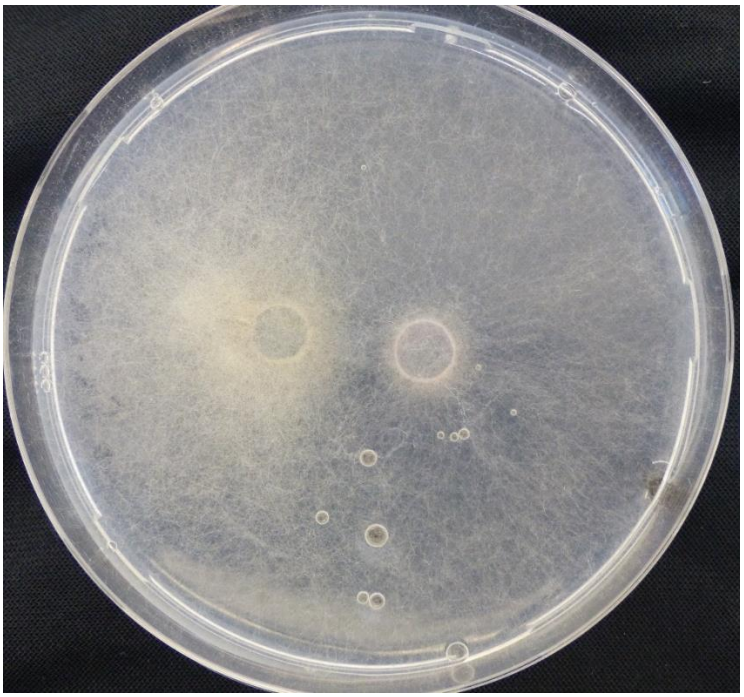
Bacterial consortium associated with the saprotrophic fungus *Mucor hiemalis*: Friends or foes?



MSc thesis Biology (GEN-80430)
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Summary

Fungal-bacterial interactions are important and abundant in nature and agricultural soils, with interactions comprising a continuum between mutualism and antagonism (De Boer et al. 2005, Frey-Klett et al. 2011). The most intimate interactions are ectosymbiosis and endosymbiosis, which occur abundantly on and in fungi (Hoffman and Arnold 2010, Scheublin et al. 2010, Rudnick et al. 2015). These bacteria can affect pathogenesis (Partida-Martinez and Hertweck 2005, Minerdi et al. 2008), mycorrhization (Bonfante and Anca 2009) and metabolism (Schroeckh et al. 2009, Benoit et al. 2015) of their host. In this project the mucormycotina *Mucor hiemalis* was hypothesized to host endobacteria that migrate out of the fungus under nutrient poor conditions. This hypothesis was based on a slime layer appearing around the hyphae after re-plating 3-5 times under nutrient poor conditions. It was aimed to confirm this observation, to isolate, visualize and identify the bacteria and to provide indications for the causes of this migration behavior.

Here, *M. hiemalis* was shown to be associated with a diverse bacterial consortium. The original isolate M0 and the antibiotic treated isolate M9 were associated with a distinct bacterial community. The treatment and change in community affected morphology, pigmentation, hyphal extension and volatile profile of the fungus. Hyphal extension was promoted for M0 compared to M9, but final biomass was not significantly different. Interestingly, after 3-5 times re-plating the fungus under nutrient poor conditions, a slime layer appeared around the hyphae. A new protocol was developed for the isolation of bacteria associated with fungi using antifungal volatiles, which yielded bacterial colonies. The original and antibiotic treated isolates were placed together to facilitate bacterial exchange. Morphological differences did not merge including the slime layer, however with time the differences described above slowly started to disappear.

M. hiemalis isolates M0 and M9 both host bacterial consortium which community differed due to an antibiotic and washing treatment. This selection is hypothesized to be based on resistance or host protection. Both antibiotic resistance and host protection remain to be determined in the future. The role of the bacteria for the fungus remains unclear, since no clear benefits or costs could be observed. The bacteria may benefit by promoting hyphal extension to facilitate their migration over air filled soil pores. Such migration has been described by Warmink et al. (2011). Further elucidation of the bacterial roles can be archieved by re-introducing the bacterial isolates and by the use of molecular methods. In further studies it will be interesting to screen more fungal cultures for bacterial presence and the effects of these bacteria. In soils fungi are often intimately associated with bacteria, so it is very likely that more fungi in collections are associated with bacteria.

Introduction

Bacterial-fungal interactions are abundant and important in nature, with interactions ranging from mutualism to antagonism (De Boer et al. 2005, Kobayashi and Crouch 2009). These interactions are increasingly considered to be important because of its potential impact on food, agriculture and medicine (Peleg et al. 2010, Frey-Klett et al. 2011, Scherlach et al. 2013). Among bacterial-fungal interactions are bacteria that can exploit fungal hyphae for bacterial dispersal over soil pores (Warmink et al. 2011), antibiosis (De Boer et al. 2005) and mycophagy: the consumption of fungal derived substrates (De Boer et al. 2004, Leveau and Preston 2008). Recent advances in this field have identified the importance of volatile organic compounds (VOCs) for these interactions as signaling, inhibitory and growth promoting agents (Effmert et al. 2012, Schmidt et al. 2015). An interesting case is the consortium of *Fusarium oxysporum* and associated bacteria (*Serratia, Achromobacter, Bacillus and Stenotrophomonas*). These associated bacteria transform the pathogenic *F. oxysporum* into a non-pathogenic biocontrol stain, showing antagonistic activity against pathogenic *F. oxysporum* strains via the production of volatiles (Minerdi et al. 2008, Minerdi et al. 2009)

