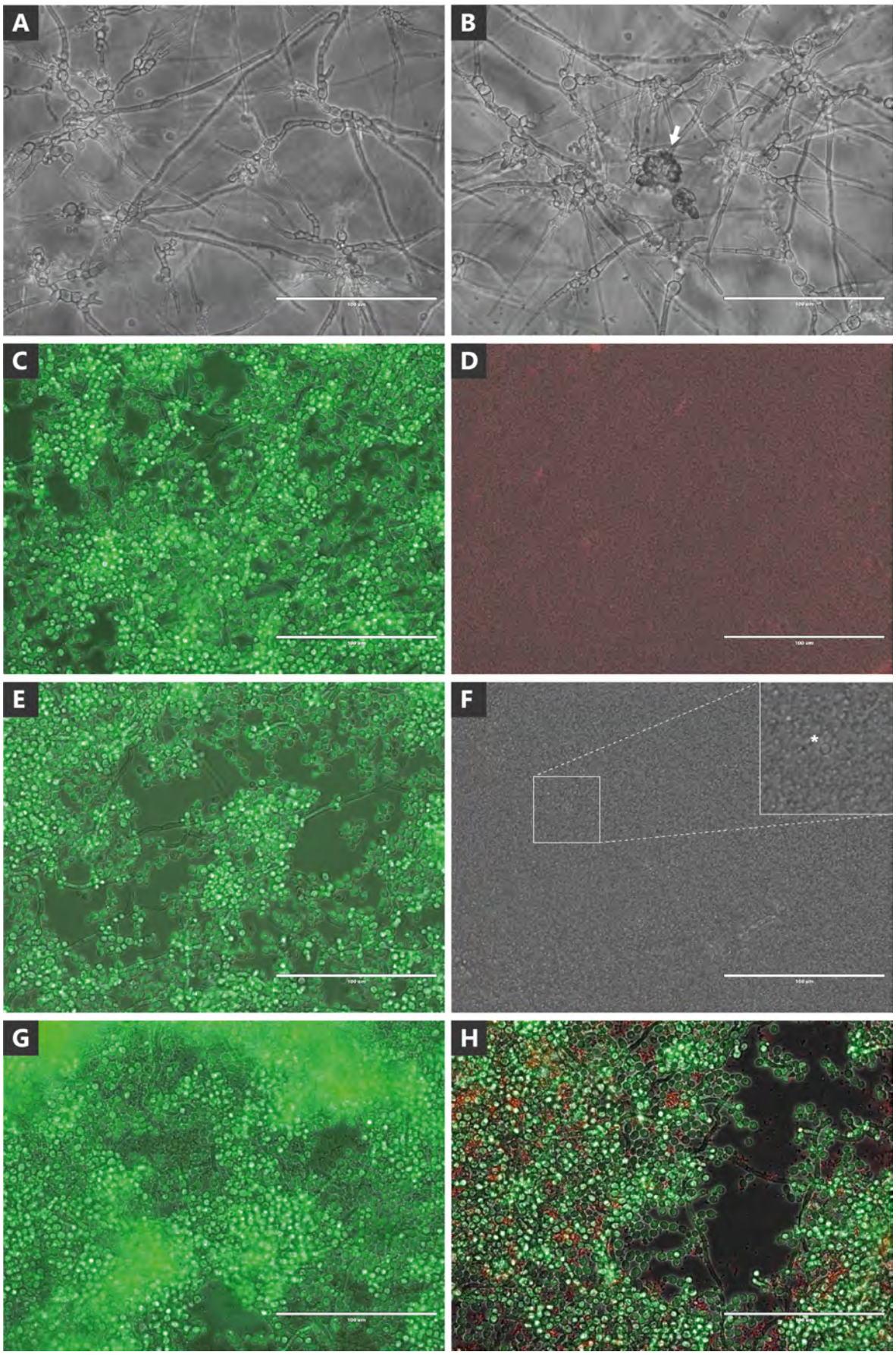


Fig. 3. Fluorescence microscopy images of the simultaneous inoculation interaction assay. (A) *A. niger* alone. (B) *A. niger* with calcium oxalate crystals (white arrow). (C) *C. albicans* alone. (D) *C. oxalaticus* alone. (E) *A. niger* with *C. albicans*. (F) *A. niger* with *C. oxalaticus*. The insert is a zoom to show an *A. niger* coinidium, whose germination was inhibited by *C. oxalaticus*. (G) *C. albicans* with *C. oxalaticus*. (H) *A. niger* with *C. albicans* and *C. oxalaticus*. Scale bars = 100 µm.

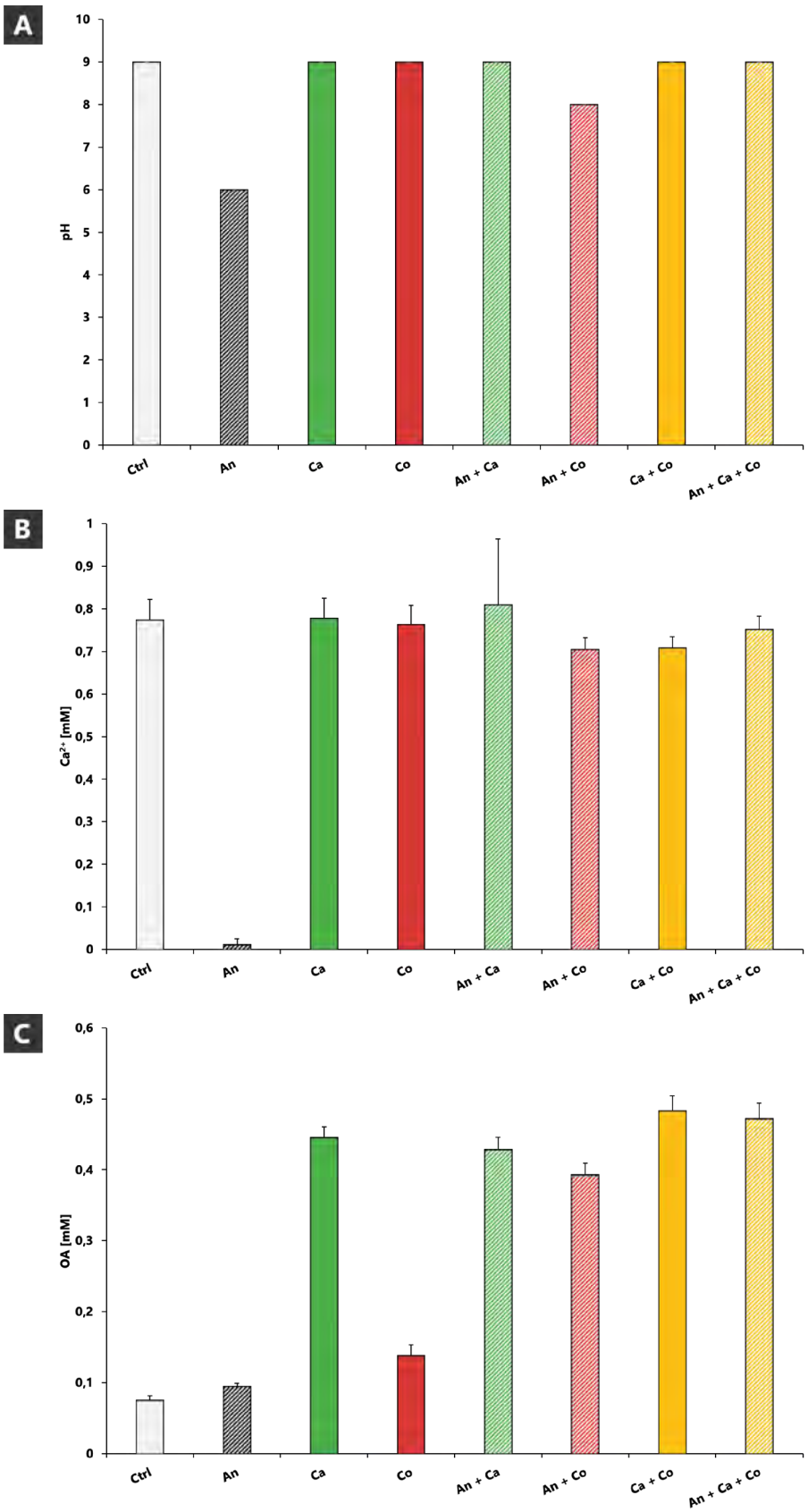


Fig. 4. pH, free calcium and free oxalic acid concentrations measurements of the simultaneous inoculation interaction assay. (A) pH measurements. (B) Free calcium concentration measurements. (C) Free oxalic acid concentration measurements. The bar represent the mean of three independent biological replicates + sd. The p-values of the individual comparison between treatments are presented in Table 3.

Table 3. p-values of the statistical analyses performed on the calcium and oxalic acid quantification data

	Simultaneous				Sequential			
	Calcium		Oxalic acid		Calcium		Oxalic acid	
Tukey's multiple comparisons test	**Summary**	**Adjusted P Value**	**Summary**	**Adjusted P Value**	**Summary**	**Adjusted P Value**	**Summary**	**Adjusted P Value**
Ctrl vs. An	****	<0.0001	ns	0.3947	****	<0.0001	ns	>0.9999
Ctrl vs. Ca	ns	>0.9999	****	<0.0001	ns	>0.9999	****	<0.0001
Ctrl vs. Co	ns	>0.9999	****	<0.0001	ns	>0.9999	****	<0.0001
Ctrl vs. An + Ca	ns	0.9781	****	<0.0001	*	0.0435	****	<0.0001
Ctrl vs. An + Co	ns	0.5943	****	<0.0001	****	<0.0001	****	<0.0001
Ctrl vs. Ca + Co	ns	0.6645	****	<0.0001	ns	0.9399	****	<0.0001
Ctrl vs. An + Ca + Co	ns	0.9988	****	<0.0001	ns	0.9854	****	<0.0001
An vs. Ca	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
An vs. Co	****	<0.0001	***	0.0004	****	<0.0001	****	<0.0001
An vs. An + Ca	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
An vs. An + Co	****	<0.0001	****	<0.0001	ns	>0.9999	****	<0.0001
An vs. Ca + Co	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
An vs. An + Ca + Co	****	<0.0001	****	<0.0001	****	<0.0001	****	<0.0001
Ca vs. Co	ns	>0.9999	****	<0.0001	ns	>0.9999	ns	0.9906
Ca vs. An + Ca	ns	0.9886	ns	0.5428	*	0.0444	ns	0.9894
Ca vs. An + Co	ns	0.5277	****	<0.0001	****	<0.0001	****	<0.0001
Ca vs. Ca + Co	ns	0.5985	**	0.0032	ns	0.9420	ns	>0.9999
Ca vs. An + Ca + Co	ns	0.9967	ns	0.0836	ns	0.9862	ns	0.2433
Co vs. An + Ca	ns	0.9122	****	<0.0001	ns	0.0796	ns	0.7096
Co vs. An + Co	ns	0.7743	****	<0.0001	****	<0.0001	****	<0.0001
Co vs. Ca + Co	ns	0.8314	****	<0.0001	ns	0.9843	ns	0.9858
Co vs. An + Ca + Co	ns	>0.9999	****	<0.0001	ns	0.9981	ns	0.7233
An + Ca vs. An + Co	ns	0.1236	**	0.0075	****	<0.0001	****	<0.0001
An + Ca vs. Ca + Co	ns	0.1552	****	<0.0001	ns	0.4332	ns	0.9932
An + Ca vs. An + Ca + Co	ns	0.7778	***	0.0004	ns	0.2863	*	0.0404
An + Co vs. Ca + Co	ns	>0.9999	****	<0.0001	****	<0.0001	****	<0.0001
An + Co vs. An + Ca + Co	ns	0.9100	****	<0.0001	****	<0.0001	****	<0.0001
Ca + Co vs. An + Ca + Co	ns	0.9425	ns	0.9151	ns	>0.9999	ns	0.2182

6.3.3. Cross-inoculation of *C. albicans* and *A. niger* in spent medium of the other partner

Given the inhibitory effect observed on *A. niger* conidia germination by *C. albicans* when co-cultured together, we decided to use filtered spent medium of each of the partners and to inoculate it with the other partner (cross-inoculation), in order to see if the inhibition is due to factors released by either fungus in the culture medium. When cultured in *A. niger*-ALI spent medium, *C. albicans* growth seemed to be inhibited (Fig. 5A), as compared to when cultured in fresh ALI medium or in the co-inoculation scheme (Fig. 5B). Coleman *et al.* (36) showed that cell free-supernatant of *A. fumigatus* inhibited the growth of *C. albicans*. Inhibition has been shown to be caused by gliotoxin, a known secondary metabolite and virulence factor produced by *A. fumigatus* (36). Gliotoxin is also produced by other *Aspergillus* spp. associated with aspergillosis, such as *A. niger*, *A. flavus*, *A. terreus* (36-38), but also by few *C. albicans* strains (39). However, gliotoxin production has not been assessed in our *A. niger* strain. As *A. niger*-spent medium was acidic due to oxalic acid production (cf. previous experiments), the growth inhibition of *C. albicans* might be due to the low pH of the culture medium. Even if *C. albicans* has been shown to be able to rapidly alkalinize its environment through ammonium secretion upon hyphal growth shift (12), this might not be visible in the conditions tested here because other environemental cues (i.e. 37°C-incubation temperature) for yeast-to-hyphal growth shift might not be favourable. Inhibition could also be caused by the scarcity in free calcium in the growth medium. Indeed, calcium has been found to be important not only for yeast growth, but also for directed hyphal growth in *C. albicans* (40-42).

The fact that oxalic acid production by *A. niger* led to the precipitation of calcium ions in the form of calcium oxalate crystals might explain the growth inhibition of *C. albicans* in yeast form, but also the absence of filamentation when grown in *A. niger*-spent medium. In the case of *A. niger* conidia and microcolonies inoculated in *C. albicans*-spent medium (Fig. 5C & 5F), both of them seem to be inhibited by the secreted metabolites of *C. albicans*, as compared to when inoculated in fresh ALI medium (Fig. 5D & 5G). As explained above, inhibition of *A. fumigatus* hyphal growth by *C. albicans* has previously been reported, but most probably through a volatile compound (35). Therefore, the nature of the inhibitory metabolites still need to be investigated.

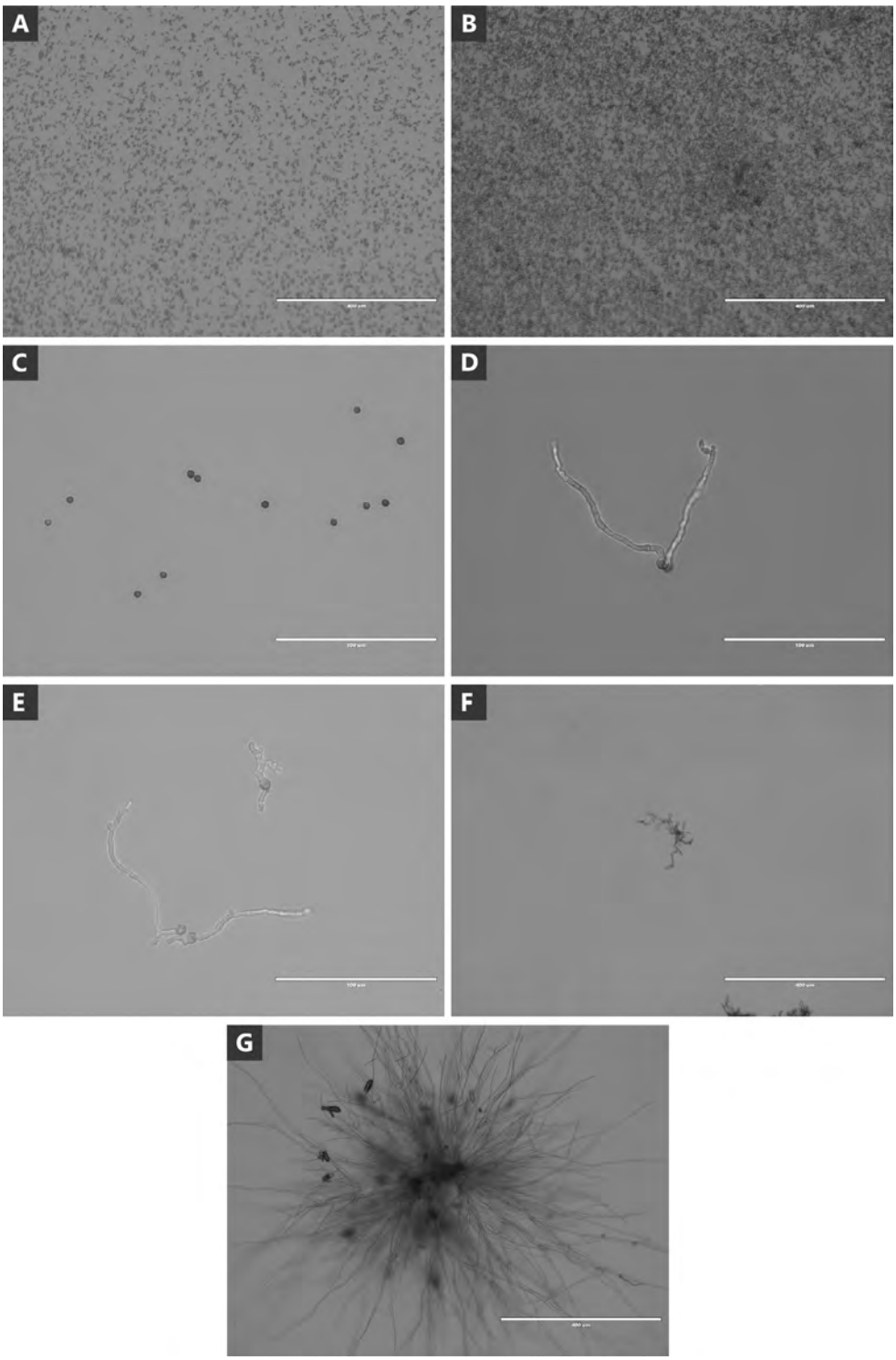


Fig. 5. Microscopy images of the cross-inoculation assay. (A) *C. albicans* in *A. niger*-ALI spent medium. (B) *C. albicans* in fresh ALI medium. (C) *A. niger* spores in *C. albicans*-ALI spent medium. (D) *A. niger* spores in fresh ALI medium. (E) *A. niger* germinated spores before changing media. (F) *A. niger* in *C. albicans*-ALI spent medium. (G) *A. niger* in fresh ALI medium. Scale bars = 400 µm for (A), (B), (F) and (G); and 100 µm for (C), (D) and (E).

6.3.4. *Candida-Aspergillus-Cupriavidus* tripartite interaction – sequential inoculation

As we could not test the triggering effect of oxalic acid produced by *A. niger* on the growth shift from yeast to hyphae in *C. albicans* in the previous experiment, the next step was to perform an interaction assay with sequential inoculation. First *A. niger* was inoculated alone, and 24 h after, *C. albicans* and *C. oxalaticus* were added (individually or combined). As in the case of simultaneous inoculation, after 72h of incubation, the wells inoculated with *A. niger* alone were yellow, indicating a pH shift. The same was true for the wells first inoculated with *A. niger* and then with *C. oxalaticus* (Fig. 6).

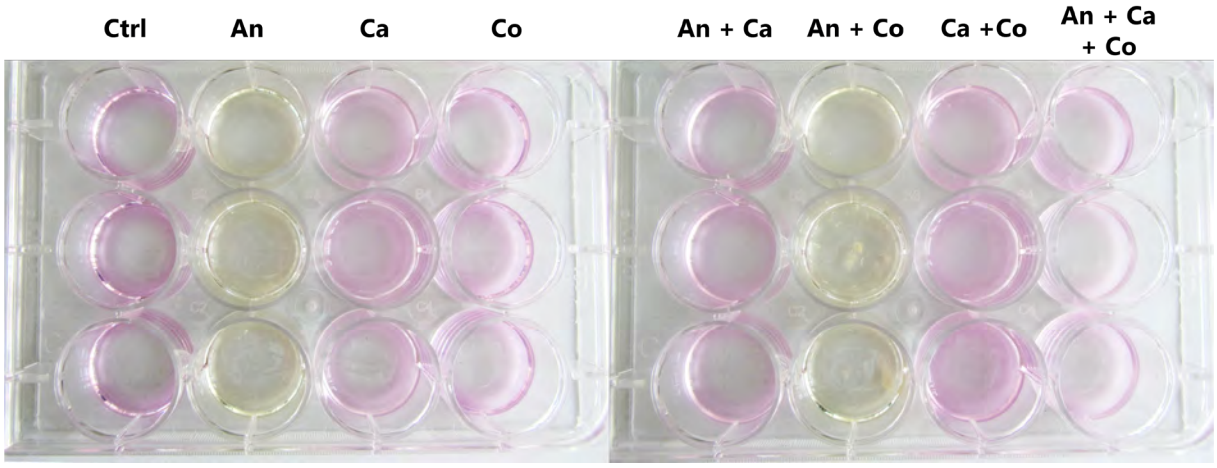


Fig. 5. Picture of the plates of the sequential interaction after 72h. A yellow color indicates a pH < 7, and a pink color a pH > 7. Ctrl = control; An = *A. niger*; Ca = *C. albicans*; Co = *C. oxalaticus*; An + Ca = *A. niger* + *C. albicans*; An + Co = *A. niger* + *C. oxalaticus*; Ca + Co = *C. albicans* + *C. oxalaticus*; An + Ca + Co = *A. niger* + *C. albicans* + *C. oxalaticus*.

This color shift in the pH indicator of the medium was once more correlated with the growth of *A. niger* (Fig. 7A), and resulted in a pH drop from 9 to 6 (Fig. 8A). This pH drop was accompanied by a drop in free calcium and free oxalic acid concentrations (Fig. 8B & C), indicating the sequestration of calcium by oxalic acid. Unlike the case when *C. oxalaticus* is co-inoculated with *A. niger* at the same time (Fig. 3F), *C. oxalaticus* seems to be completely inhibited by *A. niger* when conidia have already germinated (Fig. 7E). This explains the drop in pH and free calcium concentration in the co-culture treatment (Fig. 8A & B). However, *A. niger* seems to produce oxalic acid in excess (Fig. 8C). The fact that in the sequential inoculation of *C. oxalaticus,* conidia germination and growth of *A. niger* were not controlled by the bacterium shows the importance of inhibiting this pathogen before germination. This is what usually happens in healthy individuals in which inhaled *Aspergillus* conidia are readily eliminated either by mucocilliary clearance, or by phagocytosis by macrophages (20). In case of germination, *Aspergillus* germlings can still be eradicated by neutrophils or dendritic cells. Once these defenses are passed, it is usually too late and *Aspergillus* infects its host (20). Nevertheless, it is important to indicate that another reason for the failure of *C. oxalaticus* to control *A. niger* could be the low cell density at which the

bacterium was inoculated. A dose-reponse assay would have to be performed in order to identify the minimal dose at which *C. oxalaticus* is able to inhibit the mycelial growth of *A. niger*.

When co-cultured with *A. niger*, *C. albicans* formed a few hyphae, but grew mostly in its yeast form (Fig. 7D). This can be explained by a decrease in calcium ions concentration in the culture medium. Moreover, *C. albicans* seems to control the pH of the medium that returned to a neutral value (Fig. 8A). This could be achieved either by the consumption of oxalic acid, or by producing alkalinizing molecules. As indicated above, genes related to oxalate transport have been found in the genome of *C. albicans*, indicating that *C. albicans* is able to import oxalic acid in its cells and use it potentially as a carbon source. Additionally, *C. albicans* has been shown to excrete ammonia in order to modify its environement and self-induce the shift from yeast-to-hyphal growth (12). However, the exact mechanism by which *C. albicans* control the pH of the medium when co-cultured with *A. niger* still needs to be investigated.

As indicated above, neither *C. albicans*, nor *C. oxalaticus* seem to influence the pH of the medium, or the free calcium concentration (Fig. 8A & B), but appear to produce oxalic acid (Fig. 8C). As in the case where *C. albicans* is co-cultured with *A. niger*, *C. albicans* hyphae were visible when co-cultured with *C. oxalaticus* (Fig. 7F). Moreover, the fact that the calcium concentration is higher in the *A. niger*-*C. albicans* co-culture condition, as compared to the conditions when *A. niger* is cultured alone (Fig. 8B) suggests that *C. albicans* might remobilize precipitated calcium in the form of calcium oxalate by calcium oxalate degradation. Indeed, genes related to oxalate transport and catabolism have been found in the genomes of *C. albicans* and *Candida glabrata*, respectively, in the *Candida* Genome Database. Moreover, no calcium oxalate crystals were visible in the *A. niger*-*C. albicans* co-culture (Fig. 7D).

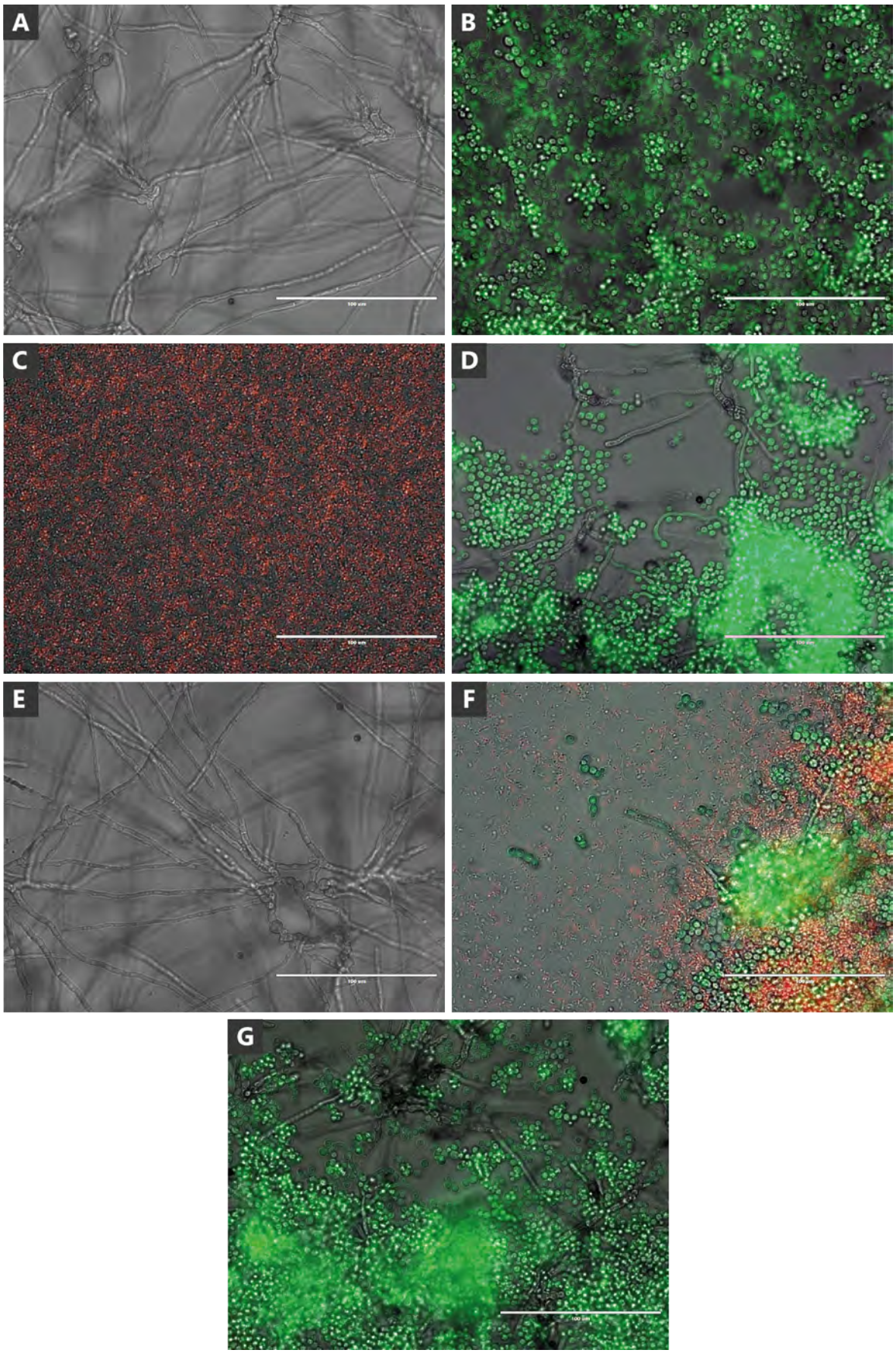


Fig. 7. Fluorescence microscopy images of the sequential inoculation interaction assay. (A) *A. niger* alone. (B) *C. albicans* alone. (C) *C. oxalaticus* alone. (D) *A. niger* with *C. albicans*. (E) *A. niger* with *C. oxalaticus*. (F) *C. albicans* with *C. oxalaticus*. (G) *A. niger* with *C. albicans* and *C. oxalaticus*. Scale bars = 100 µm.

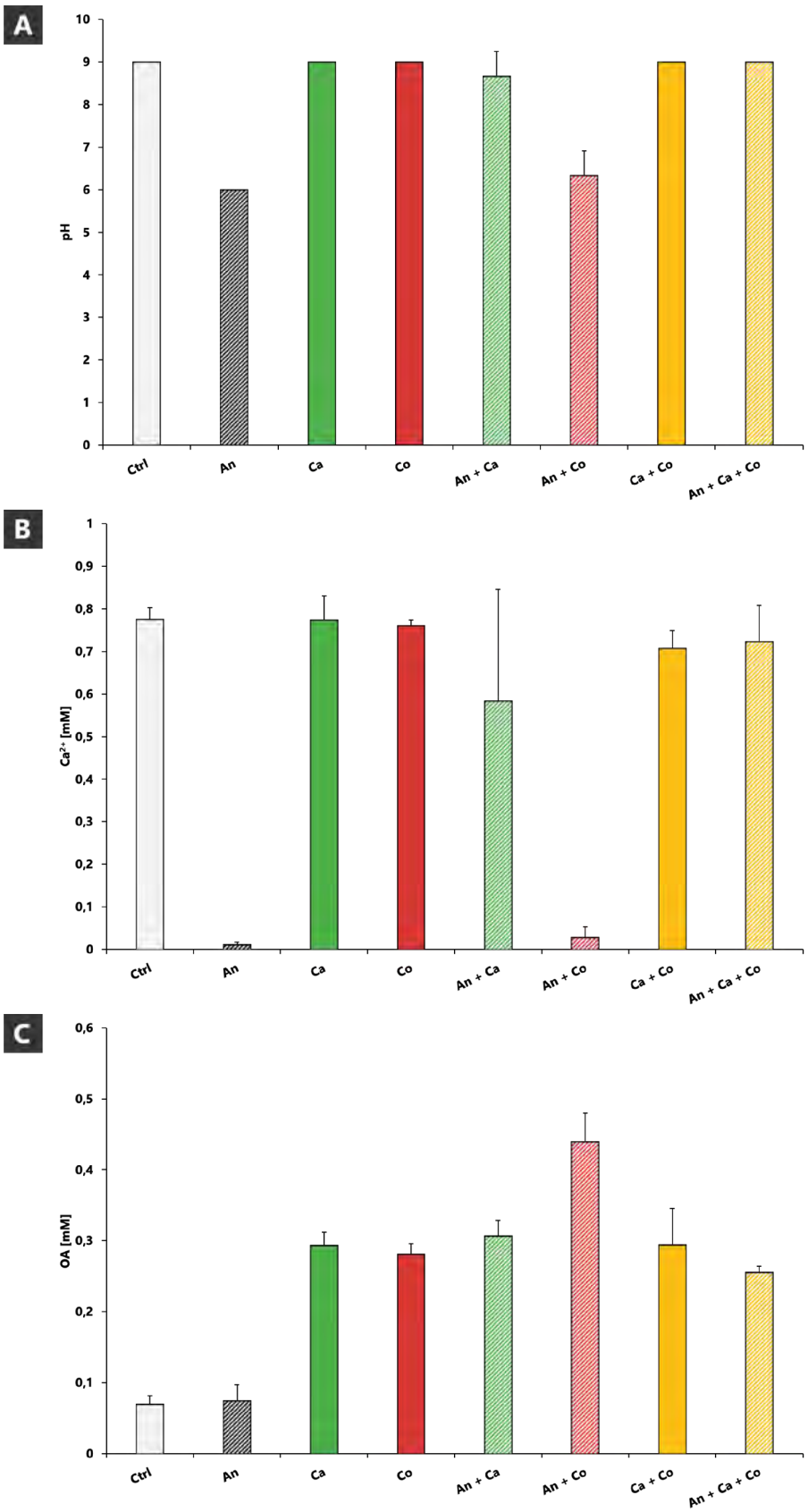


Fig. 8. pH, free calcium and free oxalic acid concentrations measurements of the sequential inoculation interaction assay. (A) pH measurements. (B) Free calcium concentration measurements. (C) Free oxalic acid concentration measurements. The bars represent the mean of three independent biological replicates + sd. The p-values of the individual comparison between treatments are presented in Table 3.

6.4. Conclusion

Here we present a first exploratory study of the tripartite interaction between *A. niger*, *C. albicans* and *C. oxalaticus*. The hypothesis by which *A. niger* will induce the shift from yeast to hyphal growth in *C. albicans* by producting oxalic acid could not be verified. However, as calcium ions have been shown to have a key role in hyphal growth in *C. albicans*, the absence of filamentation in *C. albicans* can be explained by the precipitation of calcium ions in the form of calcium oxalate crystals by *A. niger*. Additionally, we also observed an inhibitory effect of *A. niger* conidia germination, as well as hyphal growth, by *C. albicans*, in cell-free supernatants. Additionnal experiments are required in order to identify the secondary metabolites responsible for the inhibition of *A. niger*. Nevertheless, further invertigations in 3D-lung cell cultures might be worth in order to better understand this interaction in the presence of host cells.

We then assessed the production of several LMWOAs by *C. albicans* in different nutritional conditions. Although a more extensive study would have to be performed in order to decipher the exact conditions that influence the type and concentration of organic acid produced by *C. albicans*, production of acetic acid was identified in various conditions. As organic acids are known to play a role in fungal pathogenesis, for instance, by facilitating the activity of enzymes required for the infection, the role of acetic acid in the pathogenesis of *C. albicans* should be addressed in the future. Moreover, we also observed the environmental pH control exerted by *C. albicans* when it is co-cultured with *A. niger*. Although, we were not able to determine the specific mechanism by which *C. albicans* control the pH, when co-cultured with *A. niger*, we observed the remobilization of chelated calcium from calcium oxalate crystals. Finally, the specific effect of the *A. niger*-*C. albicans* interaction on the concentrations of oxalic acid needs to be investigated further.

Acknowledgments

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References

1. Underhill DM, Iliev ID. The mycobiota: interactions between commensal fungi and the host immune system. Nat Rev Immunol. 2014;14(6):405-16.

2. Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. Candida albicans morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol. 2011;10(2):112-22.

3. Noble SM, Gianetti BA, Witchley JN. Candida albicans cell-type switching and functional plasticity in the mammalian host. Nat Rev Microbiol. 2017;15(2):96-108.

4. Kim J, Sudbery P. Candida albicans, a major human fungal pathogen. J Microbiol. 2011;49(2):171-7.

5. Bongomin F, Gago S, Oladele RO, Denning DW. Global and multi-national prevalence of fungal diseases—estimate precision. 2017.

6. d'Enfert C, Kaune AK, Alaban LR, Chakraborty S, Cole N, Delavy M, et al. The impact of the Fungus-Host-Microbiota interplay upon Candida albicans infections: current knowledge and new perspectives. FEMS Microbiol Rev. 2020.

7. Chen H, Zhou X, Ren B, Cheng L. The regulation of hyphae growth in Candida albicans. Virulence. 2020;11(1):337-48.

8. Di Carlo P, Di Vita G, Guadagnino G, Cocorullo G, D'Arpa F, Salamone G, et al. Surgical pathology and the diagnosis of invasive visceral yeast infection: two case reports and literature review. World J Emerg Surg. 2013;8(1):38.

9. Chin VK, Foong KJ, Maha A, Rusliza B, Norhafizah M, Chong PP. Multi-step pathogenesis and induction of local immune response by systemic Candida albicans infection in an intravenous challenge mouse model. Int J Mol Sci. 2014;15(8):14848-67.

10. Gupta K. Fungal infections and the kidney. Indian J Nephrol. 2001;11.

11. Mba IE, Nweze EI. Mechanism of Candida pathogenesis: revisiting the vital drivers. Eur J Clin Microbiol Infect Dis. 2020;39(10):1797-819.

12. Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H, Lorenz MC. The fungal pathogen Candida albicans autoinduces hyphal morphogenesis by raising extracellular pH. mBio. 2011;2(3):e00055-11.

13. Rane HS, Hayek SR, Frye JE, Abeyta EL, Bernardo SM, Parra KJ, et al. Candida albicans Pma1p Contributes to Growth, pH Homeostasis, and Hyphal Formation. Front Microbiol. 2019;10:1012.

14. Tsuboi R, Matsuda K, Ko IJ, Ogawa H. Correlation between culture medium pH, extracellular proteinase activity, and cell growth of Candida albicans in insoluble stratum corneum-supplemented media. Arch Dermatol Res. 1989;281(5):342-5.

15. Samaranayake LP, Geddes DAM, Weetman DA, MacFarlane TW. Growth and acid production of Candida albicans in carbohydrate supplemented media. Microbios. 1983;37(148):105-15.

16. Dutton MV, Evans CS. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology. 1996;42(9):881-95.

17. de Oliveira Ceita G, Macêdo JNA, Santos TB, Alemanno L, da Silva Gesteira A, Micheli F, et al. Involvement of calcium oxalate degradation during programmed cell death in Theobroma cacao tissues triggered by the hemibiotrophic fungus Moniliophthora perniciosa. Plant Science. 2007;173(2):106-17.

18. Takeuchi H, Konishi T, Tomoyoshi T. Detection by light microscopy of candida in thin sections of bladder stone. Urology. 1989;34(6):385-7.

19. Muntz FHA. Oxalate-producing pulmonary aspergillosis in an alpaca. Veterinary Pathology. 1999;36(6):631-2.

20. Latge JP, Chamilos G. Aspergillus fumigatus and Aspergillosis in 2019. Clin Microbiol Rev. 2019;33(1).

21. Gharieb MM, Gadd GM. Influence of nitrogen source on the solubilization of natural gypsum (CaSO4. 2H2O) and the formation of calcium oxalate by different oxalic and citric acid-producing fungi. Mycological Research. 1999;103(4):473-81.

22. Gharieb MM. Nutritional effects on oxalic acid production and solubilization of gypsum by Aspergillus niger. Mycological Research. 2000;104(5):550-6.

23. Ruijter GJG, van de Vondervoort PJI, Visser J. Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology. 1999;145(9):2569-76.

24. Jaya S, Vipparti H. Mixed fungal lung infection with Aspergillus fumigatus and Candida albicans in a immunocomprimised patient: case report. J Clin Diagn Res. 2014;8(4):DD08-10.

25. Kim SH, Clark ST, Surendra A, Copeland JK, Wang PW, Ammar R, et al. Global Analysis of the Fungal Microbiome in Cystic Fibrosis Patients Reveals Loss of Function of the Transcriptional Repressor Nrg1 as a Mechanism of Pathogen Adaptation. PLoS Pathog. 2015;11(11):e1005308.

26. Bonhomme J, Chauvel M, Goyard S, Roux P, Rossignol T, d'Enfert C. Contribution of the glycolytic flux and hypoxia adaptation to efficient biofilm formation by Candida albicans. Mol Microbiol. 2011;80(4):995-1013.

27. Imeria Ferro K. The impact of oxalogenic plants on soil carbon dynamics - formation of a millennium carbon storage as calcium carbonate. Neuchâtel: University of Neuchâtel; 2012.

28. Şahin N, Işik K, Tamer AÜ, Goodfellow M. Taxonomic position of 'Pseudomonas oxalaticus' strain Ox1(T) (DSM 110(T)) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia oxalatica comb, nov. Systematic and Applied Microbiology. 2000;23(2):206-9.

29. Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, Randell SH. Well-differentiated human airway epithelial cell cultures. Methods in molecular medicine. 2005;107:183-206.

30. Angle JS, McGrath SP, Chaney RL. New Culture Medium Containing Ionic Concentrations of Nutrients Similar to Concentrations Found in the Soil Solution. Applied and Environmental Microbiology. 1991;57(12):3674.

31. Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. Applied and Environmental Microbiology. 1985;49(1):1-7.

32. Plassard C, Fransson P. Regulation of low-molecular weight organic acid production in fungi. 2009. p. 30-9.

33. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. In: Gadd GM, Sariaslani S, editors. 106: Academic Press; 2019. p. 49-77.

34. Punja ZK, Jenkins SF. Influence of Medium Composition on Mycelial Growth and Oxalic Acid Production in Sclerotium rolfsii. Mycologia. 1984;76(5):947-50.

35. Randhawa HSS, R. S. Kowshik, T. In vitro inhibition of Aspergillus fumigatus by Candida species, especially C. albicans and C. glabrata. Current Science. 2002;Vol. 82(No. 7):pp. 860-5.

36. Coleman JJ, Ghosh S, Okoli I, Mylonakis E. Antifungal activity of microbial secondary metabolites. PLoS One. 2011;6(9):e25321.

37. Lewis RE, Wiederhold NP, Lionakis MS, Prince RA, Kontoyiannis DP. Frequency and species distribution of gliotoxin-producing Aspergillus isolates recovered from patients at a tertiary-care cancer center. J Clin Microbiol. 2005;43(12):6120-2.

38. Kupfahl C, Michalka A, Lass-Florl C, Fischer G, Haase G, Ruppert T, et al. Gliotoxin production by clinical and environmental Aspergillus fumigatus strains. Int J Med Microbiol. 2008;298(3-4):319-27.

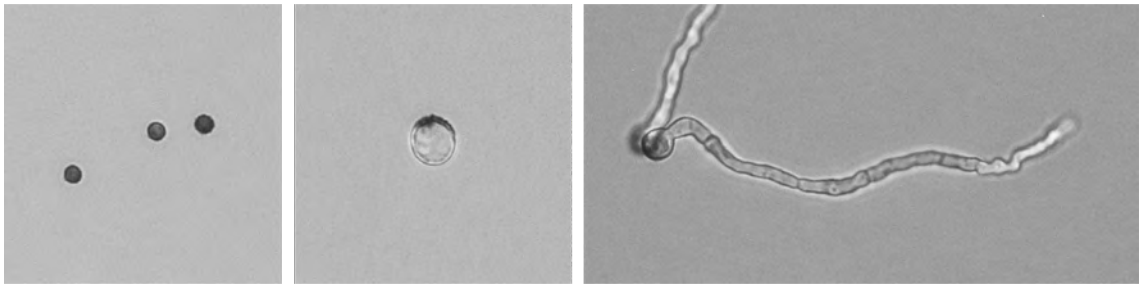
39. Shah DT, Glover DD, Larsen B. In situ Mycotoxin Production by Candida albicans in Women with Vaginitis. Gynecologic and Obstetric Investigation. 1995;39(1):67-9.

40. Peng L, Yu Q, Wei H, Zhu N, Ren T, Liang C, et al. The TRP Ca(2+) channel Yvc1 regulates hyphal reactive oxygen species gradient for maintenance of polarized growth in Candida albicans. Fungal Genet Biol. 2019;133:103282.

41. Brand A, Shanks S, Duncan VM, Yang M, Mackenzie K, Gow NA. Hyphal orientation of Candida albicans is regulated by a calcium-dependent mechanism. Curr Biol. 2007;17(4):347-52.

42. Sakaguchi S, Shibuya K, Iida H, Anraku Y, Suzuki T. Roles of Ca2+ in hyphal and yeast-form growth in Candida albicans. Growth regulation by altered extracellular and intracellular free Ca2+ concentrations. Mycoscience. 1997;38(2):215-25.

CHAPTER 7



Synthesis, general discussion, perspectives and conclusions

Foreword

In this chapter, I present a summary synthesizing the findings of each chapter of this dissertation, a general discussion of the implications of these findings, future perspectives of research and a conclusion.

7. Synthesis, general discussion, perspectives and conclusions

7.1. Synthesis

The general aim of this thesis was to develop a novel paradigm regarding the virulence and infection mechanisms in *Aspergillus* spp., namely the manipulation of the host environmental pH (acidification) via oxalic acid production. The second objective was to develop and assess a novel biocontrol approach based on the environment manipulation through bacterial oxalotrophy, a process we have named *environmental interference*, for the treatment of pulmonary aspergillosis. In this study we provided the first demonstration and *in-vitro* proof of this concept in different culture settings including a 3D lung tissue model.

7.1.1. Chapter 3

The specific aims of this chapter were first to identify a fungal model with relevance to human health based on the ability to produce oxalic acid (or LMWOAs) in different culture media; and second, to test the impact of the environmental (trophic) conditions on the interaction between fungi and bacteria (oxalotrophic and non-oxalotrophic). The importance of identifying the conditions favoring the biocontrol bacterium over the pathogenic fungus was discussed, as well as the implications of this approach from a biocontrol stand point.

The underlying hypothesis tested in this chapter was that trophic conditions have an effect on LMWOA production, as well as on the interaction with bacteria (oxalotrophic and non-oxalotrophic). Because of its consistent acidification behavior due to the production of LMWOAs, *A. niger* was selected as a model human fungal pathogen. Representatives of this genus have been reported as agents of pulmonary aspergillosis, with calcium oxalate crystals being observed in infected lung tissues. Moreover, we confirmed that trophic conditions have an effect on LMWOA production, and on the interaction between fungi and bacteria. Additionally, oxalotrophic bacteria controlled acidification by *A. niger* in pH-indicator containing media, as well as fungal growth in media with poor nutrient content.

7.1.2. Chapter 4

This chapter presented the first demonstration of the biocontrol activity of oxalotrophic bacteria over *A. niger* in *in vitro* conditions (Petri dishes and 3D-lung cell tissues). Our hypothesis was that oxalic acid production is needed for *Aspergillus* spp. to manipulate pH during infection of lung tissues and thus interfering with this process through bacterial oxalotrophy (consumption of oxalate) may limit its growth potential.

Overall, we validated our hypothesis indicating that oxalotrophic bacteria can be used to manipulate the microenvironment created by *A. niger* during the infection of bronchial epithelial tissue. However, we could not assess the specific contribution of oxalic acid secretion in the infectious potential of *A. niger*. In this context, generating and testing a non-oxalogenic mutant *A. niger* strain is needed. Moreover, the extension of the present environmental interference concept through oxalotrophy to *A. fumigatus*, as well as its validation in a pre-clinical murine model is crucial for demonstrating the *in vivo* potential of oxalotrophy to interfere with *Aspergillus* lung infection.

7.1.3. Chapter 5

In the previous chapter, we showed that the addition of *C. oxalaticus*, a bacterial species that is completely foreign from the lung environment, induced non-negligeable cellular damage to the lung epithelial cells. Therefore, the use of living bacteria, at least non-endemic lung bacteria, would not be a viable therapeutic option. In this chapter, we investigated oxalotrophy in the model bacterium *C. oxalaticus* by analyzing its genome. Another goal was to identify markers to assess oxalotrophy in the lung bacterial microbiota.

Overall, we could show that *C. oxalaticus* Ox1 possesses all the genes known to be required for oxalotrophy, and that they seem to be arranged in an operon. Moreover, transcriptional regulatory genes were also found in the vicinity of these genes. The exact role of these transcriptional regulators in oxalotrophy would have to be addressed in the future. More in-depth genomic analyses of the oxalate degradation operon containing the *oxc*, *frc* and *oxlT* genes, as well as the transcriptional regulators, need to be performed in order to define the minimal set of genes required for a completely abiotic enzymatic system for oxalotrophy.

7.1.4. Chapter 6

The aim of this last chapter was to explore for the first time a Fungal-Fungal-Bacterial interaction involving *Candida albicans*, *A. niger*, and *C. oxalaticus*. Indeed, fungal co-infections can be frequent in immunocompromised patients, and in patients with cystic fibrosis. Another aim was to investigate the production of organic acids (i.e. LMWOAs) by *C. albicans* in different culture media. The hypothesis was that oxalate produced by *A. niger* could trigger the shift from yeast to hyphal growth in *C. albicans*, and thus by inhibiting *A. niger* using oxalotrophic bacteria would trigger a return to yeast growth.

Overall, our hypothesis that *A. niger* triggers the shift from yeast-to-hyphal growth in *C. albicans* through oxalic acid production, and that the co-inoculation with *C. oxalaticus* would inhibit both fungal

pathogens, could not be validated. Indeed, tripartite interactions cultures could not be performed because of the inhibition of *A. niger* by *C. albicans*, and of *C. oxalaticus* by growing *A. niger*. In-vitro inhibition of *A. fumigatus* by *C. albicans* and *C. glabrata* has been reported in the literature. However, investigating this interaction in 3D-lung cell cultures could be worth, since some studies reported mixed *Aspergillus-Candida* infections. We also investigated for the first time LMWOA production by *C. albicans* in different culture media at 24 and 37°C and showed that *C. albicans* produced oxalic, formic and acetic acids in different concentrations depending on the culture conditions. Further investigations on LMWOA production by *C. albicans* in-vitro in 3D-lung cell cultures, and in-vivo could be performed, along with transcriptomics analyses.