Interspecies interactions in the broad sense are also referred to as symbiosis, comprising a continuum between mutualism and parasitism (Wilkinson 2001, Newton et al. 2010). The most intimate of these interactions is endosymbiosis, when the smaller partner occurs intracellular in the host (Margulis and Chapman 1998). Another intimate interaction is ectosymbiosis, when the bacterial partner occurs adhering to fungal hyphae consuming fungal exudates (Warmink et al. 2009, Stopnisek et al. 2015). This ability to adhere to fungal hyphae is a widespread trait among bacteria (Scheublin et al. 2010, Rudnick et al. 2015).
In the model species *Aspergillus nidulans* and *A. niger* ectobacteria, being several actinomycetes and *Bacillus subtillus* respectively, were shown to affect primary and secondary metabolism (Schroeckh et al. 2009, Benoit et al. 2015). The best characterized fungi adhering bacteria are specific rhizosphere bacteria called mycorrhizal helper bacteria (MHB), who colonize mycorrhizal hyphae thereby forming the mycorrhizosphere. (Frey-Klett et al. 2007, Bonfante and Anca 2009, Scheublin et al. 2010). MHB promote the functioning of the mycorrhizal-plant symbiosis as a fungal extension of the rhizosphere (Frey-Klett et al. 2007, Bonfante and Anca 2009). Recently, it has been shown that the ectomycorrhizal *Laccaria bicolor* can distinguish between beneficial, neutral and antagonistic bacteria and respond accordingly (Deveau et al. 2015).

Endosymbiosis are known to be widespread in fungi, which started with the discovery of bacteria like organisms in several arbuscular mycorrhizal (AM) fungi (Artursson et al. 2006). Among which the nitrogen fixing *Nostoc punctiforme* living in the non-mycorrhizal Glomeromycete *Geosiphon pyriforme* (Kluge et al. 1992) and phosphate solubilizing bacteria in *Glomus mosseae* (Mirabal-Alonso et al. 2007)**.** An extensive study by Hoffman and Arnold (2010) screened many plant epiphytic fungi and found that many host endobacteria as well, among which *L. bicolor (Bertaux et al. 2003, Bertaux et al. 2005)* and *Tuber borchii* (Barbieri et al. 2000).
A more intensively studied example, is the arbuscular mycorrhiza *Gigaspora margarita* with its vertically transmitted partner *Candidatus Glomeribacter gigasporarum* (called candidatus because of its unculturabillity) (Bonfante and Anca 2009). Curing *G. margarita* of its endosymbiont results in retarded presymbiotic growth and altered spore morphology as well as altered metabolism (Lumini et al. 2007, Salvioli et al. 2010), demonstrating an important role of the endobacteria for the functioning of *G. margarita* (Lumini et al. 2007). Nowadays, the genome of this endobacterium is described, revealing the ability to produce VitaminB12 as well as a pathogenesis island encoding secretion systems (Ghignone et al. 2012). Next to the endobacterium *Glomeribacter*, *G. margarita* has been shown to host other endobacteria related to the Mollicutes (Desirò et al. 2014). So, the AM fungus hosts a diverse endobacterial community, a microbiome.

Another well-studied example is a member of the Mucorales: the rice pathogen *Rhizopus microsporus* (Partida-Martinez and Hertweck 2005, Lackner et al. 2009). This fungus contains endobacteria named *Burkholderia rhizoxinica* and *Burkholderia endofungorum* (Partida-Martinez et al. 2007a), which have been shown to produce a potent toxin involved in host pathogenesis (Partida-Martinez and Hertweck 2005, Gee et al. 2011). Interestingly, the endobacteria enforce their vertical transmission by controlling host sporulation making use of a hrp type III secretion system (Partida-Martinez et al. 2007b, Lackner et al. 2011a). As a result the host is not able to reproduce in absence of its endosymbiont, thereby ensuring maintenance of the symbiosis (Partida-Martinez et al. 2007b). One of the two endobacteria, *B. rhizoxinica,* is the first endobacterium that is sequenced providing interesting insights in the endosymbiosis (Lackner et al. 2011b). One of these insights is the presence of pathogenesis related genes like type II, type III and type IV secretion systems, as well as an interesting secretome including chitinases, chitosanases and other effector proteins (Lackner et al. 2011b). These effector proteins facilitate the active infection of host cells by destabilizing the fungal cell wall (Moebius et al. 2014).

Mucor hiemalis, a mucormycotina (formerly zygomycete), is a fast growing coenocytic saprotroph commonly found in soil. It is observed in litter, decaying fruits, dung and in the rhizosphere of many plants (Domsch et al. 2007). Recently an interesting observation has been done on *M. hiemalis* isolated from the rhizosphere of *Carex arenaria* (sand sedge) *(De Rooij-van der Goes et al. 1995)*. The fungus appeared to be in pure culture when grown on rich medium, but when changed to nutrient poor medium endobacteria moved out of the fungus causing a slime layer around the hyphae (Paolina Garbeva and Wietse de Boer, pers comm) (fig. 1) The effect of nutritional conditions of the host fungus on outward migration of endobacteria has not been described before and might provide interesting insights on the development of fungal-endosymbiontic relationships. This observation raises interesting questions. Who are the bacteria moving out and are they able to live seprately? Once the bacteria are outside the fungus, can they infect their host or different eukaryotic hosts? It will also be interesting to elucidate the role of these endobacteria for the fungus. The current project will examine the factors that cause the release of bacteria from the fungus. These bacteria will be identified and indications for their role will be provided.