7.2. General discussion

7.2.1. Environmental interference as a new paradigm of biocontrol strategy of human fungal pathogens

In the present thesis, we tested a new paradigm of biocontrol strategy of *Aspergillus* spp. based on the modification of the environment in which the pathogen thrives, a concept we called ***environmental interference***. In order for a disease to occur, three factors have to interact simultaneously: a pathogen, a susceptible host, and a favorable environment. These three factors constitute the disease triangle (Fig. 1A), which dictates the occurrence of a disease caused by a particular pathogen in a susceptible host in a particular environmental setting (1). The disease triangle concept is often used in plant disease management (2). The main disease management strategy generally applied to control fungal infections is the use of antifungal compounds (Fig. 1B). By acting on the pathogen, the interaction between the three pillars of the disease triangle is disrupted, and onset of the disease cannot occur. However, the overuse of antifungal compounds, as well as their simultaneous use in agriculture and human health, have foster the emergence of cross-resistance to these antimicrobial compounds in fungal pathogens (3). Therefore, in order to slow down the pace of emergence of antifungal resistance, new fungal disease control strategies are badly needed. The one pointed out in this thesis is a biocontrol strategy based on the "environment" corner of the disease triangle (Fig. 1C).

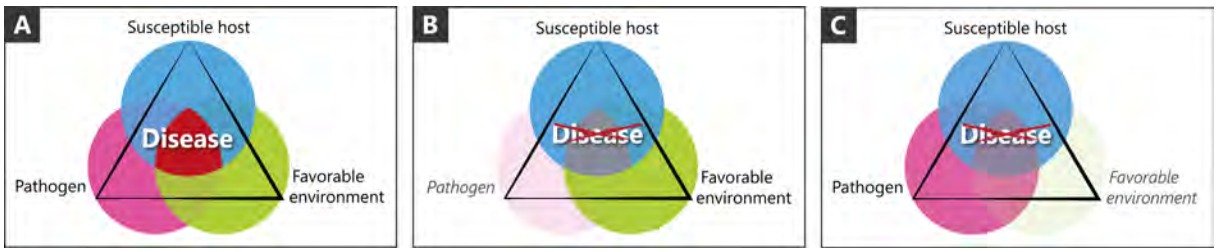


Fig. 1. Disease triangle and biocontrol strategy for the fight against fungal pathogens.

Biological control, or biocontrol, is defined as a process in which an organism is used to inhibit the infection, growth or reproduction of a pathogen. The first organism is often referred to as biological control agent (BCA) (4). In our case, the oxalotrophic bacterium *C. oxalaticus* is used as a BCA to control the growth of the fungal pathogen *A. niger*. By degrading the oxalic acid produced by *A. niger* in order to acidify its environment and make it more favorable for the infection, *C. oxalaticus* manipulates the pH, reverting it back to a more neutral, physiological value, and thus inhibits the growth of *A. niger*. To the best of our knowledge, our study is the first demonstration of such biocontrol strategy for the control of opportunistic human fungal pathogens such as *A. niger*. A similar approach has already been proposed in the case of the biocontrol of *C. albicans* growth by lactic acid bacteria (e.g. *Lactobacillus* spp.) (5, 6). Indeed, *C. albicans* has been described to alkalinize the host tissues through the secretion of ammonia to shift from yeast to hyphal growth form during the infection process (7, 8). In the same way, interfering with this alkalinization process, as well as the yeast-to-hyphae growth form shift, could limit *C. albicans* growth. *Lactobacillus* spp. could potentially limit the growth of *C. albicans* through the secretion of lactic acid and subsequent environmental pH manipulation. However, acidification through lactic acid secretion has not been shown to be correlated to the inhibition of *Candida* spp. by *Lactobacillus* strains (5, 6). *C. albicans* has also been shown to consume lactic acid, which prevents the acidification of its environment (6). Aside from lactic acid, *Lactobacillus* spp. are known to produce other antifungal compounds such as hydrogen peroxide, bacteriocins and biosurfactants (6, 9). However, lactic acid production by *Lactobacillus* spp., and subsequent environment acidification, might confer protection against *C. albicans* pathogenicity *in-vivo* (9). Biocontrol through oxalate degradation by bacteria has already been proposed and assessed in the case of plant fungal pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (10-12). Therefore, one can argue that the overall theme of pH manipulation as a target of environmental control of disease might be a very promising strategy in the case of fungal pathogens.

The environmental interference principle developed in this thesis could be potentially extended to other environmental factors having a significant role in fungal pathogenesis, such as for instance iron. Indeed, iron is an essential nutrient that is usually limiting, and thus its acquisition is crucial for pathogen's virulence (13). Ghio *et al.* (14) reported ferric iron (Fe^{3+}) complexation at the surface of calcium oxalate crystals associated with *A. niger* infection, resulting in lung tissue injury via the generation of oxidants. However, fungal, as well as bacterial pathogens, are known to acquire iron through the secretion of siderophores (15, 16). Loss in the ability to produce siderophores, and thus to acquire iron, has been shown to be detrimental for *A. fumigatus in vivo* (17). Moreover, interfering with the acquisition of iron by the use of chelators has been shown to inhibit the growth of *A. fumigatus* in a cornea infection model

in mice (18). Thus, developing a bacterial biocontrol system based on the interference of *Aspergillus* spp. iron acquisition could be another solution exploiting the principle of interfering with the environment. This could be achieved either by direct competition between *Aspergillus* spp. and the biocontrol bacteria, where this latter would produce a siderophore with a higher affinity for iron than the fungal siderophore, allowing them to better acquire iron than the fungus, or by "cheating" from the bacteria through stealing of the siderophores produced by *Aspergillus* spp. Indeed, non-siderophore producing bacteria are known to steal other species siderophores through the use of a matching receptor (19). Therefore, screening of bacterial members of the lung microbiota for iron-mediated growth inhibition of *Aspergillus* spp. could be a strategy to investigate in the future.

One aspect that has been completely overlooked in the original disease triangle concept is the presence of the host microbiota and its role in disease development. A paper from Bernardo-Cravo *et al.* (20) recently highlighted the necessity of including the host microbiome as a fourth factor influencing the onset of a disease, as it plays a very important role in host immunity. Therefore, the disease triangle should become a disease pyramid (Fig. 2) and one should take into account all four factors, i.e., the host, the host microbiome, the environment, and the pathogen, when thinking about disease management.

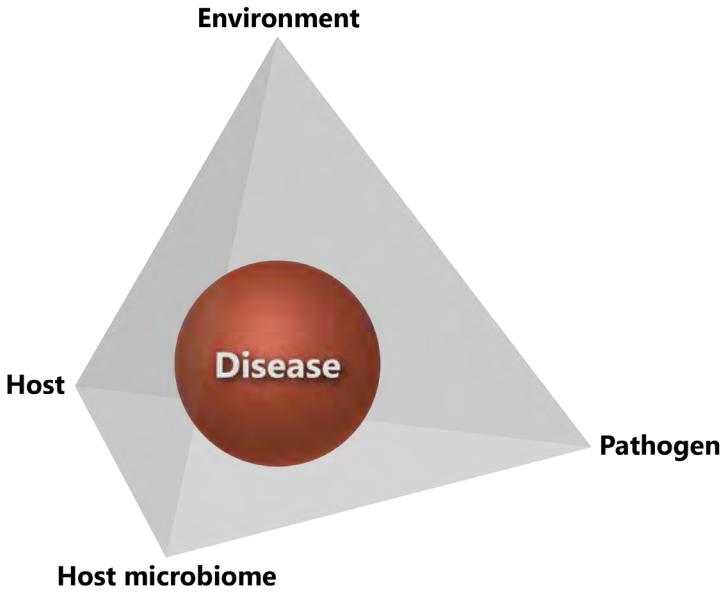


Fig. 2. Disease pyramid concept – including the host microbiome in the factors influencing the onset of disease (adapted from Bernardo-Cravo et al. (20).

Single microbial species, or microbial communities have been shown to inhibit the development of pathogens through a mechanism called colonization resistance (21, 22). A recent paper from Yildiz *et al.* (23) reported host protection against *Streptococcus pneumoniae* colonization of mouse lungs by probiotic *Lactobacillus murinus* strains from the local microbiota. Such lung microbiota-based biocontrol strategy could be applied against aspergillosis, as lung microbiota has been suggested to prevent the

establishment of *Aspergillus* spp. in the lungs (24).

The implementation of environmental interference poses several challenges. First, the concept developed in this thesis needs to be validated in a pre-clinical murine infection model. Indeed, even though the environmental interference concept works in *in-vitro* 3D lung cells tissue systems, its validation in an *in-vivo* model is essential to assess its potential to be applied in a clinical setting. Second, the exogenous *C. oxalaticus* bacterial strain used to control the growth of *A. niger* caused damages to the lung cells. There is thus a need to develop mono-species lung probiotic strains, lung bacterial communities, or a cell-free enzymatic system to prevent cellular damage. Moreover, the efficacy of the developed product needs to be assessed for potential use as a therapeutic application. Furthermore, the live biotherapeutic product (LBP) or enzymatic system could also be used in combination with antifungal compounds for a greater efficacy or synergistic effect. Indeed, there is an increasing interest in using combinations of probiotics and antimicrobial compounds against gut bacterial pathogens such as *Helicobacter pylori* (25). This could also be applied in the fight against lung fungal pathogens. Third, in case of the administration of a single-species lung probiotic or a simplified consortium/community, its ability to establish, and thus overcome colonization resistance, in the resident microbiota should be assessed. Other important components to consider when speaking about a medical treatment are the administration dose and frequency. Indeed, the minimal dose of the therapeutic product will have to be optimized to maximize the chances of success to hinder the infection of the fungal pathogen. Moreover, the optimal frequency of administration of the therapeutic product should also be defined. Finally, it is also important to ensure that the LBP not only causes minimal damage to lung tissues, but that it is also the least immunogenic possible.

7.2.2. pH modulation in fungal pathogenesis

Environmental pH is an extremely important factor influencing not only fungal growth and development, but also fungal physiology (8, 26). Indeed, pH modulation has been shown to affect fungal enzymes activity (27). Moreover, pH has also been pointed out as a crucial element controlling fungal pathogenicity. Indeed, fungal infections are often accompanied with a shift in pH in the surrounding host tissue (26) , either through the secretion of acids, or alkali (8). Fungi are known to produce low molecular weight organic acids (LMWOAs) such as for instance oxalate, citrate, malate, formate, acetate, or succinate, that contribute to the modulation of pH (28, 29). Pathogenic fungi acidify their environment in order to enhance the activity of enzymes, as well as to damage the host tissues (8, 30). This the case of the oxalate-producing phytopathogenic fungi *S. sclerotiorum* and *B. cinerea* (10, 28, 31). Oxalic acid is a known pathogenicity factor for *S. sclerotiorum* and *B. cinerea*, where both acidification and calcium ions

sequestration weaken the cells wall structure, facilitate infection of the host cells, inhibit plant defenses, and induce apoptosis (1, 28, 32-34). Although it has been widely recognized that oxalic acid plays a role in the pathogenesis of plant fungal pathogens, the same is not true for human opportunistic fungal pathogens such as *Aspergillus* spp. In the present thesis, we evaluated the potential role of oxalic acid secretion by the opportunistic fungal pathogen *A. niger* in the infection of lung cells. We highlighted that acidification through oxalic acid production was accompanied by a drop in free calcium concentration, which was confirmed by the presence of calcium oxalate crystals. However, the specific contribution of oxalic acid production in the pathogenesis of *A. niger* could not be addressed, as the use of a non-oxalate producer mutant is required for this. We also tested *F. oxysporum* because it has been reported to produce oxalic acid (35). However, we failed to detect oxalic acid through UHPLC in the culture media tested. As it has been shown by several studies, the profile of the LMWOAs produced by a certain fungal strain depend on the culture conditions (nutrients, pH), and is strain-specific (36-43). Moreover, oxalotrophic bacteria failed to inhibit the growth of *F. oxysporum* on Angle medium supplemented with a pH indicator (bromocresol purple), the only medium in which LMWOA production was detected. This may indicate that the acid produced is not oxalic acid. Indeed, *Fusarium* spp. are known to produce fusaric acid, which is a recognized virulence factor of this fungal genus (44). In a tomato infection model, supplementation of copper, iron or zinc was found to be protective against *F. oxysporum* infection, something indicating that fusaric acid might chelate those metals (44). However, the role of fusaric acid has not been yet elucidated in the infection of a mammalian host. Even if *Fusarium* spp. might acquire metal ions through more specific uptake mechanisms such as siderophores in the case of iron, fusaric acid-mediated metals chelation might have a role in pathogenesis in animal hosts (44), something that need to be investigated.

C. albicans is also known to acidify its environment through the secretion of acetate from sucrose- and glucose-containing culture media, which allow the production of aspartyl proteases (8, 45-48). We could confirm the production of acetate (which was the most abundant LMWOA produced by *C. albicans*), oxalate and formate in different culture media and incubation temperature through UHPLC analyses. However, the specific contribution of the LMWOAs produced by *C. albicans* needs to be further investigated.

Other fungal pathogens manipulate the pH of their environment through alkalinization. This is notably the case of the dimorphic opportunistic human fungal pathogen *C. albicans*. Alkalinization facilitates the invasion of the host tissues, and the evasion of the immune system (47). Alkalinization of the host environment occurs through the release and accumulation of ammonia (NH_3), which is then converted into ammonium ions (NH_4^+) by the urease (8, 47). Moreover, a lack of carbon is required for ammonia-

mediated alkalinization to occur (26). *C. albicans* has been shown to auto-induce its switch to the hyphal growth form through the release of ammonia (7).

Even if this pH modulation strategy through alkalinization of the host environment by *C. albicans* has not been covered in this thesis, the specific role of the lung microbiota in the establishment of *C. albicans* infection should be further investigated.

7.2.3. Towards a potential therapeutic solution – Isolation of oxalotrophic bacteria from the lung microbiome & development of an enzymatic system for oxalate degradation *in-vivo*

The introduction of an exogenous bacterial strain in the lung ecosystem to control *Aspergillus* spp. growth might not be viable as it induced adverse effects, measured as lactate dehydrogenase (LDH) leakage, to the lung cells, as seen in Chapter 4. This made us consider a more feasible alternative therapeutic solutions to develop in the future. One of them is the enrichment and isolation of oxalate-degrading bacteria directly from the lung microbiota. Indeed, while oxalate-degrading bacteria are well characterized in the gut microbiome, for instance *Oxalobacter formigenes*, *Lactobacillus* spp. and *Bifidobacterium* spp. (49), which have been used as probiotics for the treatment of hyperoxaluria (high oxalate in urine) and the management of kidney stones (49, 50), this is not the case in the case of the lung microbiome. Based on the most abundant genera of the core lung microbiota of transplanted lungs identified in a recent study from Das *et al.* (51), the following genera have previously been reported to have an oxalotrophic activity: *Lactobacillus* (52), *Streptococcus* (53), *Prevotella* (54, 55) and *Veillonella* (55). All these reports indicate a potential oxalate-degrading activity in the lung microbiome. However, assessing this oxalotrophic potential and enriching and isolating oxalate-degrading bacteria still needs to be done (cf. 7.3.4.).

The generation of engineered live biotherapeutic strains could also be a solution. Live biotherapeutic products are defined by the Food and Drug Administration (FDA) as "*a biological product that (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine*" (56). The advances of synthetic biology led to the development of live biotherapeutic strains genetically engineered to target specific diseases. One of the most known and used is the *Escherichia coli* Nissle 1917 strain which has been shown to be active against cancer (57, 58), to inhibit the growth of pathogens, such as vancomycin-resistant *Enterococcus* (59) or *Salmonella* (60), to sense and kill *P. aeruginosa* (61), and even to diagnose or reduce inflammation (62-64). According to the guidelines of the FDA and the European Pharmacopoeia Commission (EPC) in terms of live biotherapeutic organisms, engineered probiotics should possess certain features for a successful

drug development, and be safe (non-pathogenic) for human and environmental health (64). First, the engineered probiotic strain should lack antibiotic resistance plasmids in order to avoid its dissemination in the host microbiota and the environment. Second, the gene or metabolic pathway of interest should be integrated in the genome with an inducer/repressor tunable system, as well as a biocontainment module (i.e., cell death module), preventing the probiotic strain to grow in the environment. In our case, the association of an enzymatic pathway with a live engineered probiotic strain could be the way to go. We previously managed to enrich and isolate soil spore-forming bacteria that germinate in response to oxalate following pasteurization (29). Spore-forming bacteria have been shown to be highly prevalent in the gut microbiome (65), but their prevalence in the lung microbiota has not been investigated yet. Bacterial spores constitute an interesting delivery system as they are an efficient dissemination cell type and could be ideal for supplementing the airway microbiota (66). As future perspective, we propose to enrich and isolate spore-forming bacteria from BALF samples from transplanted lung through culture and Raman sorting (cf. 7.3.4.). Mohamed *et al.* (67) developed a heterologous protein expression and release system from the spore core of *Bacillus subtilis* spores, with an autolytic mechanism upon germination and release of the produced protein. This might be interesting to use for the production and delivery of enzymes involved in the degradation of oxalate.

Another option to eliminate living bacteria is the development of a completely enzymatic system. The genomic analysis of our *C. oxalaticus* Ox1 showed and confirmed that our strain possesses all the necessary genes for oxalate degradation, which are the oxalyl-CoA decarboxylase, encoded by the *oxc* gene, the formyl-CoA transferase, encoded by the *frc* gene, and the oxalate:formate antiporter, encoded by the *oxlT* gene. Several patents are published on the use of oxalate-degrading enzymes systems for the treatment of hyperoxaluria and the management of kidney stones. Allison (68) developed an encapsulated lyophilized *Oxalobacter formigenes* cell extract containing the enzymes Oxalyl-CoA decarboxylase and the Formyl-CoA transferase and supplemented with Oxalyl-CoA, $MgCl_2$ and Thiamine-pyrophosphate (ThPP) as cofactors in a hard gelatin drug capsule. The two other patents (69, 70) are based on a system containing a recombinant oxalate decarboxylase (OxDC) from *Bacillus subtilis* and expressed heterologously in *E. coli*, either encapsulated (69) or in the form of nano- or micro-agglomerates (70). These systems are developed for oral administration and are adapted for an optimal activity of the enzymes in the gut environment in order to reduce dietary oxalate. In our case, a system similar to the one developed by Allison (68) would be ideal, as the Oxalyl-CoA decarboxylase and Formyl-CoA transferase found in the genome of *C. oxalaticus* Ox1 are orthologous of *O. formigenes* ones. An important difference between the anaerobic oxalotrophy pathway found in *O. formigenes*, compared to the aerobic pathway in *C. oxalaticus* Ox1, is that in the former, formate is further oxidized

to CO_2 by the formate dehydrogenase instead of being exported outside the cell, as in the latter (29). Another point to take into account is the delivery of the enzymatic system, which would have to be in the form of an aerosol in order to be carried efficiently into the lung. One solution would be the use of gold nanoparticles which have already been used for lung drug delivery (71), as well as nanocarrier of therapeutic enzymes at their surface (72). Future perspective on the development of an enzymatic formulation for future testing on 3D-lung cell tissues is described in 7.3.6.

7.3. Perspectives

Most of the proposed perspectives are not just research ideas, but will actually be addressed in the next few years during my post-doc. The proposed follow-up work will pave the way for a more holistic and ecological approach to human fungal diseases management, taking into consideration the interplay between the pathogen, the host, and its microbiota in the onset of pulmonary aspergillosis. This will allow us to further understand the factors that render the lung ecosystem, and its associated microbiota, more permissive to fungal infections, and thus to develop and propose lung probiotic products to hamper fungal development. Moreover, the proposed follow-up work could also be extended and extrapolated to tackle other fungal diseases, such as mucormycosis.

7.3.1. Genome analysis of *A. niger* and construction of a non-oxalogenic mutant

In order to validate our model supposing a significant role of the production of oxalic acid in the pathogenesis of *A. niger*, the generation of a mutant deficient in oxalic acid production is essential. There are a few reports of such a mutant (73-75), but despite our efforts, none of the research groups concerned had replied to our requests. Therefore, we propose to generate such a mutant by using a recently developed CRISPR/Cas9 system for *A. fumigatus* (76). We will thus first sequence the whole genome of our *A. niger* strain NEU M8, and annotate it thanks to transcriptomics data (RNA sequencing) under different conditions (see Chapter 4). This mutant will be used in the Petri dish, 3D-lung cell culture on Transwells® inserts and on lung-on-a-chip systems in order to establish the effect of suppressing oxalic acid production on pathogenesis as well as in competition with oxalotrophic bacteria.

7.3.2. Extension of the environmental interference concept to *A. fumigatus*

Given the clinical relevance of *A. fumigatus*, as this species is responsible for 90% of the reported cases of aspergillosis (77), the translation of the environmental interference principle initially developed with *A. niger* to *A. fumigatus* is crucial. For this, we will first confirm the production of oxalic acid by *A. fumigatus* in different culture media, including the ALI medium. Then, confrontation experiments will be performed

using *C. oxalaticus* as oxalotrophic bacterium, from *in-vitro* Petri dish to 3D-lung cell tissue systems using the SiMPLInext smart transwell insert system, replicating those already carried out between this bacterium and *A. niger*, as presented in Chapter 4.

7.3.3. Validation of the environmental interference concept against aspergillosis in a pre-clinical murine infection model

While animal models are frequently used and critical for advancing our understanding of microbial pathogenesis, their use raises a number of ethical concerns (78). Nowadays, all animal experimentation studies need to comply with the "3R principles of animal experimentation" (Reduction, Refinement, Replacement), that were originally formulated by Russel and Burch in 1959 (79). Nevertheless, using an animal model is an important step forward to the development of a potential clinical application. Indeed, validating the results presented in this thesis for the biocontrol of *A. niger* in a pre-clinical *in-vivo* murine model, and extending this concept to *A. fumigatus* is crucial.

A pre-clinical *in-vivo* immunocompromised murine model will be developed following available protocols (80-82) in collaboration with the Department of Pulmonology, Lausanne University Hospital (CHUV). For that, adult BALB/cJ mice will be pretreated with a combination of cortisone and cyclophosphamide, in order to lead to an immunocompromised state in mice that is similar to the transplant setting (80). Mice will be infected through oropharyngeal instillation with different doses of *A. niger* NEU M8 (collection of UNINE) (83) or *A. fumigatus* CEA10 (CBS 144.89) or vehicle control. We propose to use bioluminescent strains of *A. niger* and *A. fumigatus* in order to be able to monitor *Aspergillus* spp. infection and biocontrol efficacy of oxalotrophic bacteria *in vivo*. These bioluminescent strains will be constructed following published protocols (84-86), or already published strains will be requested (84, 85). *In-vivo* imaging of *Aspergillus*-infected mice will be performed on a Bruker *in-vivo* Xtreme II Preclinical Optical/X-ray Imaging System. After 48-72h of infection, animals will be sacrificed and lung samples will be analyzed for signs of infection via culture, tissue damage via histological assessment of formalin fixed paraffin embedded lungs, and immune cell composition will be determined in BALF and whole lung tissue by flow cytometry. Oxalic acid concentrations will be measured in BALF in order to validate an increased oxalic acid production in the lungs due to *Aspergillus* spp. infection in the developed murine pre-clinical model of pulmonary aspergillosis. In the clinical setting, *Aspergillus* infection is often associated with a decline in lung function (87). To determine whether this is replicated in our pre-clinical model, the airway resistance, tissue elastance, and forced expiratory volume will be measured (88). In order to assess the potential of oxalotrophic bacteria to decrease the burden of *A. niger* or *A. fumigatus* infection, mice will be pretreated with *C. oxalaticus* (89) by intranasal instillation before infection with *Aspergillus* spp. A

dose-titration and time course will be performed to establish optimal conditions. To assess the effects on disease pathology, we will perform the measurements described in Table 1.

Table 1. Summary table of the parameters that will be assessed in the pre-clinical murine infection model

Focus	Parameter	Method
Environmental parameters	pH	i-STAT CG8+
	Calcium	i-STAT CG8+
	Oxalic acid	Colorimetric assay
Aspergillus	Conidia germination & mycelial growth	Bioluminescent imaging
	Systematic infection markers	Measurement of plasma beta-D-glucan & galactomannan
Air-blood barrier	Permeability	Intravenous infusion of 150 kDA FITC-dextran & 4.4 kDA TRITC-dextran; immunoblot analysis of BALF albumin
	Tissue damage	BALF and blood lactate dehydrogenase
	Cohesive strength	Immunofluorescence for cadherin-17 (adherens junction) and desmoglein-2 (desmosome)
	Antifungal defense & inflammatory cell requirement	qPCR and/or Luminex assays for Dectin-1, TLR2, TLR4, beta defensins (hBD2, hBD7), calprotectin (S100A8, S100A9), IL17F receptor (IL17RA, IL17RC), mucin (MUC5AC) and IL-8 (CXCL8)
Pathology	Histopathology	Microscopic examination of lung tissue sections
	Inflammation & immune response	Differential cell couts in BALF; qPCR-based analysis of gene expression profiles of BALF cells and whole lung; flow cytometry analysis of BALF cells; peripheral blood mononuclear cells and bone marrow precursors involved in airway inflammation
	Respiratory function	FlexiVent measurements of Forced Expiratory Volume (FEV) & Forced Vital Capacity (FVC)
Lung microbiota	Lung bacterial and fungal microbiota composition & biomass	16S/ITS amplicon sequencing & qPCR

7.3.4. Enrichment and isolation of lung oxalotrophic bacterial strains from BALF from transplanted lung

Because of the cellular damages caused by *C. oxalaticus*, a soil bacterial species completely foreign to the lung ecosystem, to control *A. niger* in 3D-lung cell tissues, we will enrich and isolate lung oxalotrophic bacterial isolates directly from Broncho-alveolar lavage fluids (BALF) samples from transplanted lungs, in collaboration with the CHUV. The CHUV routinely collects BALF samples through bronchoscopy between 0.5 and 48 months after transplantation, as part of their post-transplant monitoring of recipients, allowing

them to investigate the dynamics of the bacterial microbiota of the lung. We received 20 samples of BALF with a total volume of 1'600µl (1'200µl BALF + 400µl glycerol).

The enrichment procedure will be adapted from Tamer *et al.* (90). "Crude" as well as pasteurized (80°C for 10 min) (91) BALF samples will be used. Pasteurization will be used in order to favor the enrichment of spore-forming bacteria that germinate in response to oxalate. Spore-forming bacteria are highly prevalent in the gut microbiome (65) and are interesting because spores are an efficient dissemination cell type that could be an ideal material for supplementing the airway microbiota (66). A first test will be performed on 2 samples with different volumes of inoculum in order to determine the adequate inoculation volume to use for the enrichment and isolation procedure. Indeed, as the metabolism we are trying to enrich is extremely specific, there is a risk of getting no results if the inoculum volume is too small. Briefly, 100, 200, and 500 µl of crude or pasteurized BALF samples will be inoculated in 3mL of enrichment medium. The enrichment cultures will be incubated at 37°C under agitation (150 rpm) for 5-7 days. This enrichment process is repeated 3 times by transferring 250 µl of the culture in 5 mL of enrichment medium, either Schlegel-AB medium supplemented with 0.4% calcium oxalate (SchAB + CaOx), or RPMI 1640 medium supplemented with 0.4% calcium oxalate (RPMI + CaOx). Despite the fact that SchAB + 0.4% KOx medium is routinely used as an enrichment medium for soil oxalotrophic bacteria (90), we will use instead the RPMI 1640 medium supplemented with 0.4% potassium oxalate (RPMI + KOx) in order to increases the chances to enrich for oxalotrophic bacteria present in the lung microbiota. Indeed, the RPMI 1640 medium resembles the composition of the deep lung fluids (51, 92), which make it more suitable for the isolation of oxalotrophic bacteria from the lung microbiota than the Schlegel-AB medium. Isolation of potential oxalotrophic bacteria will be carried out by plating 100 µl of enrichment cultures onto bilayered-solid RPMI 1640 agar medium containing 0.4% CaOx in the top layer. Oxalate consumption will be visually assessed by the formation of dissolution halos around growing colonies after 5-7 days of incubation at 37°C. The isolated oxalotrophic bacterial strains will be tested in the Petri dish, 3D-lung cell culture on Transwells® inserts and on lung-on-a-chip systems in order to assess their ability to inhibit *A. niger*/*A. fumigatus* conidia germination and development.

Additionally, we will attempt to sort bacterial spores from BALF using Raman-activated microbial cell sorting (93) in collaboration with Andrea Corona Ramirez, a PhD student in our laboratory working on the development of new methods for the study of spore-forming bacteria in natural communities, and the ETH Zurich. Compared to vegetative cells, bacterial endospores contain dipicolinic acid (66), giving them a unique spectroscopic signature when analyzed with Raman Spectroscopy. This gives the opportunity to use that biosignature to sort them from vegetative cells. As this "enrichment" method is non-destructive for the sample, culture-based assays can be easily performed. The aim would be to

apply this technique to natural communities, and more specifically to the lung microbiota. Indeed, the prevalence of endospore-forming bacteria (Firmicutes) in the lung microbiota has never been assessed. Germination assays in the presence of oxalic acid as a trigger factor can then be performed on the sorted bacterial spores in order to isolate potential lung oxalotrophic probiotics for the supplementation of the lung microbiota. Additionally, single-cell sequencing of sorted spores will also be performed.

7.3.5. Assessing the potential of artificial communities of the lung microbiota with and without oxalotrophic strains to inhibit *A. niger* and *A. fumigatus* conidia germination, or to strengthen the epithelial barrier

In addition to the enrichment and isolation of lung oxalotrophic bacterial strains from the lung microbiota, the potential of artificial communities of the lung microbiota, supplemented or not with oxalotrophic strains to inhibit *A. niger* and *A. fumigatus* conidia germination (suppressive vs. conducive communities), or to strengthen the epithelial barrier, will be assessed. To do so, a bank of 215 lung bacterial isolates representing 47 deeply characterized phylotypes of the human lung microbiota (collaboration with Prof. Philip Engel, Department of Fundamental Microbiology, University of Lausanne) will be used to assemble random lung communities by keeping the ratio between the predominant phyla of the core lung bacterial microbiota (51). Moreover, the isolated lung oxalotrophic isolates from BALF, or alternatively the existing oxalotrophic bacterial strain collection of the LAMUN, which count around 20 strains, will be used to supplement the randomly assembled artificial lung bacterial communities. These artificial communities will be tested in confrontation cultures with *A. niger*/*A. fumigatus* in traditional 2D-cell cultures in a dose-specific manner to get dose-response values for minimized cell damage, as well as in 3D-lung cell culture on Transwells® inserts and on lung-on-a-chip systems in order to assess their ability to inhibit *A. niger*/*A. fumigatus* conidia germination and development, or to strengthen the epithelial barrier. The integrity of the epithelial barrier will be assessed by measuring the trans-epithelial electrical resistance (TEER), the permeability by detecting the leakage of FITC-sodium (0.4 kDa) and RITC-dextran (70 kDa) through the epithelial barrier, and by immunofluorescent staining for E-cadherin (adherens junctions) and Zo-1 protein (tight junctions) (94, 95).

7.3.6. Development of an entirely enzymatic delivery system of key enzymes of the oxalotrophy pathway

Based on the genomic sequences of the two main key oxalotrophy genes of *C. oxalaticus* Ox1, i.e. the oxalyl-CoA decarboxylase (*oxc*), and the formyl-CoA transferase (*frc*), we could overexpress the Oxc and Frc proteins in *E. coli*, then extract and purify them. In terms of the formulation of the enzymatic system,

one has to think about the other elements to include in the formulation. In addition of the Oxc and Frc proteins, thiamine pyrophosphate (ThPP) will have to be added as it is a co-factor of the Oxc. Moreover, CoA:oxalate CoA transferase will also have to be added as oxalate needs to be activated by the addition of a CoA before being decarboxylated by the Oxc (29). Finally, in order to completely degrade oxalate into CO_2, formate dehydrogenase (Fdh) will be added, along with NAD^+ to allow its reduction into NADH + H^+. This enzymatic formulation will then be tested *in-vitro* in confrontation with *A. niger/A. fumigatus* to replace living bacteria.

7.3.7. Construction of a non-oxalotrophic mutant of *C. oxalaticus* Ox1 by CRISPR-Cas9

In order to understand more deeply the oxalotrophy metabolism, *C. oxalaticus* Ox1 will be used as a model bacterium for the construction of a non-oxalotrophic mutant by CRISPR-Cas9, following published protocols (96-98). To do so, following the genome analysis of the *C. oxalaticus* strain Ox1, and more specifically the genomic organization of the genes related to oxalotrophy, the oxalyl-CoA decarboxylase (*oxc*), the formyl-CoA transferase (*frc*) and the oxalate:formate antiporter (*oxlT*) genes will be knocked-out individually, in pairs, or altogether, and the ability of the mutants generated to degrade calcium oxalate crystals will be assessed on bilayered-solid SchAB agar medium supplemented with 0.4% CaOx. Moreover, as the above-mentioned oxalotrophy genes seem to be organized in an operon, along with several transcriptional regulatory genes, knock-outs of these genes will also be performed in order to assess their role in the oxalotrophy metabolism. Finally, the generated mutants will be tested in-vitro in confrontation with *A. niger* to see if their ability to control the growth of this fungus is lost upon loss of their oxalate-degradation capabilities.

7.3.8. Effect of biogenic calcium oxalate formation on the immune response associated to invasive aspergillosis

The results presented in Chapter 4 suggest a potential role of oxalic acid not only in the infection of the lung tissue by *A. niger*, but also in the inhibition of the immune response. Indeed, the chelation of Ca^{2+} by oxalic acid, and the subsequent formation of biogenic calcium oxalate crystals might have a very significant consequence on the immune response of the host, as calcium ions are essential components of the signaling cascade leading to the inflammatory response (99-101). Therefore, the aim of this project is to demonstrate the effect of oxalic acid secretion by *A. niger* (including biogenic calcium oxalate crystal formation) on blocking the inflammation signaling cascade in monocytes and their derived macrophages and dendritic cells. This project has been submitted to the Novartis Foundation, and we obtained funding to acquire the material and equipment to perform the experiments to achieve

this goal.

The hypotheses that will be tested are:

- Oxalic acid secreted by *A. niger* will interfere with the inflammation signaling cascade in monocytes, macrophages and dendritic cells, by sequestrating Ca^{2+} in an insoluble form (biogenic calcium oxalate crystals).
- Co-culturing of the fungal pathogen with the oxalotrophic bacterium *Cupriavidus oxalaticus* will restore Ca^{2+} levels to a physiological level and restore the immune response.

Accordingly, the specific aims of this proposal are the following:

- To evaluate the influence of oxalic acid in the generation of the innate immune response (inflammation).
- To evaluate the potential of bacterial oxalotrophy as a rescue mechanism of the innate immune system for the clearance of the fungus by monocytes, macrophages and dendritic cells.

In order to assess the above-mentioned hypotheses and aims, we will use a human monocytic leukemic cell line (THP-1) (102), along with *A. niger* as fungal pathogen and *C. oxalaticus* as biocontrol agent. More specifically, the engineered THP1-Blue™ NF-κB (Cat. N° thp-nfkb, InvivoGen) cells were chosen, as the induction of the NF-κB signal transduction pathway leading to inflammation, through the stimulation of Pattern Recognition Receptors (PRRs), can be detected and monitored using colorimetry (103).

The first step will be to differentiate the THP-1 and primary monocytes into either monocyte-derived macrophages (mo-MΦ) or monocyte-derived dendritic cells (mo-DC), both of which are found in the lung in case of a fungal infection (104).

THP1-Blue™ NF-κB monocytes will be maintained in RPMI 1640 medium containing 2 mM L-glutamine, 25 mM HEPES and 10% heat-inactivated fetal bovine serum (FBS) in an incubator at 37°C and 5% CO_2, following the instructions of the manufacturer. Cells will be used between passages 3 to 6 to preserve as much efficiency as possible (105). Frozen stocks containing $5\text{-}7\times10^6$ cell/mL will be prepared in a freezing medium composed of 90% FBS and 10% DMSO.

THP-1 monocytes will be differentiated in M0 macrophages by exposing them to 100 ng/mL PMA (phorbol 12-myristate 13-acetate) during 24 to 48h. These M0 macrophages will be then activated into classical inflammatory M1 macrophages with IFN-γ and LPS (103, 106). Immature dendritic cells (iDC) will be obtained by exposing THP-1 cells to rH IL-4 (100 ng/mL) and rH GM-CSF (100 ng/mL) in RPMI + 10% FBS for 5 days. Mature DCs will be obtained following 1-3 days exposure to rH IL-4 (200

ng/mL), rH GM-CSF (100 ng/mL), rH TNFα (20 ng/mL) and 200 ng/mL ionomycin in serum-free RPMI (103, 107). The differentiation will be checked by the detection of specific markers for MΦ and DC by immunofluorescence and subsequent observation in fluorescence confocal microscopy: CD68, CD71 and CD36 for MΦ, and CD80 and CD86 for DC (106, 107). The presence of the CD14 marker, characteristic for monocytic cells, should be lower when differentiated into mo-MΦ or mo-DC.

The TLR assay using the QUANTI-Blue™ solution (Cat. N° rep-qbs, InvivoGen) will be used to assess the activation of the innate immune response by all three cell types. The following experimental treatments will be tested:

- THP-1 cells (monocytes, mo-MΦ or mo-DC) + *A. niger*
- THP-1 cells (monocytes, mo-MΦ or mo-DC) + *C. oxalaticus*
- THP-1 cells (monocytes, mo-MΦ or mo-DC) + *A. niger* + *C. oxalaticus*

The following control treatments will be tested:

- Culture medium (blank control)
- THP-1 cells (monocytes, mo-MΦ or mo-DC) (negative control)
- *A. niger*
- *C. oxalaticus*
- THP-1 cells (monocytes, mo-MΦ or mo-DC) + LPS (positive control)

All treatments will be run in triplicate for 24 h and incubated at 37°C and 5% CO_2. Spectrophotometer measurements will be done at 620 nm. Dose-response preliminary tests will be performed to determine the optimal cell density of THP-1 cells, *A. niger* conidia and *C. oxalaticus* cells.

Additionally, pH will be monitored using Self-adhesive pH Sensor Spots (PreSens, Germany). Ca^{2+} and oxalic acid will be also measured using colorimetric assay kits from Sigma-Merck. Inflammatory cytokines will be quantified using the Inflammation 20-Plex Human ProcartaPlex™ Panel from ThermoFisher (Cat. N° EPX200-12185-901).

7.3.9. Biocontrol of aspergillosis in cystic fibrosis

Fungal infections are known to affect patients suffering from cystic fibrosis (CF), with *Aspergillus* spp. being one of the most commonly isolated genera in those patients (108). Moreover, polymicrobial trans-kingdom infections are also increasingly frequent (109). For instance, this is particularly true in the case of the fungal-bacterial interaction between *Aspergillus fumigatus* and *Pseudomonas aeruginosa* (110, 111). In addition, *Aspergillus* spp. and *Candida* spp. co-infections could also be frequent in CF

patients, as these two genera are frequently co-isolated from sputum (112). By developing a CF 3D-lung tissue model using either the SiMPLInext platform, or a lung-on-a-chip system, we could investigate the dynamics of the polymicrobial infections between *Aspergillus* spp. and lung bacteria, or other fungi such as *Candida* spp. Moreover, the environmental interference concept through bacterial oxalotrophy could also be tested in these systems. Furthermore, the potential of previously assembled disease-suppressive communities to control *Aspergillus* spp. conidia germination and development could also be assessed. For that, CF diseased human bronchial/tracheal epithelial cells available at Lonza (Cat. # 00196979) will be used and cultured in BEGM™ Bronchial Epithelial Cell Growth Medium BulletKit™ (Cat. # CC-3170).

7.3.10. Aspergillosis and the gut-lung axis – Development of a dual gut-lung-a-chip

The gut and the lung compartments have been shown to mutually influence each other through the transfer of metabolites, such as short chain fatty acids (SCFAs), or direct seeding of bacteria from the gut to the lungs (113). This crosstalk between the gut and the lung is the so-called gut-lung axis. Moreover, as the lung microbiota is thought to have a potential impact on the establishment, and the host immune regulation against *Aspergillus* spp., one could think that the gut microbiota could have as well an influence on the immune modulation against pulmonary aspergillosis (24). Very recently, Mateos-Hernandez *et al.* (114) reported that the administration of a gut microbiota bacterial strain (*E. coli* O86:B7) decreased the inflammatory response and conferred protection against pulmonary aspergillosis in poultry. To our knowledge, this is the only experimental evidence of a protective and regulatory immune role against pulmonary aspergillosis. However, the specific crosstalk between the gut microbiota and the lung microbiota and its role in the permissiveness of the lung environment to *Aspergillus* spp., and the strengthening of the epithelial barrier still need to be investigated. To do so, a dual gut and lung organ-a-chip system model will be developed by using for example the HUMIMIC Chip2 system from TissUse (Berlin, Germany) that allows the simultaneous culture of two different organs. Moreover, immune cells will also be added in order to assess as well the immune response against *Aspergillus* spp.

7.4. Conclusions

In summary, we first selected *A. niger* as an opportunistic fungal pathogen model based on its ability to produce oxalic acid as the sole LMWOA in different culture media, and we investigated the potential role of oxalic acid as a pathogenicity factor in the onset of pulmonary aspergillosis. We then could show that oxalic acid production by *A. niger* conducted to a dramatic drop in pH, free calcium and free oxalic acid concentrations, which was associated with the precipitation of calcium oxalate crystals. We also demonstrated for the first time a new proof-of-concept biocontrol strategy based on the manipulation

of the environment in which the pathogen thrives, something we called environmental interference. By using the oxalate-degrading bacterial strain *C. oxalaticus* Ox1, we could maintain pH, free calcium and free oxalic acid concentrations at physiological levels, suggesting that *A. niger* conidia germination and hyphal growth were inhibited in 3D-lung cell tissues. As expected, the addition of a foreign bacterial species on 3D-lung cell tissues induced non-negligeable cell damage, something we will address in the future with the isolation of oxalotrophic bacterial strains from the lung microbiota, or by developing an entirely abiotic enzymatic system thanks to the genomic analysis of the oxalate operon of our *C. oxalaticus* Ox1 strain. Finally, we could also investigate for the first time fungal:fungal:bacterial interaction between *A. niger, C. albicans* and *C. oxalaticus in-vitro* mimicking the case of a mixed fungal infection as observed in immunocompromised patients and patients with cystic fibrosis. To conclude, although the presented results on the environmental interference through oxalotrophy are promising, the development of a pre-clinical *in-vivo* animal model and the subsequent validation of the proposed approach is needed in order to translate these results into a potential clinical application. Indeed, a more holistic approach with the integration of the immune system component will be key to understand the interplay between the host, the lung microbiota and the pathogen.

References

1. Moore D, Robson G, Trinci T. 21st Century Guidebook to Fungi. Cambridge: Cambridge University Press; 2011. 627- p.

2. Scholthof KB. The disease triangle: pathogens, the environment and society. Nat Rev Microbiol. 2007;5(2):152-6.

3. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science. 2018;360(6390):739-42.

4. Heydari A, Pessarakli M. A Review on Biological Control of Fungal Plant Pathogens Using Microbial Antagonists. Journal of Biological Sciences. 2010;10(4):273-90.

5. Parolin C, Marangoni A, Laghi L, Foschi C, Nahui Palomino RA, Calonghi N, et al. Isolation of Vaginal Lactobacilli and Characterization of Anti-Candida Activity. PLoS One. 2015;10(6):e0131220.

6. Zangl I, Pap IJ, Aspock C, Schuller C. The role of Lactobacillus species in the control of Candida via biotrophic interactions. Microb Cell. 2019;7(1):1-14.

7. Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H, Lorenz MC. The fungal pathogen Candida albicans autoinduces hyphal morphogenesis by raising extracellular pH. mBio. 2011;2(3):e00055-11.

8. Vylkova S. Environmental pH modulation by pathogenic fungi as a strategy to conquer the host. PLoS Pathog. 2017;13(2):e1006149.

9. Graf K, Last A, Gratz R, Allert S, Linde S, Westermann M, et al. Keeping Candida commensal: how lactobacilli antagonize pathogenicity of Candida albicans in an in vitro gut model. Dis Model Mech. 2019;12(9).

10. Schoonbeek H-j, Jacquat-Bovet A-C, Mascher F, Métraux J-P. Oxalate-degrading bacteria can protect Arabidopsis thaliana and crop plants against Botrytis cinerea. Molecular plant-microbe interactions. 2007;20(12):1535-44.

11. Marina M, Romero FM, Villarreal NM, Medina AJ, Gárriz A, Rossi FR, et al. Mechanisms of plant protection against two oxalate-producing fungal pathogens by oxalotrophic strains of Stenotrophomonas spp. Plant Molecular Biology. 2019;100(6):659-74.

12. Dickman MB, Chet I. Biodegradation of oxalic acid: A potential new approach to biological control. Soil Biology & Biochemistry. 1998;30(8-9):1195-7.

13. Misslinger M, Hortschansky P, Brakhage AA, Haas H. Fungal iron homeostasis with a focus on Aspergillus fumigatus. Biochim Biophys Acta Mol Cell Res. 2021;1868(1):118885.

14. Ghio AJ, Peterseim DS, Roggli VL, Piantadosi CA. Pulmonary oxalate deposition associated with Aspergillus niger infection. An oxidant hypothesis of toxicity. Am Rev Respir Dis. 1992;145(6):1499-502.

15. Aznar A, Dellagi A. New insights into the role of siderophores as triggers of plant immunity: what can we learn from animals? J Exp Bot. 2015;66(11):3001-10.

16. Bairwa G, Hee Jung W, Kronstad JW. Iron acquisition in fungal pathogens of humans. Metallomics. 2017;9(3):215-27.

17. Matthaiou EI, Sass G, Stevens DA, Hsu JL. Iron: an essential nutrient for Aspergillus fumigatus and a fulcrum for pathogenesis. Curr Opin Infect Dis. 2018;31(6):506-11.

18. Leal SM, Jr., Roy S, Vareechon C, Carrion S, Clark H, Lopez-Berges MS, et al. Targeting iron acquisition blocks infection with the fungal pathogens Aspergillus fumigatus and Fusarium oxysporum. PLoS Pathog. 2013;9(7):e1003436.

19. Kramer J, Ozkaya O, Kummerli R. Bacterial siderophores in community and host interactions. Nat Rev Microbiol. 2020;18(3):152-63.

20. Bernardo-Cravo AP, Schmeller DS, Chatzinotas A, Vredenburg VT, Loyau A. Environmental Factors and Host Microbiomes Shape Host-Pathogen Dynamics. Trends Parasitol. 2020;36(7):616-33.

21. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. Nature. 2015;517(7533):205-8.

22. Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, et al. Targeted Restoration of the Intestinal Microbiota with a Simple, Defined Bacteriotherapy Resolves Relapsing Clostridium difficile Disease in Mice. PLoS Pathogens. 2012;8(10):e1002995-e.

23. Yildiz S, Pereira Bonifacio Lopes JP, Berge M, Gonzalez-Ruiz V, Baud D, Kloehn J, et al. Respiratory tissue-associated commensal bacteria offer therapeutic potential against pneumococcal colonization. Elife. 2020;9.

24. Kolwijck E, van de Veerdonk FL. The potential impact of the pulmonary microbiome on immunopathogenesis of Aspergillus-related lung disease. European Journal of Immunology. 2014;44(11):3156-65.

25. Silva DR, Sardi JdCO, Pitangui NdS, Roque SM, Silva ACBd, Rosalen PL. Probiotics as an alternative antimicrobial therapy: Current reality and future directions. Journal of Functional Foods. 2020;73.

26. Fernandes TR, Segorbe D, Prusky D, Di Pietro A. How alkalinization drives fungal pathogenicity. PLoS Pathog. 2017;13(11):e1006621.

27. Alkan N, Espeso EA, Prusky D. Virulence regulation of phytopathogenic fungi by pH. Antioxid Redox Signal. 2013;19(9):1012-25.

28. Dutton MV, Evans CS. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology. 1996;42(9):881-95.

29. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. In: Gadd GM, Sariaslani S, editors. 106: Academic Press; 2019. p. 49-77.

30. Prusky D, Yakoby N. Pathogenic fungi: leading or led by ambient pH? Mol Plant Pathol. 2003;4(6):509-16.

31. Mbengue M, Navaud O, Peyraud R, Barascud M, Badet T, Vincent R, et al. Emerging trends in molecular interactions between plants and the broad host range fungal pathogens botrytis cinerea and sclerotinia sclerotiorum. 2016. p. 422-.

32. van Kan JAL. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends in Plant Science. 2006;11(5):247-53.

33. Cessna SG, Sears VE, Dickman MB, Low PS. Oxalic acid, a pathogenicity factor for Sclerotinia sclerotiorum, suppresses the oxidative burst of the host plant. Plant Cell. 2000;12(11):2191-9.

34. Heller A, Witt-Geiges T. Oxalic Acid Has an Additional, Detoxifying Function in Sclerotinia sclerotiorum Pathogenesis. PLoS ONE. 2013;8(8):1-17.