Hypothesis

The fungus hosts endobacteria that migrate out of the cell due to nutrient limitation. If these partners can grow independently, this would suggest a facultative or cyclic symbiosis. It is hypothesized that the endobacteria escape from the fungus because the host can't provide sufficient nutrients. An alternative is that the fungus is trying to eject its endobacteria because of the associated costs.

Objective

The objective is to understand why the endobacteria of *Mucor hiemalis* migrate out of the fungus. To provide an answer, following objectives will be pursued.

1. Confirming that *Mucor hiemalis* contain endobacteria migrating out of the fungus under nutrient poor conditions.
2. Isolating, visualizing and identifying endobacteria.
3. Establish whether bacteria affect fungal growth, by comparing the isolated fungus with the cured fungus under different nutrient conditions.

At first it will be tested whether the observation is reproducible to verify if endobacteria move out of four different isolates of *M. hiemalis.* One of these isolates was treated extensively with antibiotics and will be included for comparison. The influence of nutrient conditions will be tested, as well as a comparison among the different isolates.

If these bacteria move out, they will be used for identification by PCR amplification. This will indicate whether the fungus hosts one bacterium or a diverse microbiome. When the endobacteria are moving out, some endobacteria may remain inside the fungus, those bacteria will be identified as well. The fraction of these bacteria remaining in the fungus may be quantified. Next to identification, the endobacteria present inside the hyphae will be visualised by microscopic methods.
An attempt will be made to isolate the endobacteria in pure culture. If the endobacteria can be isolated, they will be plated together with cured *M. hiemalis* to see if the bacteria are able to reinfect the fungus.

Another aim is to cure *M. hiemalis* of its endosymbionts and to compare growth and morphology of wild-type and cured fungus. The secondary metabolite profile will also be compared, since the emission profile can be affected by symbiotic bacteria (Partida-Martinez and Hertweck 2005, Minerdi et al. 2009). This may provide further indications for the role of the endobacteria.

Materials and methods

Fungal strains

4 *Mucor hiemalis* isolates (M0, M10, M21 and M23) were used. All isolates originated from the rhizosphere of *Carex arenaria* (Sand sedge) collected from two sandy dune soil locations in the Netherlands (De Rooij-van der Goes et al. 1995, De Boer et al. 2008). An antibiotic treated isolate (M9) derived of the isolate M0 was used: M0 was plated on King's B agar with antibiotics (rifampicin and kanamycin), transferred to Water Yeast Agar and Oatmeal agar. The spores were collected, washed over glass wool with sterile demiwater and stored at -80°C.

Fungal growth conditions

All isolates were grown on 5 different media ranging from nutrient rich to nutrient poor: 0.5x Potato dextrose agar (PDA), 0.1x Tryptone soy broth agar (TSB), Water-yeast agar +$(NH_4)_2SO_4$ (WAYN), Water-yeast agar (WAY) and Water agar +$(NH_4)_2SO_4$ (WA) (Table 1). The media were adjusted to pH 6.7, except PDA (pH 5.5-6) and autoclaved at 121°C for 20 minutes.

Table 1 Test media

Test Media	Abbreviation	Composition per liter demiwater (grams)
0.5x Potato dextrose agar	PDA	19.5 Potato dextrose agar (Oxoid), 7.5 agar (Merck).
0.1x Tryptone soy broth agar	TSB	20 agar (CMM van Boom), 5 NaCl (Merck), 3 Tryptone soy broth (Oxoid), 1 KH_2PO_4 (Sigma-Aldrich), 0.1 $(NH_4)_2SO_4$. (VWR)
Water agar +$(NH_4)_2SO_4$	WA	20 agar (CMM van Boom), 5 NaCl (Merck), 1 KH_2PO_4 (Sigma-Aldrich), 0.1 $(NH_4)_2SO_4$ (VWR)
Water-yeast agar +$(NH_4)_2SO_4$	WAYN	20 agar (CMM van Boom), 5 NaCl (Merck), 1 KH_2PO_4 (Sigma-Aldrich), 0.1 yeast extract (Brunschwig chemie) 0.1 $(NH_4)_2SO_4$ (VWR).
Water-yeast agar	WAY	20 agar (CMM van Boom), 5 NaCl (Merck), 1 KH_2PO_4 (Sigma-Aldrich), 0.1 yeast extract (Brunschwig chemie)

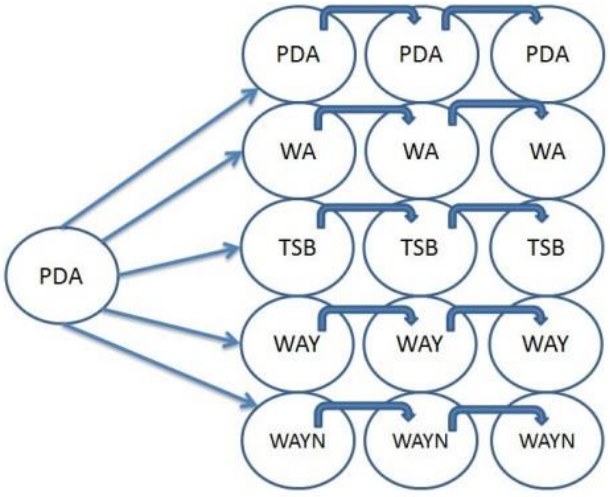


Figure 1 Work schedule

The isolates were grown on PDA, before transferring them to the test media. After 4-5 days, the strains were transferred to new plates according to the work schedule (fig 1.) The fungi were incubated in the dark at 20°C.