35. Swain MR, Ray RC. Oxalic acid production by Fusarium oxysporum Schlecht and Botryodiplodia theobromae Pat., post-harvest fungal pathogens of yams (Dioscorea rotundata L.) and detoxification by Bacillus subtilis CM1 isolated from culturable cowdung microflora. Archives Of Phytopathology And Plant Protection. 2009;42(7):666-75.

36. Gharieb MM. Nutritional effects on oxalic acid production and solubilization of gypsum by Aspergillus niger. Mycological Research. 2000;104(5):550-6.

37. Gharieb MM, Gadd GM. Influence of nitrogen source on the solubilization of natural gypsum (CaSO4. 2H2O) and the formation of calcium oxalate by different oxalic and citric acid-producing fungi. Mycological Research. 1999;103(4):473-81.

38. Marciano P, Magro P, Favaron F. Sclerotinia sclerotiorum growth and oxalic acid production on selected culture media. FEMS Microbiology Letters. 1989;61(1-2):57-9.

39. Punja ZK, Jenkins SF. Influence of Medium Composition on Mycelial Growth and Oxalic Acid Production in Sclerotium rolfsii. Mycologia. 1984;76(5):947-50.

40. Li Z, Bai T, Dai L, Wang F, Tao J, Meng S, et al. A study of organic acid production in contrasts between two phosphate solubilizing fungi: Penicillium oxalicum and Aspergillus niger. Sci Rep. 2016;6:25313.

41. Vrabl P, Fuchs V, Pichler B, Schinagl CW, Burgstaller W. Organic Acid Excretion in Penicillium ochrochloron Increases with Ambient pH. Front Microbiol. 2012;3:121.

42. Walaszczyk E, Podgorski W, Janczar-Smuga M, Dymarska E. Effect of medium pH on chemical selectivity of oxalic acid biosynthesis by Aspergillus niger W78C in submerged batch cultures with sucrose as a carbon source. Chem Zvesti. 2018;72(5):1089-93.

43. Liaud N, Giniés C, Navarro D, Fabre N, Crapart S, Gimbert IH, et al. Exploring fungal biodiversity: organic acid production by 66 strains of filamentous fungi. Fungal Biology and Biotechnology. 2014;1(1).

44. López-Díaz C, Rahjoo V, Sulyok M, Ghionna V, Martín-Vicente A, Capilla J, et al. Fusaric acid contributes to virulence of Fusarium oxysporum on plant and mammalian hosts. Molecular Plant Pathology. 2018;19(2):440-53.

45. Tsuboi R, Matsuda K, Ko IJ, Ogawa H. Correlation between culture medium pH, extracellular proteinase activity, and cell growth of Candida albicans in insoluble stratum corneum-supplemented media. Arch Dermatol Res.

1989;281(5):342-5.

46. Samaranayake LP, Geddes DAM, Weetman DA, MacFarlane TW. Growth and acid production of Candida albicans in carbohydrate supplemented media. Microbios. 1983;37(148):105-15.

47. Mba IE, Nweze EI. Mechanism of Candida pathogenesis: revisiting the vital drivers. Eur J Clin Microbiol Infect Dis. 2020;39(10):1797-819.

48. Singh DK, Nemeth T, Papp A, Toth R, Lukacsi S, Heidingsfeld O, et al. Functional Characterization of Secreted Aspartyl Proteases in Candida parapsilosis. mSphere. 2019;4(4).

49. Abratt VR, Reid SJ. Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease. 72: Academic Press; 2010. p. 63-87.

50. Hoppe B, von Unruh G, Laube N, Hesse A, Sidhu H. Oxalate degrading bacteria: new treatment option for patients with primary and secondary hyperoxaluria? Urol Res. 2005;33(5):372-5.

51. Das S, Bernasconi E, Koutsokera A, Wurlod DA, Tripathi V, Bonilla-Rosso G, et al. A prevalent and culturable microbiota links ecological balance to clinical stability of the human lung after transplantation. Nat Commun. 2021;12(1):2126.

52. Turroni S, Vitali B, Bendazzoli C, Candela M, Gotti R, Federici F, et al. Oxalate consumption by lactobacilli: Evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in Lactobacillus acidophilus. Journal of Applied Microbiology. 2007;103(5):1600-9.

53. Miller AW, Dearing D. The metabolic and ecological interactions of oxalate-degrading bacteria in the Mammalian gut. Pathogens. 2013;2(4):636-52.

54. Ticinesi A, Nouvenne A, Chiussi G, Castaldo G, Guerra A, Meschi T. Calcium Oxalate Nephrolithiasis and Gut Microbiota: Not just a Gut-Kidney Axis. A Nutritional Perspective. Nutrients. 2020;12(2).

55. Suryavanshi MV, Bhute SS, Jadhav SD, Bhatia MS, Gune RP, Shouche YS. Hyperoxaluria leads to dysbiosis and drives selective enrichment of oxalate metabolizing bacterial species in recurrent kidney stone endures. Scientific Reports. 2016;6(1).

56. Rouanet A, Bolca S, Bru A, Claes I, Cvejic H, Girgis H, et al. Live Biotherapeutic Products, A Road Map for Safety Assessment. Front Med (Lausanne). 2020;7:237.

57. Li R, Helbig L, Fu J, Bian X, Herrmann J, Baumann M, et al. Expressing cytotoxic compounds in Escherichia coli Nissle 1917 for tumor-targeting therapy. Res Microbiol. 2019;170(2):74-9.

58. He L, Yang H, Tang J, Liu Z, Chen Y, Lu B, et al. Intestinal probiotics E. coli Nissle 1917 as a targeted vehicle for delivery of p53 and Tum-5 to solid tumors for cancer therapy. J Biol Eng. 2019;13(3):58.

59. Geldart KG, Kommineni S, Forbes M, Hayward M, Dunny GM, Salzman NH, et al. Engineered E. coli Nissle 1917 for the reduction of vancomycin-resistant Enterococcus in the intestinal tract. Bioeng Transl Med. 2018;3(3):197-208.

60. Palmer JD, Piattelli E, McCormick BA, Silby MW, Brigham CJ, Bucci V. Engineered Probiotic for the Inhibition of Salmonella via Tetrathionate-Induced Production of Microcin H47. ACS Infect Dis. 2018;4(1):39-45.

61. Hwang IY, Koh E, Wong A, March JC, Bentley WE, Lee YS, et al. Engineered probiotic Escherichia coli can eliminate and prevent Pseudomonas aeruginosa gut infection in animal models. Nat Commun. 2017;8:15028.

62. Sarate PJ, Heinl S, Poiret S, Drinic M, Zwicker C, Schabussova I, et al. E. coli Nissle 1917 is a safe mucosal delivery vector for a birch-grass pollen chimera to prevent allergic poly-sensitization. Mucosal Immunol. 2019;12(1):132-44.

63. Daeffler KN, Galley JD, Sheth RU, Ortiz-Velez LC, Bibb CO, Shroyer NF, et al. Engineering bacterial thiosulfate and tetrathionate sensors for detecting gut inflammation. Mol Syst Biol. 2017;13(4):923.

64. Aggarwal N, Breedon AME, Davis CM, Hwang IY, Chang MW. Engineering probiotics for therapeutic applications: recent examples and translational outlook. Curr Opin Biotechnol. 2020;65:171-9.

65. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. Nature. 2016;533(7604):543-6.

66. Paul C, Filippidou S, Jamil I, Kooli W, House GL, Estoppey A, et al. Bacterial spores, from ecology to biotechnology. 1 ed: Elsevier Inc.; 2019. 79-111 p.

67. Mohamed MYH, Christie G. A system for the expression and release of heterologous proteins from the core of Bacillus subtilis spores. FEMS Microbiology Letters. 2018;365(23):1-7.

68. Allison MJS, Harmeet inventorOxalate-degrading microorganisms or oxalate-degrading enzymes for preventing oxalate related disease patent EP0983076B1. 2000.

69. Cowley ABC, Helena Yan, Qin Li, Qingshan inventorHigh efficiency oxalate-degrading enzymes for degradation of insoluble and soluble oxalate2016.

70. Sidhu HC, Aaron Blake Golander, Carl-Gustaf Li, Qingshan inventorPurification and isolation of recombinant oxalate degrading enzymes and spray-dried particles containing oxalate degrading enzymes. Stockholm patent US10,568,946B2. 2018.

71. Verma ML, Kumar P, Sharma S, Dhiman K, Sharma D, Verma A. Gold Nanoparticle-Mediated Delivery of Therapeutic Enzymes for Biomedical Applications. Nanoscience in Medicine Vol 1. Environmental Chemistry for a Sustainable World2020. p. 89-115.

72. Pontes JF, Grenha A. Multifunctional Nanocarriers for Lung Drug Delivery. Nanomaterials (Basel). 2020;10(2).

73. Han Y, Joosten HJ, Niu W, Zhao Z, Mariano PS, McCalman M, et al. Oxaloacetate hydrolase, the C-C bond lyase of oxalate secreting fungi. Journal of Biological Chemistry. 2007;282(13):9581-90.

74. Mela F, Fritsche K, De Boer W, Van Veen JA, De Graaff LH, Van Den Berg M, et al. Dual transcriptional profiling of a bacterial/fungal confrontation: Collimonas fungivorans versus Aspergillus niger. ISME Journal. 2011;5(9):1494-504.

75. Niu J, Arentshorst M, Nair PDS, Dai Z, Baker SE, Frisvad JC, et al. Identification of a classical mutant in the industrial host Aspergillus niger by systems genetics: LaeA is required for citric acid production and regulates the formation of some secondary metabolites. G3: Genes, Genomes, Genetics. 2016;6(1):193-204.

76. van Rhijn N, Furukawa T, Zhao C, McCann BL, Bignell E, Bromley MJ. Development of a marker-free mutagenesis system using CRISPR-Cas9 in the pathogenic mould Aspergillus fumigatus. Fungal Genet Biol. 2020;145:103479.

77. Latge JP, Chamilos G. Aspergillus fumigatus and Aspergillosis in 2019. Clin Microbiol Rev. 2019;33(1).

78. Kendall LV, Owiny JR, Dohm ED, Knapek KJ, Lee ES, Kopanke JH, et al. Replacement, Refinement, and Reduction in Animal Studies With Biohazardous Agents. ILAR journal. 2018;59(2):177-94.

79. Russell WMS, Burch RL. The principles of humane experimental technique: London: Methuen & Co. Ltd.; 1959.

80. Desoubeaux G, Cray C. Animal Models of Aspergillosis. Comparative medicine. 2018;68(2):109-23.

81. Herbst S, Shah A, Carby M, Chusney G, Kikkeri N, Dorling A, et al. A new and clinically relevant murine model of solid-organ transplant aspergillosis. DMM Disease Models and Mechanisms. 2013;6(3):643-51.

82. Sheppard DC, Graybill JR, Najvar LK, Chiang LY, Doedt T, Kirkpatrick WR, et al. Standardization of an experimental murine model of invasive pulmonary aspergillosis. Antimicrobial Agents and Chemotherapy. 2006;50(10):3501-3.

83. Imeria Ferro K. The impact of oxalogenic plants on soil carbon dynamics - formation of a millennium carbon storage as calcium carbonate. Neuchâtel: University of Neuchâtel; 2012.

84. Brock M, Jouvion G, Droin-Bergère S, Dussurget O, Nicola MA, Ibrahim-Granet O. Bioluminescent Aspergillus fumigatus, a new tool for drug efficiency testing and in vivo monitoring of invasive aspergillosis. Applied and Environmental Microbiology. 2008;74(22):7023-35.

85. Galiger C, Brock M, Jouvion G, Savers A, Parlato M, Ibrahim-Granet O. Assessment of efficacy of antifungals against Aspergillus fumigatus: value of real-time bioluminescence imaging. Antimicrob Agents Chemother. 2013;57(7):3046-59.

86. Dorsaz S, Coste AT, Sanglard D. Red-Shifted Firefly Luciferase Optimized for Candida albicans In vivo Bioluminescence Imaging. Front Microbiol. 2017;8:1478.

87. Amin R, Dupuis A, Aaron SD, Ratjen F. The effect of chronic infection with Aspergillus fumigatus on lung function and hospitalization in patients with cystic fibrosis. Chest. 2010;137(1):171-6.

88. Devos FC, Maaske A, Robichaud A, Pollaris L, Seys S, Lopez CA, et al. Forced expiration measurements in mouse models of obstructive and restrictive lung diseases. Respir Res. 2017;18(1):123.

89. Şahin N, Işik K, Tamer AÜ, Goodfellow M. Taxonomic position of 'Pseudomonas oxalaticus' strain Ox1(T) (DSM 110(T)) (Khambata and Bhat, 1953) and its description in the genus Ralstonia as Ralstonia oxalatica comb, nov. Systematic and Applied Microbiology. 2000;23(2):206-9.

90. Tamer AÜ, Aragno M. Isolement, caractérisation et essai d'identification de bactéries capables d'utiliser l'oxalate comme seule source de carbone et d'énergie. Bulletin de la Société Neuchâteloise des Sciences Naturelles. 1980;103.

91. Berge O, Mavingui P, Heulin T. Exploring Diversity of Cultivable Aerobic Endospore-forming Bacteria: From Pasteurization to Procedures Without Heat-Shock Selection. In: Logan NA, Vos P, editors. Endospore-forming Soil Bacteria. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 73-88.

92. Evans CR, Karnovsky A, Kovach MA, Standiford TJ, Burant CF, Stringer KA. Untargeted LC–MS Metabolomics of Bronchoalveolar Lavage Fluid Differentiates Acute Respiratory Distress Syndrome from Health. Journal of Proteome Research. 2014;13(2):640-9.

93. Lee KS, Palatinszky M, Pereira FC, Nguyen J, Fernandez VI, Mueller AJ, et al. An automated Raman-based platform for the sorting of live cells by functional properties. Nature Microbiology. 2019;4(6):1035-48.

94. Stucki AO, Stucki JD, Hall SR, Felder M, Mermoud Y, Schmid RA, et al. A lung-on-a-chip array with an integrated bio-inspired respiration mechanism. Lab Chip. 2015;15(5):1302-10.

95. Stucki JD, Hobi N, Galimov A, Stucki AO, Schneider-Daum N, Lehr CM, et al. Medium throughput breathing human primary cell alveolus-on-chip model. Sci Rep. 2018;8(1):14359.

96. Jiang Y, Chen B, Duan C, Sun B, Yang J, Yang S. Multigene Editing in the Escherichia coli Genome via the CRISPR-Cas9 System. Applied and Environmental Microbiology. 2015;81(7):2506.

97. Qi Lei S, Larson Matthew H, Gilbert Luke A, Doudna Jennifer A, Weissman Jonathan S, Arkin Adam P, et al. Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression. Cell. 2013;152(5):1173-83.

98. Xiong B, Li Z, Liu L, Zhao D, Zhang X, Bi C. Genome editing of Ralstonia eutropha using an electroporation-based CRISPR-Cas9 technique. Biotechnol Biofuels. 2018;11:172.

99. Vig M, Kinet JP. Calcium signaling in immune cells. 2009. p. 21-7.

100. Diamantstein T, Odenwald MV. Control of the immune response in vitro by calcium ions. I. The antagonistic action of calcium ions on cell proliferation and on cell differentiation. Immunology. 1974;27(4):531-53141.

101. Diamantstein T, Ulmer A. The control of immune response in vitro by Ca2+. II. The Ca2+ dependent period during mitogenic stimulation. Immunology. 1975;28(1):121-5.

102. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). International Journal of Cancer. 1980;26(2):171-6.

103. Chanput W, Mes JJ, Wichers HJ. THP-1 cell line: an in vitro cell model for immune modulation approach. Int Immunopharmacol. 2014;23(1):37-45.

104. Li Z, Lu G, Meng G. Pathogenic Fungal Infection in the Lung. Front Immunol. 2019;10:1524.

105. Zuliani-Alvarez L, Piccinini AM, Midwood KS. Screening for Novel Endogenous Inflammatory Stimuli Using the Secreted Embryonic Alkaline Phosphatase NF-κB Reporter Assay. Bio-protocol. 2017;7(7):e2220.

106. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC Cancer. 2015;15:577.

107. Berges C, Naujokat C, Tinapp S, Wieczorek H, Hoh A, Sadeghi M, et al. A cell line model for the differentiation of human dendritic cells. Biochem Biophys Res Commun. 2005;333(3):896-907.

108. Tracy MC, Moss RB. The myriad challenges of respiratory fungal infection in cystic fibrosis. Pediatr Pulmonol. 2018;53(S3):S75-S85.

109. Filkins LM, O'Toole GA. Cystic Fibrosis Lung Infections: Polymicrobial, Complex, and Hard to Treat. PLoS Pathog. 2015;11(12):e1005258.

110. Sass G, Nazik H, Penner J, Shah H, Ansari SR, Clemons KV, et al. Aspergillus-Pseudomonas interaction, relevant to competition in airways. Med Mycol. 2019;57(Supplement_2):S228-S32.

111. Beswick E, Amich J, Gago S. Factoring in the Complexity of the Cystic Fibrosis Lung to Understand Aspergillus fumigatus and Pseudomonas aeruginosa Interactions. Pathogens. 2020;9(8).

112. Kim SH, Clark ST, Surendra A, Copeland JK, Wang PW, Ammar R, et al. Global Analysis of the Fungal Microbiome in Cystic Fibrosis Patients Reveals Loss of Function of the Transcriptional Repressor Nrg1 as a Mechanism of Pathogen Adaptation. PLoS Pathog. 2015;11(11):e1005308.

113. Marsland BJ, Trompette A, Gollwitzer ES. The gut-lung axis in respiratory disease. Annals of the American Thoracic Society. 2015;12(November):S150-S6.

114. Mateos-Hernandez L, Risco-Castillo V, Torres-Maravilla E, Bermudez-Humaran LG, Alberdi P, Hodzic A, et al. Gut Microbiota Abrogates Anti-alpha-Gal IgA Response in Lungs and Protects against Experimental Aspergillus Infection in Poultry. Vaccines (Basel). 2020;8(2).

APPENDICES

I. Poster presentations

1. Annual PhD Students Meeting 2017, University of Neuchâtel, Neuchâtel, Switzerland, 29.03.2017.

ORGANIC ACID PRODUCTION BY FUNGI: COMPARISON ON VARIOUS MEDIA AND EFFECT OF THE INTERACTION WITH BACTERIA

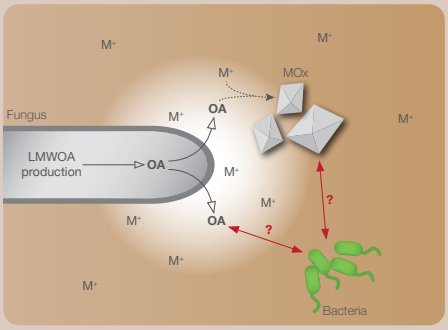
Fabio Palmieri *[1], Lindsay Pétremand [1], Saskia Bindschedler [1], Pilar Junier [1]

[1] Laboratory of Microbiology, University of Neuchâtel, Switzerland

Introduction

In fungi, **low molecular weight organic acids** (**LMWOA**) production can have important roles in processes as diverse as pathogenesis, competition, mineral weathering or lignocellulose degradation. More recently, LMWOA have been identified as being involved in the interaction between fungi and bacteria. This is particularly true in the case of oxalic acid, especially in soils. Several factors can influence LMWOA production. One of those is the availability of divalent cations. They trigger LMWOA production either as enzymatic co-factors or as a detoxification mechanism.

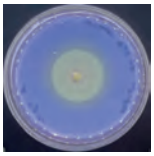
Aim:
Evaluate the production of LMWOA in fungi in different media and with the presence of bacteria.



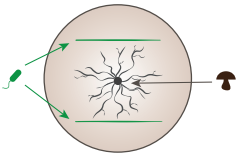
The fungi produce and excrete various organic acids (LMWOA), like oxalic acid (OA), in its environment. The OA turns into oxalate in the environment and can form insoluble oxalate crystals in presence of metal cations such as Ca^{2+} in the case of calcium oxalate (CaOx). The fungi can also interact with bacteria through the production of OA. Finally, the bacteria can also interact with the metal-oxalate crystals produced by the fungi.

Experimental design

1. Use of an **organic acid detection media containing Bromocresol Purple**
   - *Aspergillus niger*
   - *Botrytis cinerea*
   - *Rhizoctonia solani*
   - *Fusarium oxysporum*
   - *Trichoderma rossicum*

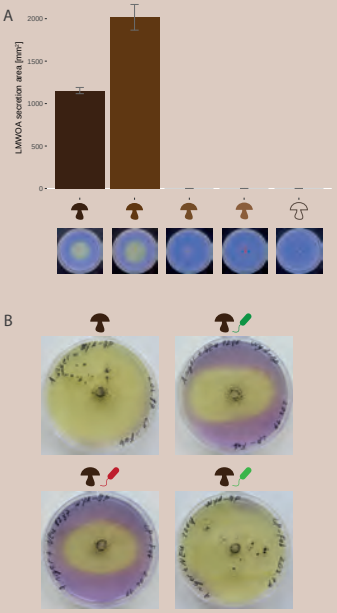


2. **Cocultures of fungi and model soil bacteria** on different media (poor, intermediate and rich nutrient medium



- *Aspergillus niger*
- *Trichoderma rossicum*
- *Cupriavidus necator* GFP
- *Cupriavidus oxalaticus* mCherry
- *Pseudomonas putida* KT2440 GFP

Organic acid detection

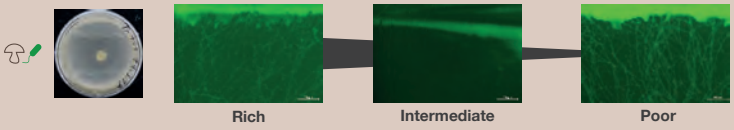


Only ***A. niger*** and ***B. cinerea*** produce **large amount of LMWOA (A)**. When A. niger is cocultured with the bacteria, both *Cupriavidus* strains seem to control LMWOA production, whereas *Pseudomonas putida* does not show any effect on the acidity **(B)**.

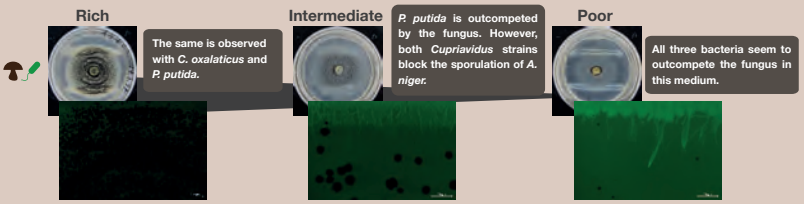
Bacterial-Fungal Interactions on different media

For *Trichoderma rossicum*

No matter the medium, **no visible effect of bacteria** on fungal growth. All three bacterial strains do **fungal highways** with the fungus



For *Aspergillus niger*



Conclusions and perspectives

With these results, we gave an insight into the interactions between a fungus that produce organic acids (i.e. *A. niger*) and a fungus that does not produce organic acids (i.e. *T. rossicum*). We also saw that the interaction change with nutrient abundance in the medium for both fungi, but with a stronger effect with *A. niger*. Further investigations are needed and LMWOA need to be quantified.

Aknowledgements
We would like to acknowledge the Novartis Research Foundation for funding this work (FreeNovation program).

*Contact: fabio.palmieri@unine.ch

2. FEMS – 7th Congress of European Microbiologists, Valencia, Spain, 09-13.07.2017.

ORGANIC ACID PRODUCTION BY FUNGI: COMPARISON ON VARIOUS MEDIA AND EFFECT OF THE INTERACTION WITH BACTERIA

Fabio Palmieri *[1], Saskia Bindschedler [1], Pilar Junier [1]

[1]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Emile-Argand 11, 2000 Neuchâtel, Switzerland

Introduction

In fungi, **low molecular weight organic acids** (**LMWOA**) production participates in processes as diverse as **pathogenesis**, **competition**, **mineral weathering** or **lignocellulose degradation** (**Fig. 1**). This is particularly true in the case of **oxalic acid** (**OA**), especially in soils. Several factors can influence LMWOA production. One of those is the availability of divalent cations (M^{2+}). They trigger LMWOA production either as enzymatic co-factors or as a detoxification mechanism (**Fig. 2**). More recently, LMWOA have been identified as being involved in the interaction between fungi and bacteria. However, the influence of LMWOA-consuming bacteria, and particularly oxalotrophic bacteria, on LMWOA production by fungi is unknown.