Fungal identification

The identity of the isolate MO was confirmed using the ITS sequences targeted by the primers ITS1 and ITS4 (White et al. 1990). Fungal DNA was extracted using the ZR fungal/bacterial miniprep (Zymo research) according to the manufacturer's instructions. Extracted DNA was amplified by PCR using a master mix containing FastStart High Fidelity Reaction Buffer (10x) (Roche) with 18 mM MgCl2 (Roche), FastStart High Fidelity Enzyme Blend (0.04 U) (Roche), DNTP's (200 µM), ITS1 primer and ITS4 primer (both 0.6 µM) and completed to a final volume of 50 µL using MilliQ water. PCR conditions were 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 72°C for 10 minutes using the PCT 200 Peltier

thermal cycler (MJ research). Product quality and size was checked by electrophoreses on a 1.5% agarose gel. PCR product was cleaned using a PCR purification kit (Qiaqen) and sent for Sanger sequencing to Macrogen (Amsterdam). Obtained sequences were checked for quality using BioEdit (Hall 1999) and aligned using the BLAST database (NCBI).

Bacterial visualization

Hyphae were collected from the plate using flame sterilized tweezers and placed on a microscopy slide. Hyphae were flame fixed and 5 µL stain was added containing 2 mg 4',6-diamidino-2 phenylindole (DAPI) (Sigma-Aldrich) per liter sterile demineralized water. The slides were stained in the dark for 15 minutes, after which a drop of sterile antifading solution (1:1 PBS: glycerol with 5% ascorbic acid) was added and visualized using the epifluorescence microscope Zeiss Axio Imager M1 (Carl Zeiss microimaging GmbH).

Staining using the life/dead backlight bacterial viability kit (Invitrogen) was done based on Partida-Martinez and Hertweck (2005). Hyphae were collected on a glass slide, after which 5 µL sterile PBS containing 1 µL of both stains was added. Staining was done as described above.

Bacterial isolation

Mycelium of M0 and M9 was collected in 7 ml sterile phosphate buffer (10 mM KH_2PO_4, pH 6.5) by using flame sterilized tweezers. A phosphate buffer control was included. Mycelium was sheared using a mixture of 0,2 mm, 0,5 mm and 1 mm silicone beads by vortexing maximal speed for 30 minutes. Resulting suspension was allowed to settle and supernatant was collected and shaken for 45 minutes. Suspension was filtrated using pore sizes 10 µM (Millipore) and 3 µM (Schleger and Schuell). 150 µL aliquots were plated on R2A medium (Difco), WAY and 1/10 TSB containing 100 mg/L filter sterilized cycloheximide (Sigma-Aldrich) and 50 mg/L thiabenzadole (Sigma-Aldrich). Three volatile compounds: dimethyldisulfide, dimethyltrisulfide and benzonitrile (Sigma-Aldrich) with known antifungal but no antibacterial activity, were added to a sterile filter paper positioned at the edge of the plate (Garbeva et al. 2014a). Plates with volatiles were kept in a protective cabinet at room temperature (+- 20°C). Obtained colonies were picked and transferred to a fresh R2A plate using a sterile inoculation loop. Picked colonies were identified as described below and stored at -80°C in glycerol.

Bacterial identification and diversity

DNA was extracted as described above from which the V6 region of the 16S rRNA was amplified using the same mastermix as described above, except that primers 968f (with GC clamp for DGGE) and 1378r (Heuer et al. 1997) were used. Mastermix for DGGE contained extra BSA (20 mg/L) and $MgCl_2$ (2.3 mM). PCR conditions were: 94°C for 2 minutes, 35 cycles (40 for DGGE) of 92°C for 30 seconds, 55°C for 1 minute, 68°C for 45 seconds and ended with 5 minutes at 68°C. Product quality and size was checked by electrophoreses on a 1.5% agarose gel.

Denaturing Gradient Gel Electrophoreses (DGGE) (Muyzer et al. 1993) was peformed as described by De Boer et al. (2003). 20 µL PCR product was electrophoresed over a denaturing gradient of 45-65% in a 6% acrylamide gel (100% denaturant was defined as 40% formamide and 7M ureum). Electrophoreses was operated for 15h at 75V in 0.5X TAE buffer at 60°C, resulting banding pattern was visualized by ethidium bromide staining.

PCR product was cleaned using a PCR purification kit (Qiaqen). Cleaned gene products were cloned into *Escherichia coli* DH5α using the pGEM-T(easy) vector kit (Promega) according to the manufacturer's instructions. Per sample, 10 clones were obtained and checked for presence of the

insert by PCR using the pGEM-T specific primers SP6 and T7 (Promega). Amplified inserts were cleaned using a PCR purification kit (Qiaqen) and sent for Sanger sequencing to Macrogen (Amsterdam). Obtained sequences were checked for quality and trimmed in Bioedit sequence alignment editor (version 7.2.5) (Hall 1999). Sequences were aligned by using the ClustalW multiple alignment tool (Larkin et al. 2007) as present in Galaxy (usegalaxy.org). Resulting sequences were identified using the NCBI BLAST nucleotide database. Phylogenetic trees were constructed using the MOLE-BLAST tool (NCBI) and the software called MEGA6 (Tamura et al. 2013).

Fungal biomass

8 mm agar disk containing mycelium were inoculated on plates containing 20 mL PDA or 12 mL WA. Fungi were incubated at 20 °C for 15 days, during which colony extension was measured. The final amount of fungal biomass was determined by ergosterol extraction as described by De Boer et al. (2007). In short, hyphae were collected by filtrating molten agar over a tea strainer. Hyphae were extracted in methanol, from which ergosterol concentration was measured by LC-MS. Differences were assessed by using paired T-tests in the software SPSS IBM 20.

Anastomosis assay

On both WA and PDA medium, agar disks of two isolates were plated together at a difference of +-1.5 cm in different combinations (M0-M0, M0-M9, M9-M9). Morphology was followed during incubation for 30 days, after which the partners were separated on new plates to examine morphology changes.

Volatile trapping and measurement

For the collection of volatiles, special glass petri dishes were used with lids with an exit to which a steel trap with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd, Llantrisant, UK) was fixed (fig. 2) (Garbeva et al. 2014c). Volatiles were collected 3 d.p.i. and 6 d.p.i, traps were removed, capped and stored at 4°C until analysis. Incubations were done in triplicate, including medium controls.

Figure 2 Petri dish with special lid containing the volatile trap. (photo Ruth Schmidt)

Trapped volatiles were desorbed using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., Llantrisant, UK) at 210 °C for 12 min (He flow 50 ml/min) and trapped on a cold trap at -10 °C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA) by heating the cold trap for 3 min to 280 °C. Split ratio was set to 1:20, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, Bellefonte, PA, USA). Temperature program used was as follows: 39 ^{o}C for 2 min, from 39 to 95°C at 3,5 °C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min and finally to 300°C at 40°C/min, hold 20 min. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (30–400 AMU, 4 spectra/s). Compounds were identified by their mass spectra using a combination of deconvolution software AMDIS and Masshunter in combination with NIST 2013 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 7th edition spectral libraries and by their linear retention indexes (lri) in the local NIOO-KNAW database.

Bacterial quantification by qPCR

16S rRNA gene counts were obtained using quantitative PCR (qPCR). DNA was extracted in triplicate as described above. All samples were diluted to 1 ng DNA / µL. Dilution series for the standard curve were prepared from 16S rRNA gene inserts in the Plasmids were extracted from overnight E. coli cultures using the Qiaprep spin miniprep kit (Qiaqen) according to the manufacturer's instructions.Extracted DNA quality and concentration was determined using nanodrop 2000 spectrophotometer (Thermo scientific).

For the qPCR reactions, 5 µL DNA template was added to a mastermix containing 10 µL 2x SensiFAST™ SYBR® No-ROX Kit (Bioline GmbH), BSA (0,25 mg/µL), primers Eub338 and Eub518 (0,5 µM each) (Lane 1991) and completed to a volume of 20 µL with MilliQ water. Reactions were done in triplicate. PCR conditions in the Rotor gene Q (Qiaqen, Venlo, the Netherlands) were: 95°C for 5 minutes, 35 cycles of 95°C for 15 seconds, 53°C for 10 sec, 72°C for 25 sec and 80°C for 15 seconds. Green fluorescence was measured at both the 72°C and 82°C step. PCR product quality was checked by melting curve analyses (53°C to 95°C, rising 1°C every 5 seconds) and electrophoreses over a 1.5% agarose gel.

Results

Fungal identification

The identity of the fungus was confirmed to be *Mucor hiemalis* based on its ITS sequences. The 600