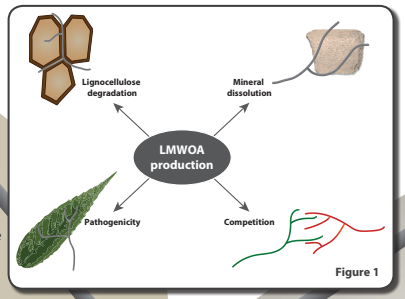


Figure 1

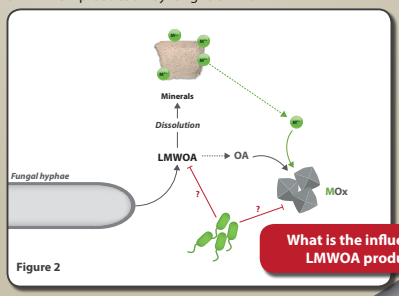


Figure 2

What is the influence of bacteria on LMWOA production by fungi?

Aim:
To evaluate the production of LMWOA in fungi in different media with the presence of bacteria.

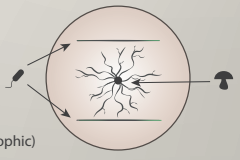
Experimental design

1. Assessment of the total **LMWOA production** on **modified Angle medium containing Bromocresol Purple** (**Angle - BP**) as pH indicator.
2. **Co-cultures of fungi and model soil bacteria** on different media.

Angle - BP | **Angle - Urea** | **Angle - $MnCl_2$** | **Angle - $CaCO_3$**

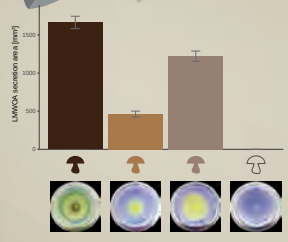
Aspergillus niger
Botrytis cinerea
Fusarium oxysporum
Trichoderma rossicum

Cupriavidus necator GFP (oxalotrophic)
Cupriavidus oxalaticus mCherry (oxalotrophic)
Pseudomonas putida KT2440 GFP (non-oxalotrophic)



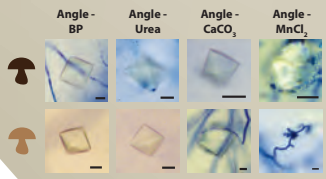
3. **Calcium oxalate (CaOx)** observation by optical microscopy.

LMWOA production



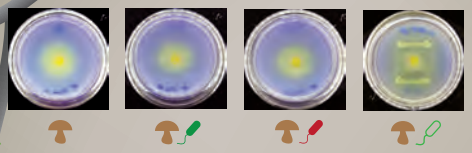
***A. niger*, *B. cinerea* and *F. oxysporum* produce a large amount of LMWOA on Angle-BP.**

CaOx crystals production



Only *A. niger* and *B. cinerea* produce calcium oxalate crystals, but Mn^{2+} cations seem to inhibit crystal production for *B. cinerea*. Bars = 10 µm

pH control by oxalotrophic bacteria



When *B. cinerea* is co-cultured with bacteria, both oxalotrophic (*Cupriavidus* spp.) strains seem to control LMWOA production, whereas *P. putida* acidifies the medium. The same was observed for *A. niger*.

Conclusions and perspectives

With these results, we gain an insight into the interactions between LMWOA-producing fungi and model soil bacteria and the effects of the latter on organic acid production by the fungus. LMWOA produced needs to be identified and quantified.

Acknoledgments

We would like to acknowledge the Novartis Research Foundation for funding this work (FreeNovation Grant).

*fabio.palmieri@unine.ch

3. FreeNovation Science Forum, Novartis Campus, Basel, Switzerland, 24.05.2018.

Bacterial oxalotrophy as a biological mechanism to control the development of fungal pathogens

Fabio Palmieri[1], Aislinn Estoppey[1], Jean Challacombe[2], Patrick S. Chain[2], Pilar Junier[1]

[1]Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, Switzerland
[2]Los Alamos National Laboratory, Los Alamos, USA

Screening

- Production of large amounts of Low Molecular Weight Organic Acids (LMWOA) by *A. niger*, *B. cinerea* and *F. oxysporum*
- Oxalic acid production by *A. niger* confirmed by HPLC
- Production of calcium oxalate crystals (CaOx) by *A. niger* and *B. cinerea*

➤ *A. niger* and *B. cinerea* were chosen as **model pathogens** of humans and plants, respectively

CaOx dissolution assay

Pseudomonas putida GFP | *Cupriavidus necator* GFP | *Cupriavidus oxalaticus* mCherry

➤ *P. putida* was chosen as model **non-oxalotrophic** bacterium

➤ *C. necator* and *C. oxalaticus* were chosen as model **oxalotrophic** bacteria

Interactions

Growth inhibition of ***A. niger***:

- Observed in R2A and WYA media
- Only with oxalotrophic bacteria
- Oxalotrophic bacteria control the pH in WYA medium (pH indicator)

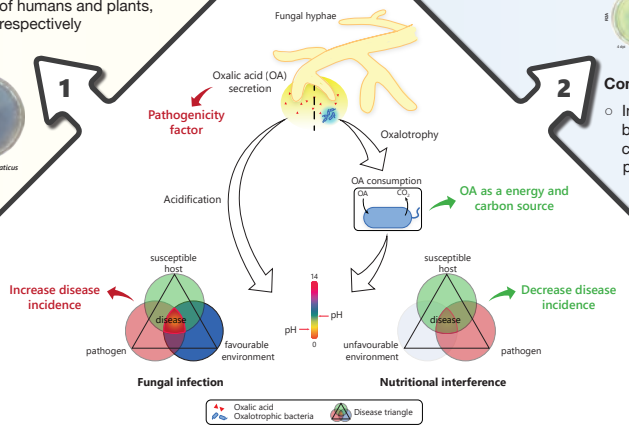
Growth inhibition of ***B. cinerea***:

- Observed in MA 1/10 and R2A media
- With oxalotrophic and non-oxalotrophic bacteria

Conclusions:

- Interactions between oxalotrophic bacteria and the pathogenic fungus change depending on the fungal partner and abiotic factors (media)
- Growth control by oxalotrophic bacteria depends on the medium
  - Control seems more effective with *C. oxalaticus*

Concept



Hypothesis:

Oxalotrophic bacteria can control the growth of human or plant pathogenic fungi by degrading oxalic acid

Aims:

- Confirm the production of oxalic acid by model pathogenic fungi
- Assess the biocontrol potential of model oxalotrophic bacteria
- Determine the conditions for an optimal control

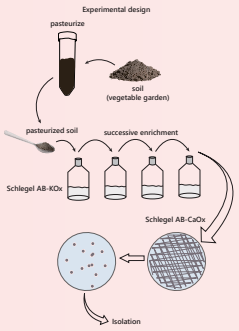
Delivery

Proposition 1:

Spores of oxalotrophic endospore-forming bacteria (EFB)

Advantages:

- Highly resistant structures
- Naturally encapsulated biocontrol agent



Hypothesis:

Spore germination of oxalotrophic EFB is triggered by oxalic acid

Aim:

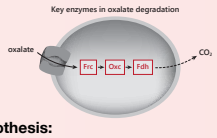
Isolate oxalotrophic EFB from soil

Proposition 2:

Key enzymes of the aerobic oxalotrophic pathway

Advantage:

- Use of enzymes
- No living organisms



Hypothesis:

Oxalate degradation by enzymes is as efficient as by living bacteria

Aim:

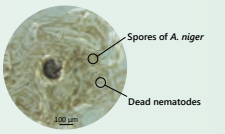
Production and purification of enzymes

Models

Animal pathosystem

Model 1: *C. elegans*

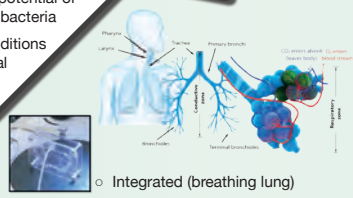
- Fast high-throughput screening method for Aspergillosis



Model 2: Lung-on-a-chip (PuLMo)

3 different bioreactors:

- Bronchioles (upper respiratory tract)
- Alveoli (deep lung)
- Integrated (breathing lung)



Plant pathosystem

Model 3: *In-vivo* microcosm experiment

- *In vivo* assay on *Lactuca sativa*

Model 4: Root-on-a-chip

- Microfluidic device including the growth of plant roots

Acknowledgements

NOVARTIS | Los Alamos NATIONAL LABORATORY EST. 1943 | INVENesis Your research partner for drug discovery | Agroscope

4. 11th International Mycological Congress (IMC11), San Juan, Puerto Rico, 16-21.07.2018, & Annual Congress of the SSM 2018, Lausanne, Switzerland, 28-30.08.2018

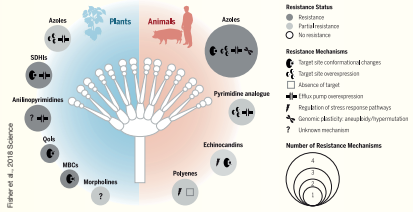
Low Molecular Weight Organic Acids as Key Molecules in Bacterial-Fungal Interactions

Fabio Palmieri[1]*, Andrea Lohberger[1], Aislinn Estoppey[1], Lindsay Pétremand[1], Jean Challacombe[2], Geoffrey L. House[2], Debora F. Rodrigues[3], Jamey D. Young[4], Saskia Bindschedler[1], Patrick S. Chain[2], Pilar Junier[1]

[1]Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, Switzerland; [2]Los Alamos National Laboratory, Los Alamos, USA; [3]Civil and Environmental Engineering, University of Houston, Houston, Texas; [4]School of Engineering Vanderbilt University, Nashville, Tennessee

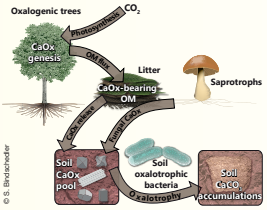
Introduction

Resistance of fungal pathogens



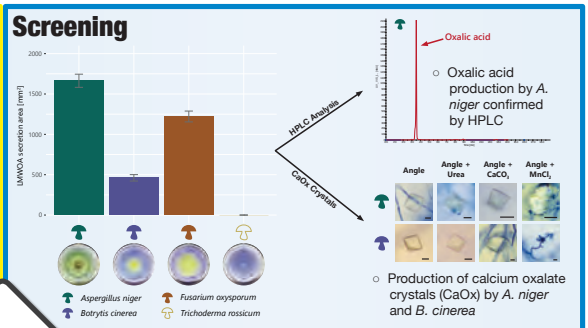
- There is an urgent need to find alternatives to fight against the emergence of **antimicrobial resistance** in both plant and human/ animal fungal pathogens

From soils to human and plant health

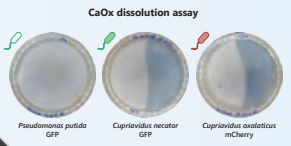


- The oxalate-carbonate pathway is a model of Bacterial-Fungal interaction in soils.
- Oxalotrophic bacteria consume the calcium oxalates (CaOx) produced by fungi.

Screening

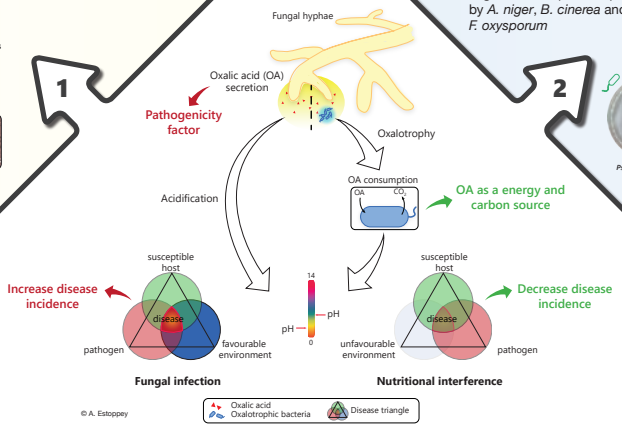


- Oxalic acid production by *A. niger* confirmed by HPLC
- Production of calcium oxalate crystals (CaOx) by *A. niger* and *B. cinerea*
- Production of large amounts of Low Molecular Weight Organic Acids (LMWOA) by *A. niger*, *B. cinerea* and *F. oxysporum*
- *A. niger* and *B. cinerea* were chosen as **model pathogens** of humans and plants, respectively



- *P. putida* was chosen as model **non-oxalotrophic** bacterium
- *C. necator* and *C. oxalaticus* were chosen as model **oxalotrophic** bacteria

Concept



Hypothesis:

Oxalotrophic bacteria can control the growth of human or plant pathogenic fungi by degrading oxalic acid

Aims:

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- Assess the biocontrol potential of model oxalotrophic bacteria
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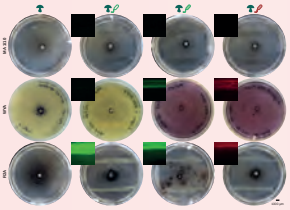
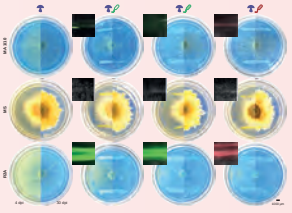


Growth inhibition of ***B. cinerea***:

- Observed in MA 1/10 and R2A media
- With oxalotrophic and non-oxalotrophic bacteria



Conclusions:

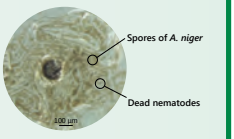
- Interactions between oxalotrophic bacteria and the pathogenic fungus change depending on the fungal partner and abiotic factors (media)
- Growth control by oxalotrophic bacteria depends on the medium
- Control seems more effective with *C. oxalaticus*

Perspectives

Animal pathosystem

Model 1: *C. elegans*

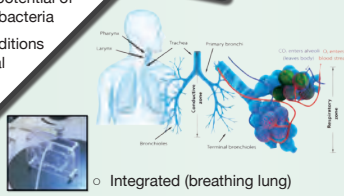
- Fast high-throughput screening method for Aspergillosis



Model 2: Lung-on-a-chip (PulMo)

3 different bioreactors:

- Bronchioles (upper respiratory tract)
- Alveoli (deep lung)



- Integrated (breathing lung)

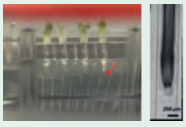
Plant pathosystem

Model 3: *In-vivo* microcosm experiment

- *In vivo* assay on *Lactuca sativa*

Model 4: Root-on-a-chip

- Microfluidic device including the growth of plant roots



Acknowledgements

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@fabio_palmieri_
*fabio.palmieri@unine.ch

5. 2019 Genomic Sciences Program Annual Principal Investigator (PI) Meeting. Tysons, VA, USA, 24-27.02.2019

Bacterial Oxalotrophy as a Biocontrol Mechanism against Fungal Pathogens

Aislinn Estoppey[1], **Fabio Palmieri[1]*** (fabio.palmieri@unine.ch), Geoffrey L. House[2], Debora F. Rodrigues[3], Jamey D. Young[4], Saskia Bindschedler[1], Pilar Junier[1], Patrick S. G. Chain[2]

[1]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland; [2]Bioscience Division, Los Alamos National Laboratory, Los Alamos, New Mexico; [3]Civil and Environmental Engineering, University of Houston, Houston, Texas; [4]School of Engineering Vanderbilt University, Nashville, Tennessee

Introduction

Because of the limited number of compounds active against fungal pathogens, this chemical arsenal is currently used both in medicine and agriculture. This has created the perfect terrain for the emergence of multi-drug resistant fungal pathogens (Figure 1; 1). Alternative control strategies are urgently needed. Biocontrol, a method in which an undesired organism is controlled using another, is one of these alternatives (2). Antagonistic bacteria or fungi are potential candidates for the biological control of fungal pathogens.

Through this Science Focus Area (SFA) project, we seek to better understand several aspects of bacterial:fungal interactions (BFI). Here, a metabolic BFI that is relevant to soil functioning is proposed to control fungal pathogens that use oxalic acid as pathogenicity factor.

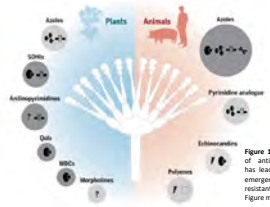


Figure 1. The intensive use of antifungal compounds has lead to the worldwide emergence or drug-resistant fungal pathogens. Figure modified from (1).

The Oxalate-Carbonate Pathway – a trophic interaction that can be harnessed to fight plant pathogens

Oxalic acid is one of the most prevalent low molecular weight organic acids produced by plants and fungi (3). In soil, it accumulates in the form of calcium oxalate crystals (CaOx). The decay of plant and fungal biomass also leads to the release of CaOx in the soil carbon pool (4). This CaOx is then consumed by oxalotrophic bacteria, leading to a local increase in soil pH, and eventually to the accumulation of calcium carbonate if the pH increases above 8. In soils the pathway describing this link between the production and consumption of CaOx and the generation of calcium carbonate is called "oxalate-carbonate pathway" (Figure 2; 5).

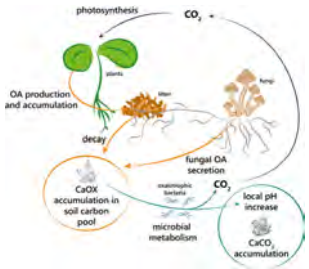


Figure 2. The Oxalate-Carbonate Pathway. In the pathway CO_2 is fixed through photosynthesis, which results in the production of the precursors of oxalic acid (OA). Fungi also produce OA. Upon decay, calcium oxalate (CaOx) accumulates in soils. Oxalotrophic bacteria consume CaOx generating a local increase of pH. This may eventually lead to calcium carbonate precipitation at pH>8. Excess CO_2 is released to the atmosphere and can enter the cycle again (3).

Oxalic acid is known to be a pathogenicity factor in some plant pathogenic fungi, such as *Sclerotinia sclerotiorum* or *Botrytis cinerea* (6, 7). This organic acid acidifies the host tissues generating a favorable environment for the infection (Figure 3A). By protecting the plant with oxalotrophic bacteria, this favorable conditions are not attained and pathogen development is controlled (Figure 3B).

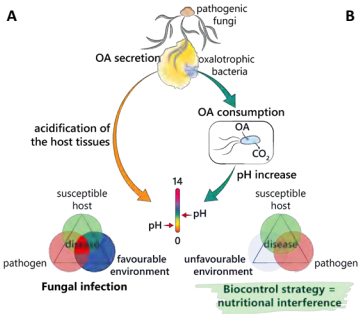


Figure 3. Working hypothesis. During a normal infection process, opportunistic fungal pathogens use oxalic acid secretion as a pathogenicity factor that renders the environment favorable for the onset of infection of a susceptible host (optimal conditions in a disease triangle). In contrast, consumption of oxalic acid by oxalotrophic bacteria renders the environmental conditions unfavorable, reducing the ability of the pathogen to attack the host.

In-vitro results of biological control

Confrontation assays between plant pathogenic fungi and bacteria have been performed in different nutritional conditions (different media) by inoculating the fungus at the center of the plate and the bacterium as two lines facing it. *T. rossicum* was used as a negative control because of its plant-growth promoting activity (8). *Cupriavidus necator*, *Cupriavidus oxalaticus* and *Burkholderia phytofirmans* are oxalotrophic, whereas *Pseudomonas putida* and the Δ*oxc*-mutant of *B. phytofirmans* were used as non-oxalotrophic controls. The table below summarizes the outcome of these confrontations.

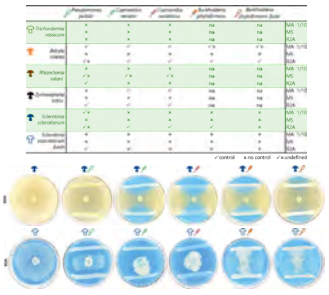


Figure 4. Confrontation assays between *S. sclerotiorum* and its Δ*oah*-mutant. The three oxalotrophic bacteria inhibited the growth of *S. sclerotiorum* and control the pH of the medium. The Δ*oxc*-mutant of *B. phytofirmans* still inhibited the growth of *S. sclerotiorum* but to a lesser extent. The Δ*oah*-mutant of *S. sclerotiorum* was controlled by all tested bacteria. A yellow color indicates the medium to be acidic (pH ≤ 3.5). A blue color indicates the medium is at a pH ≥ 6.

Confrontation *in-vitro* show that oxalotrophic bacteria control fungal growth and in consequence the pH in growth medium (Figure 4). This is consistent with the quantification of oxalic acid in the confrontation between *B. cinerea* and different bacteria, which showed that oxalotrophic bacteria reduce the concentration of oxalic acid in the medium as compared to the fungus alone (Figure 5). The same experiment will be performed for *S. sclerotiorum*.

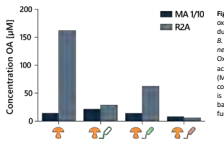


Figure 5. Quantification of oxalic acid in liquid cultures during the confrontation of *B. cinerea*, with *P. putida*, *C. necator* and *C. oxalaticus*. Oxalic acid production differs according to the medium (MA1/10 or R2A). The concentration of oxalic acid is lower in the presence of bacteria as compared to the fungus alone (first bars).

To verify the direct consumption of oxalic acid, Desorption Electrospray Ionization - Imaging Mass Spectrometry (DESI-IMS) will be used to measure the spatial pattern of oxalic acid across the confrontation area. This will allow to correlate the disappearance of oxalic acid with the presence of oxalotrophic bacteria (Figure 6). Moreover, *in-vivo* biological control (plant-bacterial-fungal interactions, PBFI) will be performed using *Lactuca sativa* (lettuce) as a model plant. Two different experimental designs will be used: magenta boxes and plant/root-on-a-chip microfluidic system (Figure 7). The magenta boxes will be used both with agar media or transparent soil (Nafion®, 9).

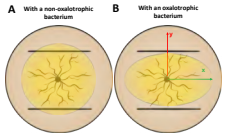


Figure 6. DESI-IMS experiment – Expected results. **A**. The presence of a non-oxalotrophic bacterium will not affect the spatial occurrence of oxalic acid. **B**. In presence of an oxalotrophic bacterium, oxalic acid concentration should decrease towards the bacterial inoculum (y-axis) and increase away from it (x-axis).

Figure 7. Experimental designs for *in-vivo* experiments. **A**. Magenta box. **B**. Plant/Root-on-a-chip microfluidic system. Collaboration with Dr. Claire Stanley.

Future directions

To Investigate the effect of oxalotrophic bacteria on the germination of reproductive structures of the two pathogens; i.e. spores (*Botrytis cinerea*) or sclerotia (*Sclerotinia sclerotorium*).