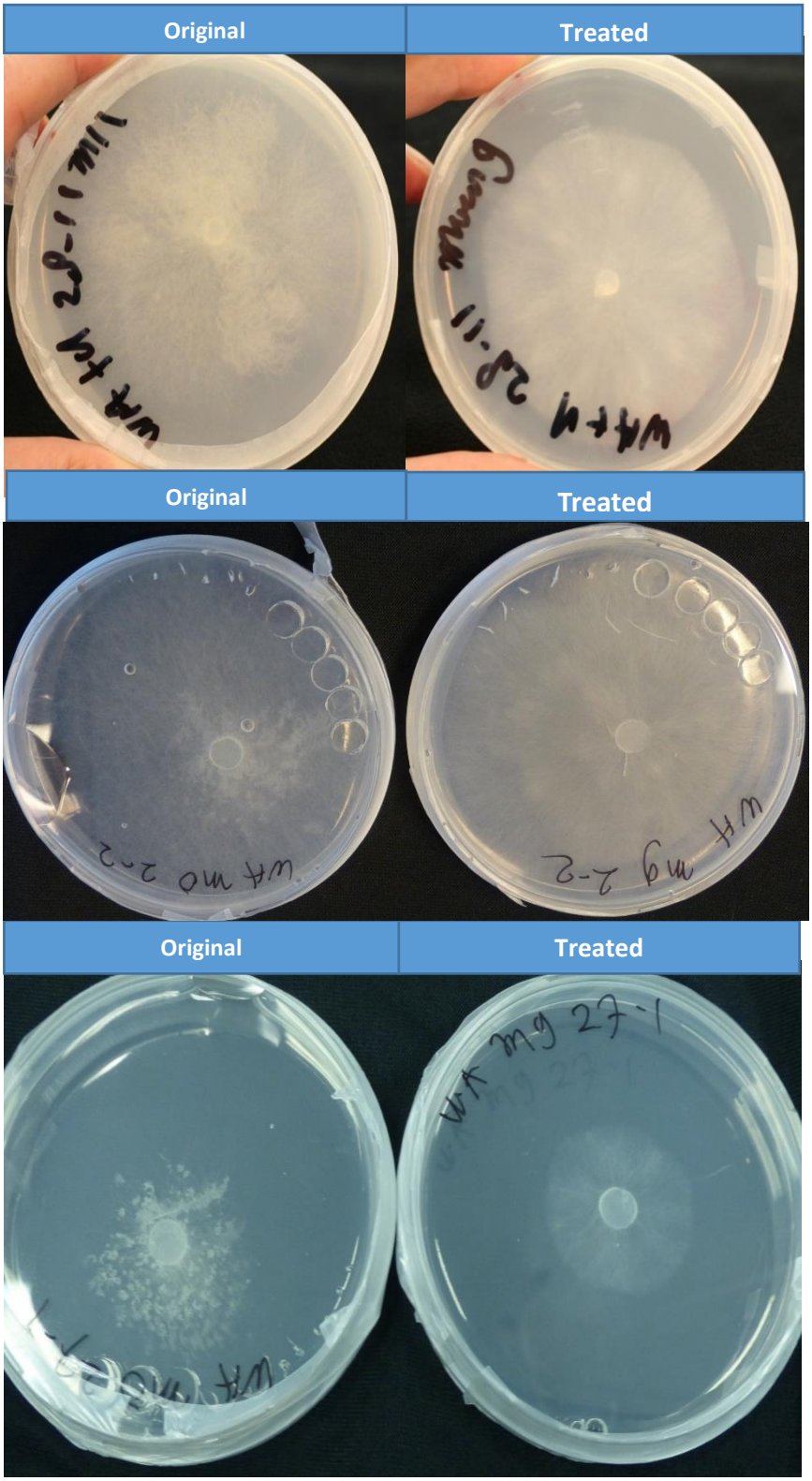
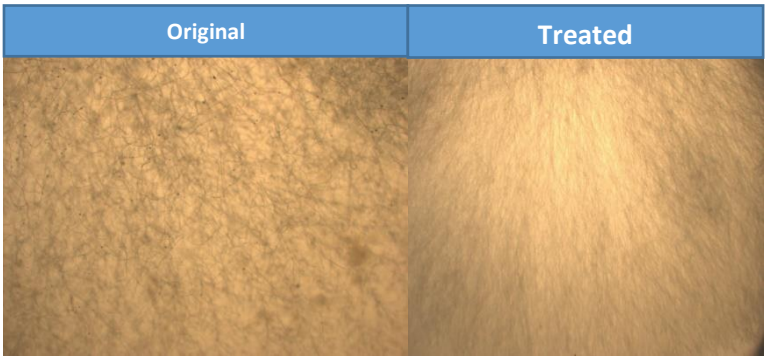


Figure 3 Morphological differences observed between the original isolate M0 (left) and the antibiotic treated isolate (right).

bp fragment revealed 100% similarity to *M. hiemalis* ITS region based on BlastN search.

Morphology

Re-plating the isolates M0 and M9 on different media ranging from nutrient poor to nutrient rich showed striking morphological differences between the original and the antibiotic treated isolates. In the poorest nutrient condition (WA) (fig. 3) the non-treated isolates M0 grew in irregular colonies with thin hyphae reaching across the whole plate (fig. 3 left). In contrast, the antibiotic treated isolate M9 grew in circular closed colonies that did not cover the whole plate (fig. 3 right). Morphological difference was also seen under the binocular (fig. 4). Interestingly, after re-plating the original isolate for several times on a nutrient-poor water agar a slime layer appeared around the hyphae, while it did not appear around the hyphae of the treated isolate (fig. 3).



Under nutrient rich conditions (PDA medium), differences in colony diameter and pigmentation were