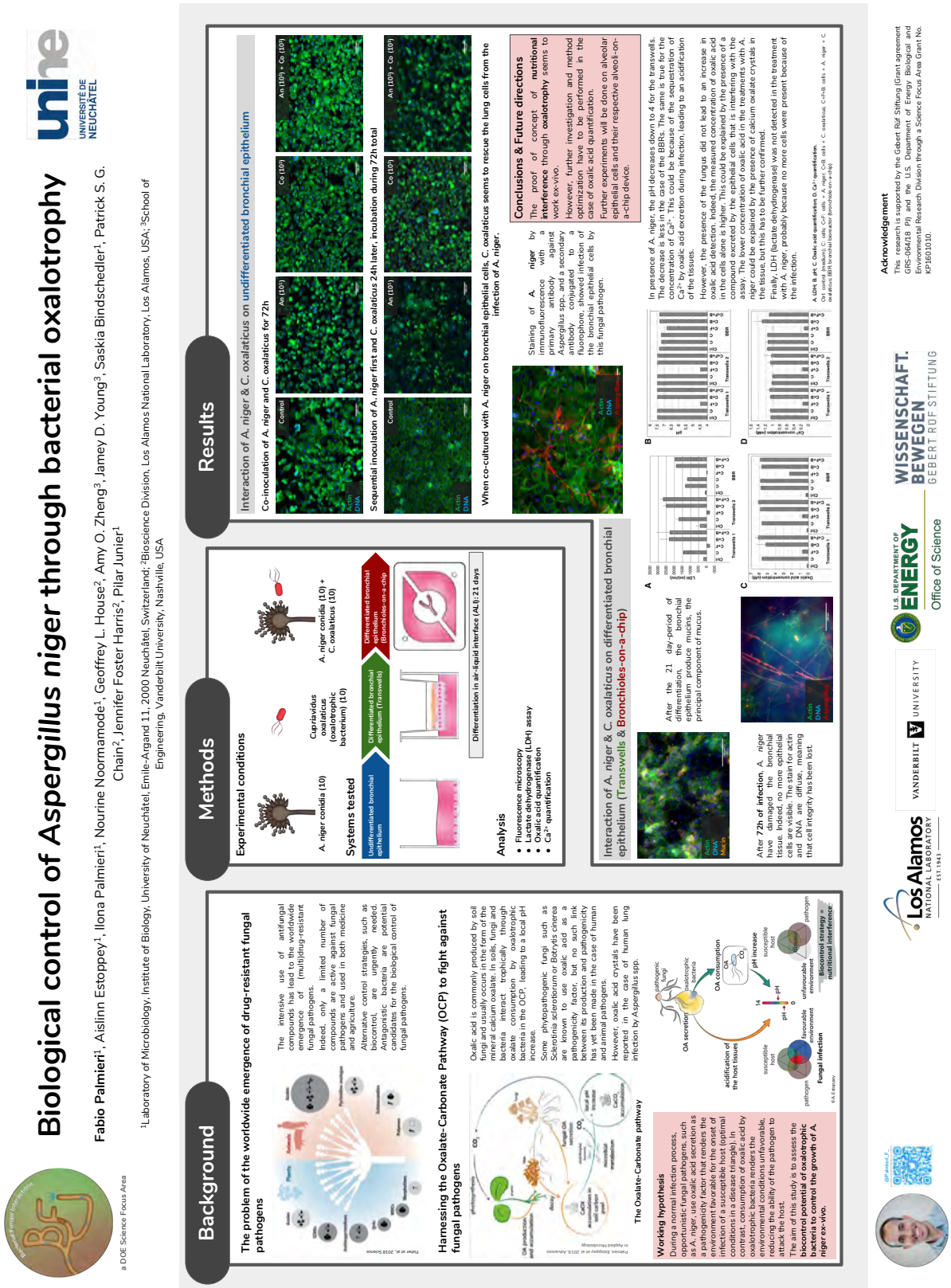
References

(1) Fisher, M. C., Hawkins, N. J., Sanglard, D. & Gurr, S. J. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science (80-.). 360, 739–742 (2018).
(2) Syed Ab Rahman, S. F., Singh, E., Pieterse, C. M. J. & Schenk, P. M. Emerging microbial biocontrol strategies for plant pathogens. Plant Sci. 267, 102–111 (2018).
(3) Palmieri, F. et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. Advances in Applied Microbiology (Elsevier Inc., 2018). doi:10.1016/bs.aambs.2018.10.001
(4) Aragno, M. & Verrecchia, E. in Microorganisms in Environmental Management: Microbes and Environment (eds. Satyanarayana, T., Prakash, A. & Johri, B. N.) 9789400722, 1–819 (2012).
(5) Martin, G. et al. Fungi, bacteria and soil pH: The oxalate-carbonate pathway as a model for metabolic interaction. Environ. Microbiol. 14, 2960–2970 (2012).
(6) Cessna, S. G., Sears, V. E., Dickman, M. B. & Low, P. S. Oxalic Acid, a Pathogenicity Factor for Sclerotinia sclerotiorum , Suppresses the Oxidative Burst of the Host Plant. Plant Cell 12, 2191–2199 (2000).
(7) Schoonbeek, H., Jacquat-Bovet, A.-C., Mascher, F. & Métraux, J.-P. Oxalate-degrading bacteria can protect Arabidopsis thaliana and crop plants against Botrytis cinerea. Mol. plant-microbe Interact. 20, 1535–1544 (2007).
(8) Saba, H. Trichoderma – a promising plant growth stimulator and biocontrol agent. Mycosphere 3, 524–531 (2012).
(9) Downie, H. et al. Transparent Soil for Imaging the Rhizosphere. PLoS One 7, e44276 (2012).

Acknowledgments

This research is supported by the Gebert Rüf Stiftung (Grant agreement GRS-064/18 PI) and the U.S. Department of Energy Biological and Environmental Research Division through a Science Focus Area Grant No. KP1601010.

6. Multi-omics for Microbiomes Conference. Pacific Northwest National Laboratory, Richland, WA, USA, 24-26.07.2019



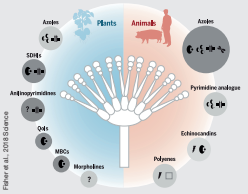
7. Annual Congress of the SSM 2019, Zürich, Switzerland, 03-04.09.2019.

Biological control of *Aspergillus niger* through bacterial oxalotrophy

Fabio Palmieri[1]*, Aislinn Estoppey[1], Ilona Palmieri[1], Nourine Noormamode[1], Geoffrey L. House[2], Jamey D. Young[3], Saskia Bindschedler[1], Patrick S. G. Chain[2], Jennifer Foster Harris[2], Pilar Junier[1]

[1]Laboratory of Microbiology, University of Neuchâtel, Neuchâtel, Switzerland; [2]Los Alamos National Laboratory, Los Alamos, USA; [3]School of Engineering, Vanderbilt University, Nashville, USA

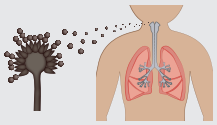
Background

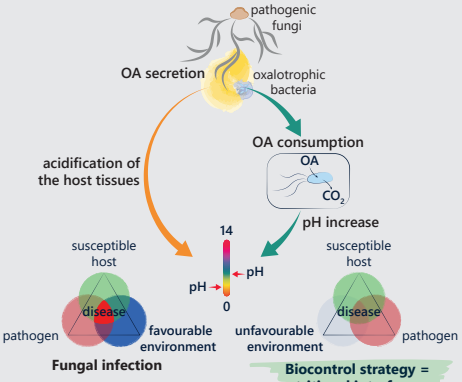


The **worldwide emergence of antifungal (multi) resistant pathogenic fungi**, such as *Aspergillus* spp., is a major threat to human health.

Aspergillosis (i.e. *Aspergillus* infection) is one of the fungal-related diseases with increased prevalence in the past few years.

In some cases of invasive pulmonay Aspergillosis, and expecially with ***A. niger***, **calcium oxalate crystals** have been observed in lung tissues.





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Working hypothesis

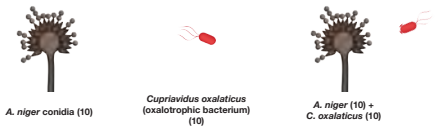
During a **normal infection process**, opportunistic fungal pathogens, such as *A. niger*, use oxalic acid secretion as a pathogenicity factor that renders the environment favorable for the onset of infection of a susceptible host (optimal conditions in a disease triangle). In contrast, **consumption of oxalic acid** by oxalotrophic bacteria renders the environmental conditions unfavorable, reducing the ability of the pathogen to attack the host.

Aim:

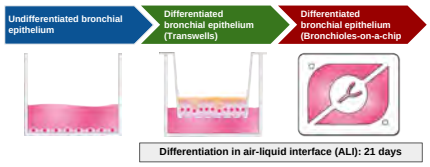
Assess the biocontrol potential of oxalotrophic bacteria to control the growth of *A. niger ex-vivo*.

Methods

Experimental conditions



Systems tested

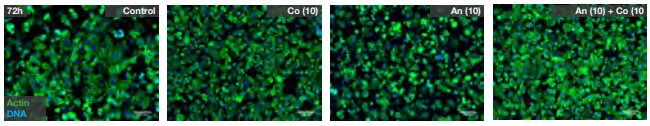


Analysis

- Fluorescence microscopy
- pH
- Ca^{2+} quantification
- Oxalic acid quantification
- Lactate dehydrogenase (LDH) assay

Results

Interaction of *A. niger* & *C. oxalaticus* on undifferentiated bronchial epithelium

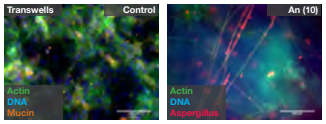


Control: bronchial cells alone; Co: cells + *C. oxalaticus*; An: cells + *A. niger*.

The numbers in brakets indicate the number of bacterial cells or conidia inoculated.

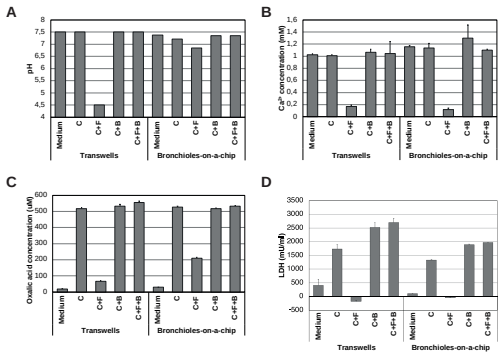
***C. oxalaticus* (Co) controls the growth of *A. niger* (An) infecting epithelial bronchial cells.**

Interaction of *A. niger* & *C. oxalaticus* on differentiated bronchial epithelium (Transwells & Bronchioles-on-a-chip)



After **72h of infection**, as small dose as 10 conidia of *A. niger* generated **major damage on differentiated bronchial epitelium**. Indeed. no more epithelial cells are visible. The stain for actin and DNA are diffuse, meaning that the cell integrity has been lost.

Control: bronchial cells alone; An (10): cells + 10 conidia of *A. niger*.



A. pH; B. Ca^{2+} quantification; C. Oxalic acid quantification; D. LDH.

C: cells; C+F: cells + *A. niger*; C+B: cells + *C. oxalaticus*; C+F+B: cells + *A. niger* + *C. oxalaticus*

In presence of *A. niger*, the pH of the culture medium decreases from 7.5 (control) to 4 for the transwells. The decrease in pH was less marked in the case of the Bronchioles-on-a-chip. This pH drop is correlated with a decrease in the concentration of Ca^{2+}. This could be explained by the **sequestration of Ca^{2+} by oxalic acid excretion during infection**, leading to an **acidification of the tissues**.

Bronchial epithelial cells seem to excrete small concentrations of oxalic acid themselves (on the order of uM). The lower concentration of oxalic acid in the treatments with *A. niger* is most likely explained by the formation of **calcium oxalate crystals in the fungal-infected tissue**, but this has to be further confirmed.

Finally, LDH (lactate dehydrogenase) was not detected in the treatment with *A. niger*, probably because no more cells were present after infection.

Conclusions & Future directions

We have demonstrated the proof of concept of **biocontrol of fungal infection through bacterial oxalotrophy**.

Further experiments will be done on the alveolar-on-a-chip biorector and in the full breathing lung-on-a-chip.

@Palmieri_F_

fabio.palmieri@unine.ch

Acknowledgements

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II. Other project and collaboration

Unprecedented bacterial diversity within the bacteriome of fungi

Aaron J. Robinson*[1], Geoffrey L. House[1], Demosthenes P. Morales[1,2], Julia M. Kelliher[1], La Verne Gallegos-Graves[1], Erick S. LeBrun[1], Karen W. Davenport[1], **Fabio Palmieri**[3], Andrea Lohberger[3], Danae Bregnard[3], Aislinn Estoppey[3], Matteo Buffi[3], Christophe Paul[3], Thomas Junier[3], Vincent Herve[3], Guillaume Cailleau[3], Simone Lupini[4], Hang N. Nguyen[4], Amy O. Zheng[5], Luciana Jandelli Gimenes[6], Saskia Bindschedler[3], Debora F. Rodrigues[4], James. H Werner[2], Jamey D. Young[5], Pilar Junier[3], and Patrick S. G. Chain[1].

[1]Biosecurity and Public Health Group, Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA, [2]Center of Integrated Nanotechnologies, Los Alamos National Laboratory, Los Alamos, NM 87545, USA, [3]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, CH-2000 Neuchâtel, Switzerland, [4]Department of Civil and Environmental Engineering. University of Houston, Houston, TX 77004, USA, [5]Department of Chemical and Biomolecular Engineering and Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37235-1604, USA, [6]Center for Environmental Research and Training, University of São Paulo, Cubatão, São Paulo 11.540 -990, Brazil.

Under Review in Communications Biology

Abstract

While fungi are an important component of the microbiota of a multitude of environments, including host organisms, they can also serve as hosts for other microorganisms such as bacteria. Knowledge of associations between fungal hosts and their bacterial associates has steadily grown in recent years as the number and diversity of examinations have increased, but current knowledge is predominantly limited to a relatively small number and diversity of fungal taxa and bacterial partners. These previous studies also focus primarily on endohyphal associates, leaving any externally-associated bacteria underexplored. Here, we screened for all potential bacterial associates in over 700 phylogenetically diverse fungal isolates, representing 367 genera, including isolates from several previously unexplored phyla. This represents nearly a tenfold increase in the total number of fungal genera previously examined. Results from two independent screens, a 16S-based exploration of fungal isolates from four distinct culture collections spanning three continents, and a screen for bacterial-specific sequences in publicly available fungal genome sequencing projects, revealed that a surprisingly diverse array of bacterial associates appear to be frequently found in otherwise axenic fungal cultures. Strain-specific associations between a particular bacterial lineage and a single fungal isolate were common, but more general associations were observed as well. Here we demonstrate that bacterial associations with diverse fungal hosts are the rule, rather than the exception, and deserve more consideration in microbiota studies and in examinations of microbial interactions.

Note

My personal contribution to this work was to perform part of the 16S screening for bacterial associates in the fungal collection of the LAMUN, as well as to perform the first exploratory analysis of the sequencing data.

III. Project highlight in the Novartis FreeNovation brochure 2021

Microbiome 2016

Oxalotrophy as a therapeutic alternative for the control of fungal pathogens

Pilar Junier and Fabio Palmieri
Laboratory of Microbiology, University of Neuchâtel

Aim and approach

Over the last three decades, society has witnessed a significant intensification in the burden of fungal diseases on human health. It is estimated that fungal pathogens, most of which are opportunistic, kill more than 1.5 million people every year. However, the limited number of options for successful treatment nowadays make the need for alternative treatments to cope with these infections an urgent global health issue. Our project aims at exploring and developing a novel therapeutic concept to fight fungal infection. More precisely, considering humans as an ecosystem, we address the environmental perturbations occurring upon the establishment of fungal infections. The originality of our project lies in our approach, which aims at targeting the environment in which the pathogen thrives, instead of targeting the pathogen itself.

We have singled out oxalic acid as the key environmental factor at the crossroads of the interaction between two key components of the human microbiome: bacteria and fungi. Oxalic acid, or its salts, is an omnipresent compound produced by a large diversity of organisms. In the particular case of fungi, secretion of oxalic acid is not only common, but also essential, as oxalic acid plays a multitude of roles such as in nutrient acquisition or pathogenesis. In the case of humans, oxalate (mainly in the form of calcium oxalate) enters our system through the diet and if not properly metabolized, it can result in hyperoxaluria (i.e., high oxalate content in urine), which in some cases ends in the formation of kidney stones. In addition, calcium oxalate crystals have been found in the lungs of immunocompromised patients suffering from aspergillosis, i.e., an infection caused by an *Asper-*

Our project aims at exploring and developing a novel therapeutic concept to fight fungal infection.

gillus mold. *Aspergillus* fungi are ubiquitous in the environment and are especially abundant in compost piles (Figure 1). They produce spores in order to disperse and colonize novel habitats. Each human being breathes thousands of spores every day, but this does not cause any harm in healthy people. However, the inhalation of spores by immunocompromised individuals can lead to the colonization of the lung tissue. In the lung, we hypothesize that the fungus will secrete oxalic acid in order to create an optimal environment for its development (Figure 1).

We have previously investigated the metabolism of oxalate in soils, in which this compound rarely accumulates. This has led to the identification of oxalotrophy, a bacterial metabolism that explains the consumption of oxalate. The knowledge gained on oxalotrophy in soils can be used as a stepping-stone for developing an alternative for the treatment of fungal pathogens using oxalate during pathogenesis. We postulated that by consuming oxalate, oxalate-degrading bacteria can reduce the growth potential of pathogenic fungi that use this compound as a pathogenicity factor. In order to test this hypothesis, we used *Aspergillus niger* as fungal pathogen and *Cupriavidus oxalaticus* as an oxalotrophic bacterium (Figure 2 A and B, respectively).

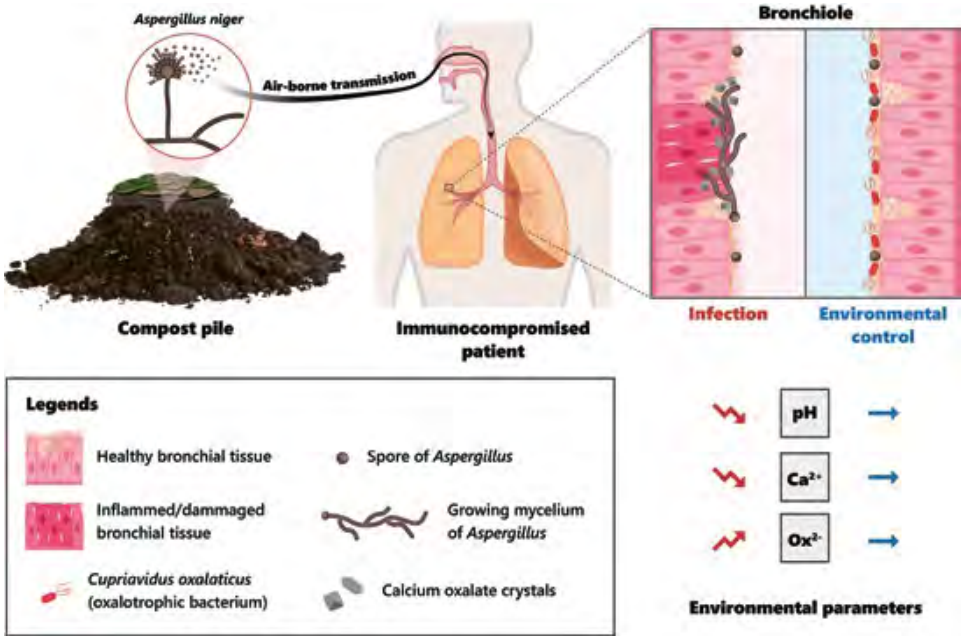

Figure 1. Graphical summary of the proposed approach to control *Aspergillus niger* infection by introducing oxalotrophic bacteria to modify the environment in which *A. niger* thrives. *A. niger* spores are highly abundant in compost piles and reach humans via airborne transmission. During a normal infection process in an immunocompromised patient, *A. niger* modifies the environment by secreting oxalic acid (or oxalate Ox^{2-}), which decreases pH and binds to calcium (Ca^{2+}) to form calcium oxalate. This creates the ideal environmental conditions for the infection of the host's tissue. The environmental control strategy proposed here takes advantage of the ability of oxalotrophic bacteria to consume calcium oxalate crystals and thus reestablish physiological pH and calcium concentrations, modifying the environment in which the pathogen would develop.

Pilar Junier
Professor, University of Neuchâtel

As an environmental microbiologist, it was unconceivable for me to obtain funding for a project related to human health. I did not expect that I will be studying humans alongside soils, geothermal sites or salt lakes. FreeNovation made me question my own view of humans and now I can see them as another ecosystem to explore and understand.

Fabio Palmieri
Ph. D. student, University of Neuchâtel

My experience with FreeNovation was very exciting and fruitful. It allowed me to explore a new area of research that I would not have been able to dive in otherwise, being an environmental microbiologist. I do really think that bringing ecology into medical microbiology and clinical research is the future.

Key results

We first cultured the fungus with and without bacteria in a medium that is used to grow lung cells. This medium contains a colored pH indicator and appears pink when it is at a physiological pH (around 7.5). In the presence of the fungus, the medium turns yellow, meaning it is acidic, as the fungus secretes oxalic acid (Figure 2 C, upper plate). However, when the fungus is cultured with bacteria, the medium stays at a physiological pH (Figure 2 D, upper plate). The concentration of oxalic acid decreased by around 90% in the presence of the bacteria (Figure 2 E). Moreover, we also saw that the presence of the bacteria inhibits the growth of the fungus (Figure 2 D, lower plate), compared to when it grows alone (Figure 2 C, lower plate).

After the experiments in Petri dishes, we wanted to see if the same happens in the presence of lung cells, a setting closer to reality. For that, we worked in an advanced *in-vitro* cell culture system containing bronchial tissue, in collaboration with a team from the Los Alamos National Laboratory, in New Mexico, US. We cultured the fungus on the bronchial tissues in presence or not of the oxalotrophic bacterium and we measured three different parameters: pH, the concentration of calcium and the concentration of oxalic acid. In the presence of the fungus alone, pH (Figure 3 A), calcium (Figure 3 B), and

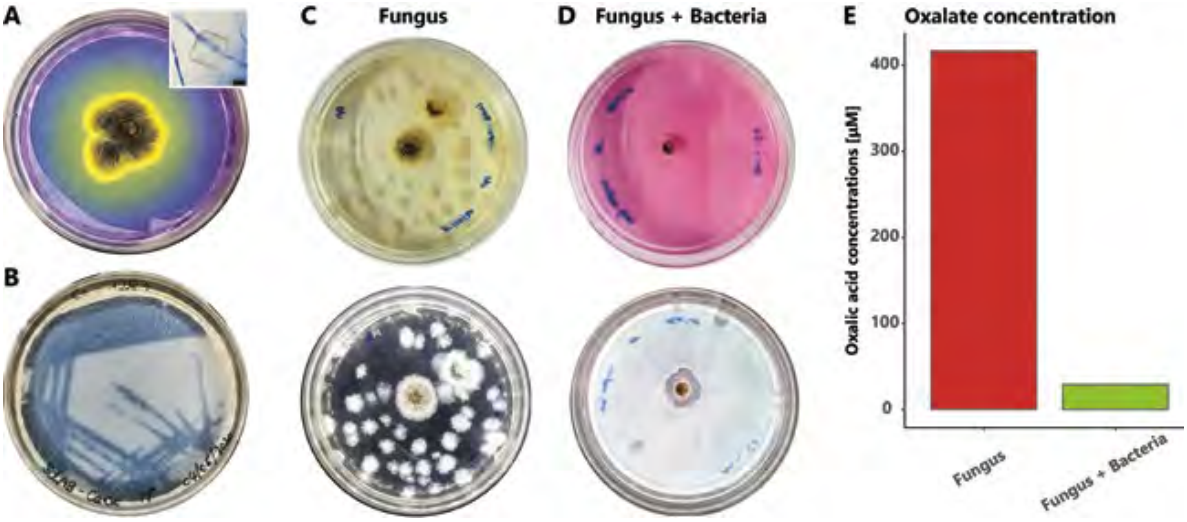

Figure 2. *In-vitro* interactions of the fungus and the bacterium. (A) Culture plate of *Aspergillus niger* grown on a medium containing a pH indicator. The yellow color indicates acidification of the medium. The insert shows an image of a calcium oxalate crystal. Scale bar = 10 µm. (B) Image showing the ability of the oxalotrophic bacterium *Cupriavidus oxalaticus*, to degrade calcium oxalate crystals and forming transparent zones in which this compound has been consumed. The top row images of (C) and (D) show the difference in pH (yellow for acidic pH and pink for physiological) after the cultivation of the fungus alone or with oxalotrophic bacteria in medium used for the cultivation of bronchial cells. The bottom row shows the effect of the presence of the bacteria on the growth of fungus (D) compared to the fungus alone (C). The graph in (E) shows the decrease of around 90% in the concentration of oxalic acid when the bacteria is cultured with the fungus, as compared to the fungus alone.

free oxalic acid (Figure 3 C) concentrations were lower as compared to when the bacteria were co-cultured with the fungus on the bronchial cells. In addition, we detected calcium oxalate crystals when the fungus was alone (Figure 3 D), but not when the fungus was cultured with bacteria (Figure 3 E). We were also able to show the aggressiveness of the fungus, which completely destroyed the bronchial tissue (Figure 4).

Altogether, our results show that using bacteria to restore the environmental parameters to a physiological level is a valid method to modify the course of fungal infection. This offers a very exciting avenue of research for an innovative therapeutic approach, centered around the isolation and characterization of new oxalotrophic bacteria from the lung microbiome, and on the validation of our findings *in-vitro* in a mouse model.

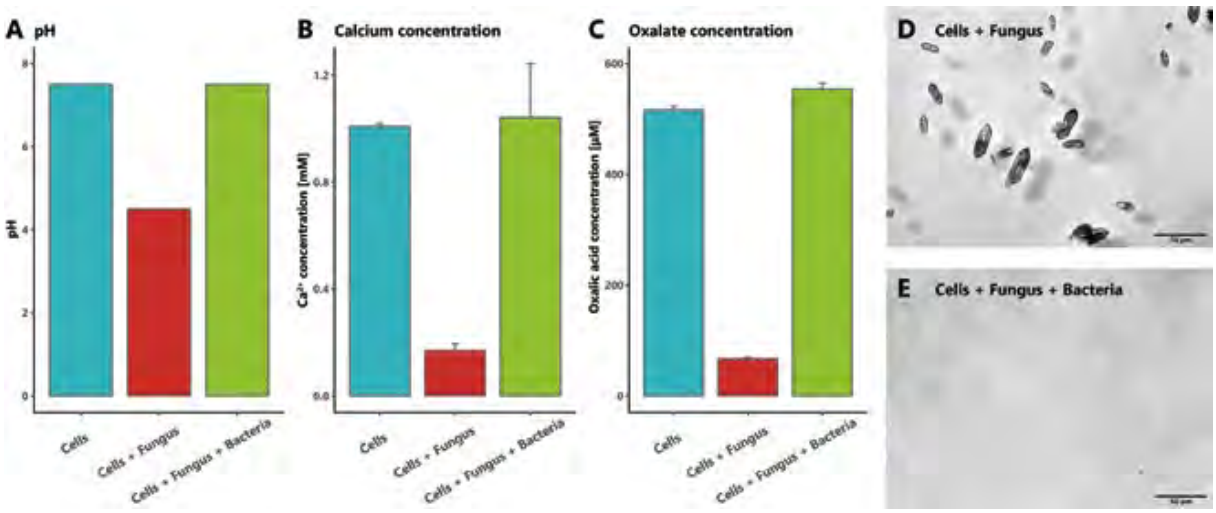

Figure 3. Environmental parameters measured in the bronchial cell cultures alone, with the fungus, or with the fungus and the bacteria: (A) pH, (B) calcium, and (C) oxalic acid concentrations. The images (D) show the presence of calcium oxalate crystals in the presence of the fungus and the absence of crystals when the bacteria were cultured with the fungus (E).