Figure 4 Morphological differences observed under the binocular between the

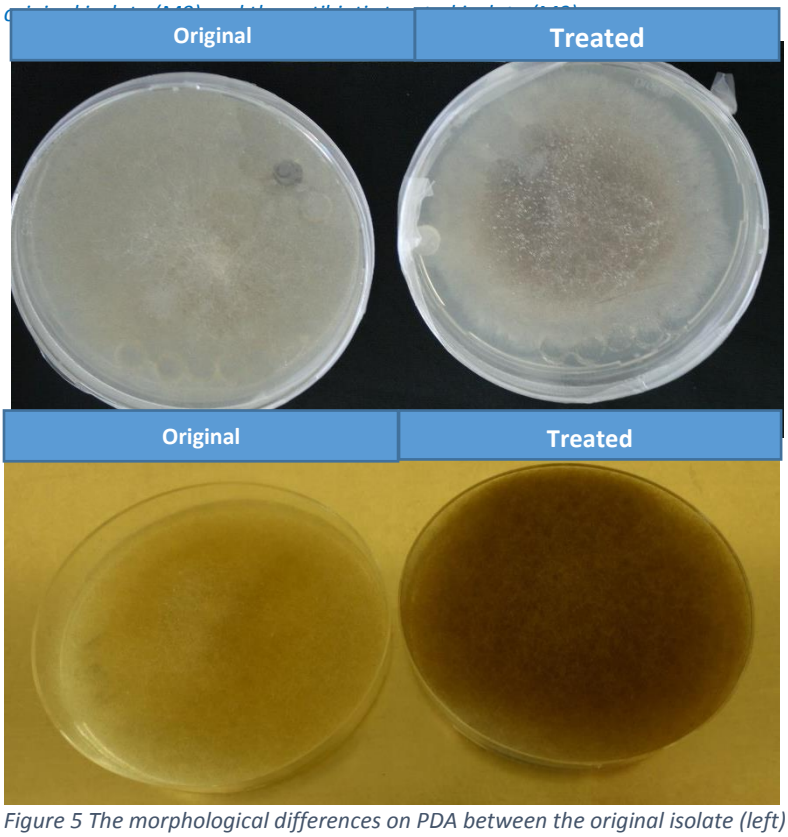
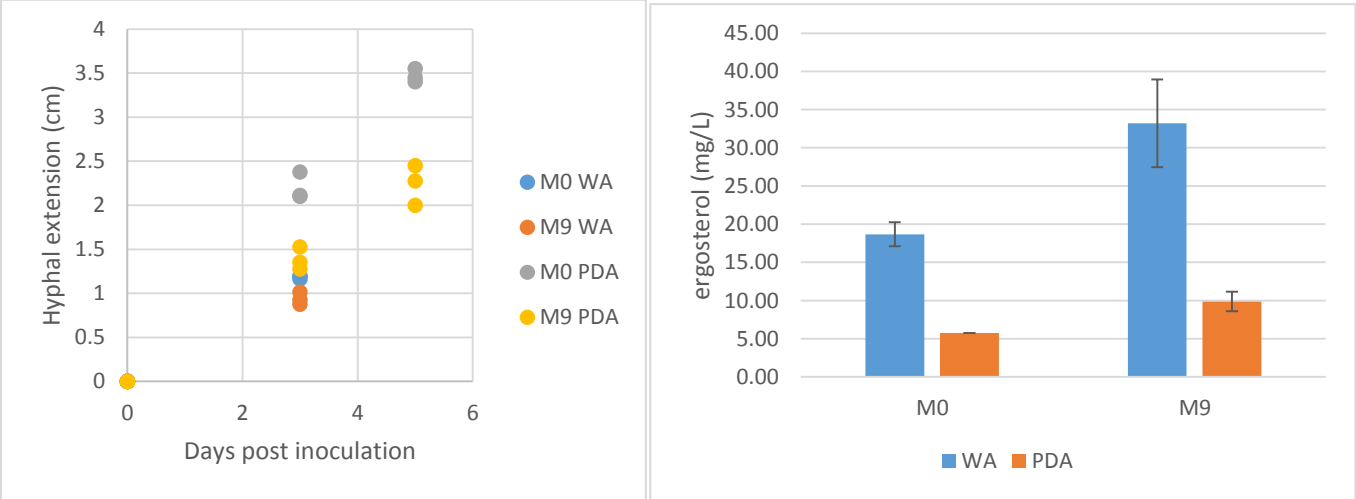


Figure 5 The morphological differences on PDA between the original isolate (left) and the cleaned isolate (right). Pictures were taken after 25 days (above) and after 40 days (below)

observed (fig 5).

Fungal biomass

Comparisons of the growth of the original and the treated isolate suggested differences in biomass between the isolates. Following the fungal growth confirmed that the colonies formed by the original M0 isolate grew faster than the antibiotic treated M9 isolates (fig. 6). This was the case for day 3 and day 5 on PDA (t-test, p=0,012 and p=0.010 respectively) and for day 3 on WA (p=0.038). Fungal biomass was measured by ergosterol extraction after 14 days, (fig. 6). On both media, the isolates did



not show significant differences in biomass (t-test, WA p=0.063 and PDA p=0,087). The original colony expanded faster over the plate compared to the treated colony, however without significant

Figure 6 Colony extension of the original isolate (M0) and the treated isolate (M9) on both nutrient poor (WA) and nutrient rich (PDA) conditions (left). The differences in biomass as measured by ergosterol extraction (right).

difference in final biomass.

Bacterial identification

To confirm the presence of bacteria, DNA was isolated and the 16S rRNA gene was amplified (fig. 7). PCR products were obtained from all *M. hiemalis* cultures including the treated isolate M9. A DGGE was performed on the 16S PCR products which resulted in a different weak banding pattern for the soil isolates compared to the antibiotic treated isolate (appendix). Identification of the associated bacteria was achieved by cloning and sequencing the 16S rRNA genes. Based on blast search at NCBI database bacteria were identified and the closest hit is presented in table 2. Results reveal distinct communities in both isolates (fig. 8). Clone library confirmed the presence of a different bacterial community in the original M0 isolate and the treated M9 isolate. Interestingly, more species were detected in the treated fungus compared to the original isolate indicating a selective influence of the antibiotic treatment.

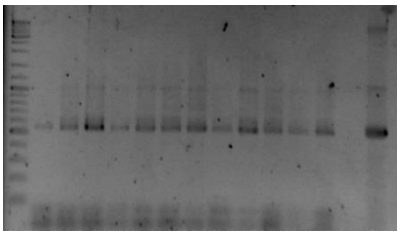


Figure 7 Agarose gel picture of PCR reaction targeting the 16S rRNA gene (primers 27F-1492R).