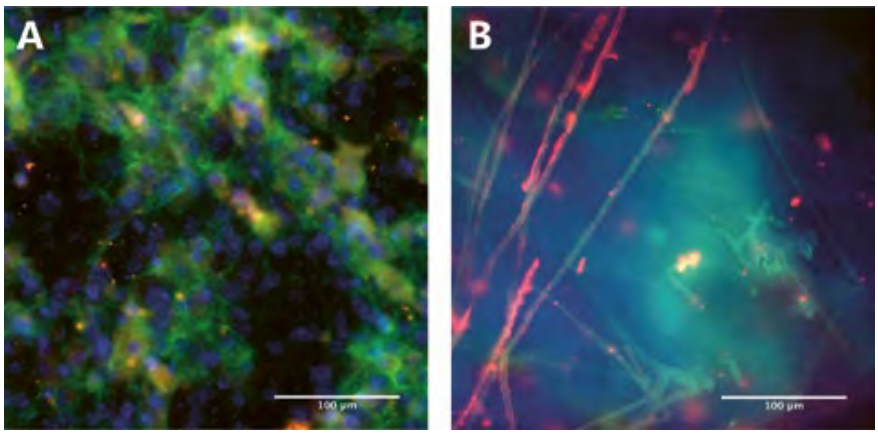

Figure 4. Images showing the aspect of a healthy bronchial cell culture (A) compared to a condition in which the cells are exposed to the fungus (B).

IV. Lay communication – Contribution to TheScienceBreaker

August 02, 2019

Microbiology

The insect microbiomes – a new hope against antimicrobial resistance?

by **Fabio Palmieri**[1] | PhD student

[1]: University of Neuchâtel, Neuchâtel, Switzerland

This Break was edited by Max Caine, *Editor-in-chief* - TheScienceBreaker

ABSTRACT

Due to the increase in antimicrobial resistance in pathogens, currently used antibiotics are becoming ineffective. Thus, new antibiotics need to be discovered. This study makes light into a yet unexploited source of potent antimicrobials: the insect microbiomes.

Image credits: Wikimedia Commons – CC-BY 2.0

Nowadays, more and more antibiotics (also referred to as antimicrobial drugs) are becoming ineffective to fight against bad bacteria and fungi because these organisms are capable of rapidly developing resistance to those compounds. These resistances arise due to the misuse and overuse of antibiotics. In order to counterbalance the emergence of new antibiotic-resistant pathogens, there is an urgent need to discover new antimicrobial compounds. Natural products are the principal source of antimicrobials and they are mainly produced by soil bacteria of the Actinobacteria group. In recent years, sampling campaigns to look for new compounds led to the rediscovery of known antimicrobial compounds. Given the need for more effective therapeutic molecules, there has been growing interest in host microbiomes as a potential source of discovery for new antibiotics. The microbiome is the collection of microorganisms, such as bacteria, fungi or viruses, colonizing a given habitat, plants or animal hosts. Indeed, antibiotics have been discovered from microbiomes of diverse animals, such as insects or humans; bacterial members of the microbiome produce antimicrobial compounds to protect the host from pathogens. There is a well-known example in the southern pine beetle where Actinobacteria, typically *Streptomyces*, produce chemical defenses to fight against infections.

Recently, a group of American scientists has investigated the potential of insect microbiomes as a valuable source of new antibiotics. They focused on

Streptomyces bacteria as they are the source of most clinically used antimicrobial drugs. Moreover, they have been shown to form beneficial associations with diverse insect hosts. Their hypothesis was that bacteria of the genus *Streptomyces* from insect microbiomes are a promising source of antimicrobial compounds, which have evolved differently from *Streptomyces* bacteria from soil.

The first thing they did was to try to isolate *Streptomyces* from diverse insect hosts, such as bees or butterflies, for example, sampled from different locations and habitats across America. The research team succeeded in isolating *Streptomyces* from insects. They showed that these bacteria were widespread across the host range and habitats investigated. For comparison, the researchers also isolated *Streptomyces* from soil and plants. By analyzing the genetic material and by comparing the evolutionary relationships of insect-, soil- and plant-*Streptomyces*, they were able to demonstrate that insect-associated bacteria evolved in distinct lineages. Of key importance, the scientists were also able to show that insect-associated *Streptomyces* harbor great potential for uncharacterized antimicrobial compound synthesis.

Investigating further, the researchers tested these insect-*Streptomyces* for their effectiveness against bacterial and fungal pathogens. By confronting the insect-associated *Streptomyces* against clinically-relevant bacteria and fungi, the researchers showed that insect-*Streptomyces* had a stronger ability to inhibit pathogens compared to soil- or plant-associated bacteria. Specifically, insect-associated *Streptomyces* had greater antifungal activity.

Thus, in order to further investigate the inhibitory potential of compounds extracted from insect-*Streptomyces*, the researchers tested their effect in a mouse infection model. First, they identified in an artificial setting (in-vitro) which extracts were able to inhibit bacterial and fungal pathogens. In the case of inhibitory activity, the extracts were analyzed in order to identify the compounds present. Unknown/novel compounds were then used in the in-vivo mouse infection model. The experimental results showed that insect-*Streptomyces* had potent inhibitory activity in-vivo, for both bacterial and fungal pathogens. Moreover, extracts coming from insect-*Streptomyces* showed no toxicity for the mouse. Finally, in one of the insect-*Streptomyces* extracts, the scientists discovered a new antimicrobial compound – *cyphomycin* – from a Brazilian *Streptomyces* isolated from the microbiome of a fungus-growing ant. Purified cyphomycin was active against multi-resistant fungal pathogens both in-vitro and in-vivo, and in low concentrations.

Through experimental and qualitative laboratory procedures, the research team confirmed their working hypothesis demonstrating that insect microbiomes are a promising source of novel antimicrobial compounds. The implications of their research open a new paradigm for the discovery of new antibiotics with exciting potential for medical applications. The promise of this new source for new antimicrobials products lies in the fact that insects, throughout their evolution, and because of the constant pressure applied by pathogens, have formed associations with *Streptomyces* bacteria that produce effective antimicrobial compounds.

In conclusion, antimicrobial products coming from insect-associated *Streptomyces* are especially suited for medical applications, as their associations with insects appear to favor low toxicity compounds to animals. Are these insect microbiome-derived antimicrobial compounds the key to our success over antimicrobial resistance? Only the future will tell us!

V. Curriculum Vitae

FABIO PALMIERI

MICROBIAL ECOLOGIST

fabio.palmieri@unine.ch
www.unine.ch/lamun

+ 41 32 718 23 34
+ 41 79 430 42 59

Laboratory of Microbiology, University of Neuchâtel,
Emile-Argand 11, 2000 Neuchâtel, Switzerland

0000-0002-5241-6898

@Palmieri_F_

Research interests

My research interests are in the field of microbial ecology, going from soil to the plant, animal and human hosts. I'm interested in studying and understanding the interactions between microbes and their environments and with each other. During my Master project, I had the opportunity to study endospore-forming bacteria in a volcanic site in Greece (Nisyros Island) by both culturable and un-culturable methods and thus evaluate the role of endospore-formation as a survival strategy to the changing environmental conditions in these habitats. For my PhD thesis, I am interested in bacterial-fungal interactions in the context of human health and how we can use bacteria as biocontrol agents against fungal pathogens. More specifically, I evaluated the biocontrol potential of oxalotrophic bacteria to inhibit the growth of *Aspergillus niger* through oxalate degradation in 3D-lung cell tissues systems. In my future work, I would like to continue to investigate host-fungal and host-microbe interactions, and more specifically the interplay of the host, the environment, and the microbiota in the establishment and growth of fungal pathogens, while keeping the link with environmental microbiology.

Education

Oct. 2016 – Present	**PhD in Microbiology,** University of Neuchâtel.
2014 – 2016	**Master of Science in Biology**, Specialization: Ecology and environment, Evolution and biodiversity, Grade cum laude, University of Neuchâtel.
2011 – 2014	**Bachelor of Science in Biology**, Grade cum laude, University of Neuchâtel.

Research experiences

Oct. 2016 – Present	**PhD Thesis**, Laboratory of Microbiology, University of Neuchâtel. "Bacterial oxalotrophy as an alternative biocontrol approach for the fight against pulmonary aspergillosis". Supervisor: Prof. Pilar Junier
Jan. – July 2019	**Visiting Research Scholar**, Host and Pathogen Biology Lab, Biosecurity and Public Health, Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM, USA. "Bacterial Oxalotrophy as a Biological Mechanism to Control Aspergillosis in a Lung-on-a-Chip System" Supervisor: Dr. Jennifer Foster Harris, Dr. Patrick S. G. Chain
April 2015 – Aug. 2016	**Master thesis**, Laboratory of Microbiology, University of Neuchâtel. "To Sporulate or not to Sporulate: Prevalence of Endospore-Forming Firmicutes along a Thermal Gradient in Alexandros Crater (Nisyros, Greece)" Supervisors: Prof. Pilar Junier, Dr. Sevasti Filippidou.
Jan. – Feb. 2015	**Internship**, Laboratory for Environmental Biotechnology (LBE), Swiss Federal Institute of Technology (EPFL). "Effect of the molecular chaperone PceT on the heterologous production of the reductive dehalogenase PceA". Supervisor: Dr. Julien Maillard.
Sept. – Dec. 2014	**Internship**, Laboratory of Microbiology, University of Neuchâtel. Internship project within the project BIOPATINA.

Teaching experiences

March – April 2021	**PhD Assistant** for the Problem-based learning in Microbiology for the 3rd year biology students, Laboratory of Microbiology, University of Neuchâtel.
Sep. – Dec. 2019	**PhD Assistant** for the practicals in microbiology for the 2nd year biology students, Laboratory of Microbiology, University of Neuchâtel.
Sep. – Dec. 2018	**PhD Assistant** for the practicals in bacteriology and mycology for the 2nd year biology students, Laboratory of Microbiology, University of Neuchâtel.
Feb. – March 2018	**PhD Assistant** for the Problem-based learning in Microbiology for the 3rd year biology students, Laboratory of Microbiology, University of Neuchâtel.
Nov. – Dec. 2017	**PhD Assistant** for the practicals in bacteriology for the 2nd year biology students, Laboratory of Microbiology, University of Neuchâtel.
Feb. – March 2017	**PhD Assistant** for the Problem-based learning in Microbiology for the 3rd year biology students, Laboratory of Microbiology, University of Neuchâtel.
Sept. – Dec. 2016	**PhD Assistant** for the practicals in bacteriology for the 2nd year biology students and for the practicals of Bioinformatics tools for the Masters students, Laboratory of Microbiology, University of Neuchâtel.
Sept. – Oct. 2015	**Student-Assistant** for the practicals in molecular biology for the 3rd year biology students (24 hours), Laboratory of Microbiology, University of Neuchâtel.
Sept. – Oct. 2014	**Student-Assistant** for the practicals in molecular biology for the 3rd year biology students (24 hours), Laboratory of Microbiology, University of Neuchâtel.

Supervising experiences

Oct. 2020 – present	**Co-advising of Laura Blanco Pérez (bachelor student),** Laboratory of Microbiology, University of Neuchâtel.
Sept. 2020 – present	**Co-advising of Pauline Udriet (biology technician trainee),** Laboratory of Microbiology, University of Neuchâtel.
Sept. 2018 – Jan. 2019	**Co-advising of Alexis Hirshi (high-school student),** Maturity project, Lycée Denis-de-Rougemont.
Sept. 2017 – June 2018	**Co-advising of Aislinn Estoppey (master student),** Laboratory of Microbiology, University of Neuchâtel.
Sept. 2016 – July 2018	**Co-advising of Lindsay Pétremand (biology technician trainee),** Laboratory of Microbiology, University of Neuchâtel.

Skills

Lab skills	Microbial cultures, strain isolation, molecular methods (DNA extraction, DNA quantification, PCR, qPCR, gel electrophoresis), biochemistry methods (protein expression induction, SDS- and Native-PAGE, cellular fractionation), cell culture methods (air-liquid interface tissue culture)
IT	Microsoft Office Suite, iWork Suite, RStudio, ArcGIS, Adobe Photoshop, Adobe Illustrator, Adobe InDesign
Languages	French (native), Italian (fluent, B2 PLIDA Certificate), English (fluent, B2 First Certificate), German (conversational).

Publications

(* equal contributions of the authors)

Peer-reviewed publications

1. Sevasti Filippidou, Marion Jaussi, Thomas Junier, Tina Wunderlin, Nicole Jeanneret, **Fabio Palmieri**, Ilona Palmieri, et al. 2016. "*Anoxybacillus geothermalis* Sp. Nov., a Facultatively Anaerobic, Endospore-Forming Bacterium Isolated from Mineral Deposits in a Geothermal Station." *International Journal of Systematic and Evolutionary Microbiology* 66 (8). Microbiology Society: 2944–51. doi:10.1099/ijsem.0.001125.

2. **Fabio Palmieri***, Aislinn Estoppey*, Geoffrey L. House, Andrea Lohberger, Saskia Bindschedler, Patrick S. G. Chain, Pilar Junier. 2019. "Oxalic Acid, a Molecule at the Crossroads of Bacterial-Fungal Interactions." *Advances in Applied Microbiology*. Vol. 106. 49-77. doi:10.1016/bs.aambs.2018.10.001.

3. Christophe Paul, Sevasti Filippidou, Isha Jamil, Wafa Kooli, Geoffrey House, Aislinn Estoppey, Mathilda Hayoz, Thomas Junier, **Fabio Palmieri**, Tina Wunderlin, Anael Lehmann, Saskia Bindschedler, Torsten Vennemann, Patrick S. G. Chain, Pilar Junier. 2019. "Bacterial Spores, from Ecology to Biotechnology." *Advances in Applied Microbiology*. Vol. 106. 79-111. doi:10.1016/bs.aambs.2018.10.002.

4. P. Junier, G. Cailleau, I. Palmieri, C. Valloton, O. C. Trautschold, T. Junier, C. Paul, D. Bregnard, **F. Palmieri**, A. Estoppey, M. Buffi, A. Lohberger, A. Robinson, J. M. Kelliher, K. Davenport, G. L. House, D. Morales, V. Gallegos-Graves, A. E. K. Dichosa, S. Lupini, H. N. Nguyen, J. D. Young, D. F. Rodrigues, A. N. G. Parra-Vasquez, S. Bindschedler, P. S. G. Chain. 2021. "Democratization of Fungal Highway Columns as a Tool to Investigate Bacteria Associated with Soil Fungi." *FEMS Microbiol Ecol*. doi:10.1093/femsec/fiab003.

Under review/Submitted

5. **Fabio Palmieri**, Ilona Palmieri, Nourine Noormamode, Aislinn Estoppey, M. Omar Ishak, Julia M. Kelliher, Armelle Vallat, Rashi Iyer, Saskia Bindschedler, Karen Davenport, Patrick S. G. Chain, Jennifer Foster Harris, Pilar Junier. 2020. "Biocontrol of *Aspergillus niger* in 3D-Lung Cell Tissues by Oxalotrophic Bacteria." *BioRxiv*. doi:10.1101/2020.08.20.259929. *Under Review In PLOS One*.

6. Aaron J. Robinson, Geoffrey L. House, Demosthenes P. Morales, Julia M. Kelliher, La Verne Gallegos-Graves, Erick S. LeBrun, Karen W. Davenport, **Fabio Palmieri**, Andrea Lohberger, Danae Bregnard, Aislinn Estoppey, Matteo Buffi, Christophe Paul, Thomas Junier, Vincent Herve, Guillaume Cailleau, Simone Lupini, Hang N. Nguyen, Amy O. Zheng, Luciana Jandelli Gimenes, Saskia Bindschedler, Debora F. Rodrigues, James. H Werner, Jamey D. Young, Pilar Junier, Patrick S. G. Chain. 2021. "Unprecedented bacterial diversity within the bacteriome of fungi". *Under Review In Communications Biology*.

7. Thierry Kuhn*, Marine Mamin*, Saskia Bindschedler, Redouan Bshary, Aislinn Estoppey, Diego Gonzalez, **Fabio Palmieri**, Pilar Junier, Xiang-Yi Li. 2021. "Bacterial competition supports Darwin's naturalization hypothesis at the local but not the regional spatial scale". *Under Review In Biology*.

In preparation

8. **Fabio Palmieri**, Pauline Udriet, Shannon L. Johnson, Karen Davenport, Patrick S. G. Chain, Saskia Bindschedler, Pilar Junier. "Complete Genome Sequence of *Cupriavidus oxalaticus* Strain Ox1, a Soil Oxalate-Degrading Species". *Microbiology Resource Announcements*.

Oral presentations (# presenting)

1. **Fabio Palmieri**#, Sevasti Filippidou, Pilar Junier. Endospore-Forming Firmicutes Dominate Bacterial Communities across Thermal Gradients in Nisyros Volcano. 74th Annual Meeting and Assembly SGM-SSM, Bern, Switzerland, 13.–15.6.2016.

2. Pilar Junier, **Fabio Palmieri**, Aislinn Estoppey. Bacterial Oxalotrophy as a Biological Mechanisn to Control the Development of Fungal Pathogens. FreeNovation Science Forum, Novartis Campus, Basel, Switzerland, 24.05.2018

3. **Fabio Palmieri**#, Andrea Lohberger, Aislinn Estoppey, Lindsay Pétremand, Jean F. Challacombe, Geoffrey L. House, Debora F. Rodrigues, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Pilar Junier. Low Molecular Weight Organic Acids as key molecules mediating Bacterial-Fungal Interactions. Bacterial-Fungal Interaction Workshop. 11th International Mycological Congress (IMC11), San Juan, Puerto Rico, 16-21.07.2018.

4. Andrea Lohberger, **Fabio Palmieri**#, Saskia Bindschedler, Geoffrey L. House, Armand Dichosa, Jean F. Challacombe, Debora F. Rodrigues, Hang Nguyen, Jamey D. Young, Patrick S. G. Chain, Eric Verrecchia, Pilar Junier. Endofungal bacteria – New insights into bacterial-fungal coexistence. 11th International Mycological Congress (IMC11), San Juan, Puerto Rico, 16-21.07.2018.

5. **Fabio Palmieri**#, Andrea Lohberger, Aislinn Estoppey, Lindsay Pétremand, Jean F. Challacombe, Geoffrey L. House, Debora F. Rodrigues, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Pilar Junier. Low Molecular Weight Organic Acids as key molecules mediating Bacterial-Fungal Interactions. Annual Congress of the SSM 2018, Lausanne, Switzerland, 28-30.08.2018

6. Andrea Lohberger#, **Fabio Palmieri**, Saskia Bindschedler, Geoffrey L. House, Armand Dichosa, Jean F. Challacombe, Debora F. Rodrigues, Hang Nguyen, Jamey D. Young, Patrick S. G. Chain, Eric Verrecchia, Pilar Junier. Endofungal bacteria – New insights into bacterial-fungal coexistence. Annual Congress of the SSM 2018, Lausanne, Switzerland, 28-30.08.2018

7. **Fabio Palmieri**#, Aislinn Estoppey, Ilona Palmieri, Nourine Noormamode, Geoffrey L. House, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Jennifer Foster Harris, Pilar Junier. Biological control of *Aspergillus niger* through bacterial oxalotrophy. Annual Congress of the SSM 2019, Zürich, Switzerland, 03-04.09.2019

Poster presentations (# presenting)

1. **Fabio Palmieri**#, Sevasti Filippidou, Pilar Junier. Endospore-Forming Firmicutes Dominate Bacterial Communities across Thermal Gradients in Nisyros Volcano. 74th Annual Meeting and Assembly SGM-SSM, Bern, Switzerland, 13.–15.06.2016.

2. **Fabio Palmieri**#, Saskia Bindschedler, Pilar Junier. Organic Acid Production by Fungi : Comparison on Various Media and Effect of the Interaction with Bacteria. Annual PhD Students Meeting 2017, Neuchâtel, Switzerland, 29.03.2017.

3. **Fabio Palmieri**#, Saskia Bindschedler, Pilar Junier. Organic Acid Production by Fungi : Comparison on Various Media and Effect of the Interaction with Bacteria. FEMS – 7th Congress of European Microbiologists, Valencia, Spain, 09-13.07.2017.

4. **Fabio Palmieri**#, Aislinn Estoppey, Jean Challacombe, Patrick S. Chain, Pilar Junier. Bacterial Oxalotrophy as a Biological Mechanisn to Control the Development of Fungal Pathogens. FreeNovation Science Forum, Novartis Campus, Basel, Switzerland, 24.05.2018.

5. **Fabio Palmieri**#, Andrea Lohberger, Aislinn Estoppey, Lindsay Pétremand, Jean F. Challacombe, Geoffrey L. House, Debora F. Rodrigues, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Pilar Junier. Low Molecular Weight Organic Acids as key molecules mediating Bacterial-Fungal Interactions. 11th International Mycological Congress (IMC11), San Juan, Puerto Rico, 16-21.07.2018.

6. **Fabio Palmieri**#, Andrea Lohberger, Aislinn Estoppey, Lindsay Pétremand, Jean F. Challacombe, Geoffrey L. House, Debora F. Rodrigues, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Pilar Junier. Low Molecular Weight Organic Acids as key molecules mediating Bacterial-Fungal Interactions. Annual Congress of the SSM 2018, Lausanne, Switzerland, 28-30.08.2018.

7. Aislinn Estoppey, **Fabio Palmieri**#, Geoffrey L. House, Debora F. Rodrigues, Jamey D. Young, Saskia Bindschedler, Pilar Junier**,** Patrick S. G. Chain. Bacterial Oxalotrophy as a Biocontrol Mechanism against Fungal Pathogens. 2019 Genomic Sciences Program Annual Principal Investigator (PI) Meeting. Tysons, VA, USA, 24-27.02.2019

8. **Fabio Palmieri**#, Aislinn Estoppey, Ilona Palmieri, Nourine Noormamode, Geoffrey L. House, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Jennifer Foster Harris, Pilar Junier. Biological control of *Aspergillus niger* through bacterial oxalotrophy. Multi-omics for Microbiomes Conference. Pacific Northwest National Laboratory, Richland, WA, USA, 24-26.07.2019

9. **Fabio Palmieri**#, Aislinn Estoppey, Ilona Palmieri, Nourine Noormamode, Geoffrey L. House, Jamey D. Young, Saskia Bindschedler, Patrick S. G. Chain, Jennifer Foster Harris, Pilar Junier. Biological control of *Aspergillus niger* through bacterial oxalotrophy. Annual Congress of the SSM 2019, Zürich, Switzerland, 03-04.09.2019. *2nd Best Poster Award*

Grants and Awards

2015	Fonds Wütrich et Matthey-Dupraz – Travel grant for the Master thesis field work in Nisyros, Greece (1000 CHF)
2019	Swiss Society for Microbiology Annual Congress – 2nd Best Poster Award (1000 CHF)
2021	Ma Thèse en 180 secondes (MT180) – Prix du public

Service-related activities

Reviewer in the following journals:

- Frontiers in Microbiology

Preprints-related activities:

- ASAPbio Ambassador (since April 2019)
- ASAPbio Fellows Program 2020 member
- preLighter at preLights (preprint highlights, since April 2020)

Lay communication activities:

- Contribution to TheScienceBreaker - Science Meets Society: «Insect microbiomes – a new hope against antimicrobial resistance?», doi: 10.25250/thescbr.brk239.
- Candidate for the 2021 edition of «Ma Thèse en 180 secondes» (MT180) – 22 April 2021.

Organization

- Member of the organization committee of Biology18, 14-16 February 2018, University of Neuchâtel.
- Co-organization of a course in the CUSO Microbial Sciences Doctoral Program – Students Choice: "Astrobiology", 29 November 2019, University of Neuchâtel.
- Organization of a course in the CUSO Microbial Sciences Doctoral Program – Preprint Journal Club, 8 September 2020, Online.

Memberships

Swiss Society for Microbiology (SSM)

Society for Applied Microbiology (SfAM)

Federation of European Microbiological Societies (FEMS)

International Society of Microbial Ecology (ISME)

American Society of Microbiology (ASM)