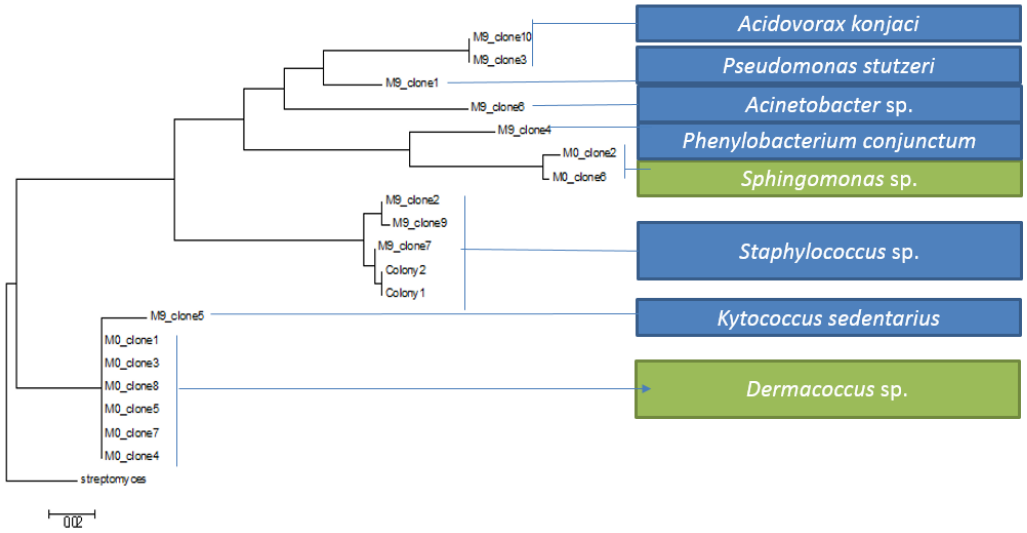


Figure 8 Maximum likelihood tree of the clone library with streptomyces coelicolor A3(2) (gene ID: 1098770) as outgroup and clones from the original isolate (green) vs clones of the treated isolate (blue)

Table 2 Identities of the bacteria associated with the original (M0) and treated isolate (M9). The identities are best hits in the NCBI blast datasbase of the V6 region of the 16S rRNA gene with identities ≥99%.

Original	Treated
Dermacoccus sp. 76H2a 16S	*Pseudomonas* sp. C2-16 16S
Dermacoccus nishinomiyaensis strain BCX-20 16S	*Acidovorax konjaci* strain ICMP 7733 16S
Sphingomonas koreensis strain NBRC 16723 16S	*Acinetobacter bouvetii* strain DSM 14964 16S
Sphingomonas dokdonensis strain DS-4 16S	*Phenylobacterium conjunctum* strain FWC 21 16S
	Kytococcus sedentarius strain DSM 20547
	Staphylococcus cohnii strain GH 137 16S
	Staphylococcus aureus subsp. aureus partial 16S

Bacterial visualization

Bacteria were observed using epifluorescence microscopy. Both DAPI staining and SYTO9 from the life/dead backlight bacterial viability kit revealed the presence of bacteria in the fungal cultures (fig. 9). No differences were observed between the M0 and M9 isolates.

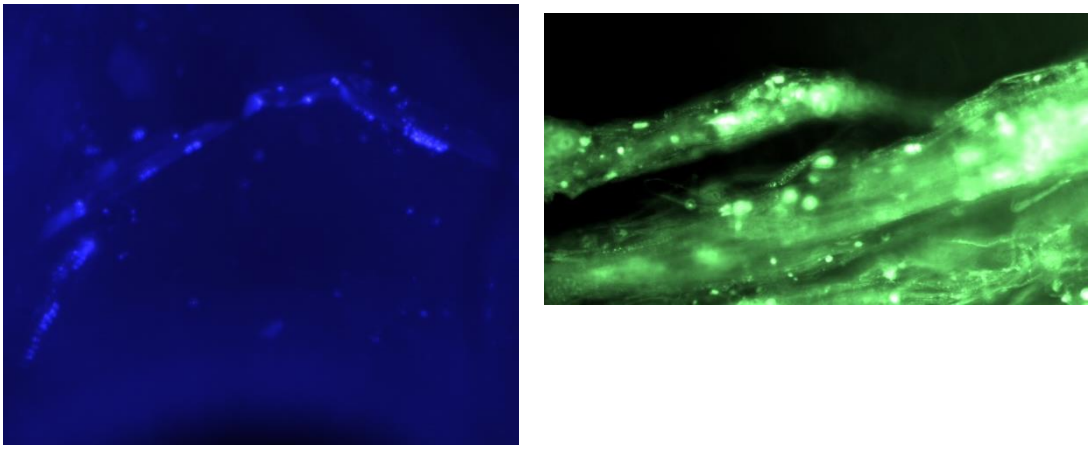


Figure 9 Microscopic view on hyphal fragments revealing bacteria (400x). Dapi staining (left) and SYTO9 (right)

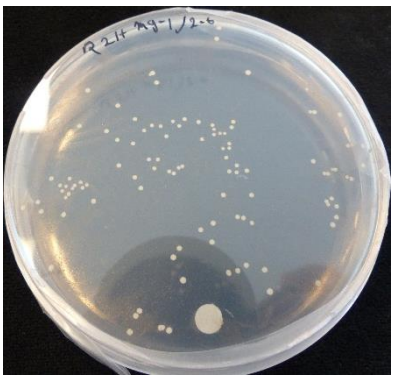


Figure 10 Bacterial colonies appearing on R2A medium incoculated with the antibiotic treated isolate

Bacterial isolation

A new protocol was developed for isolation of bacteria associated to *Mucor hiemalis*. The fungus did not respond to the convential antifungals cycloheximide and thiabenzadole. Therefore, three antifungal volatile organic compounds (benzonitrile, dimethyldisulfide and dimethyltrisulfide) were added. This combination successfully suppressed the fungus. Isolation yielded bacterial smear on R2A and TSB for the original isolate and bacterial colonies for the antibiotic treated isolate after two days (fig. 10).

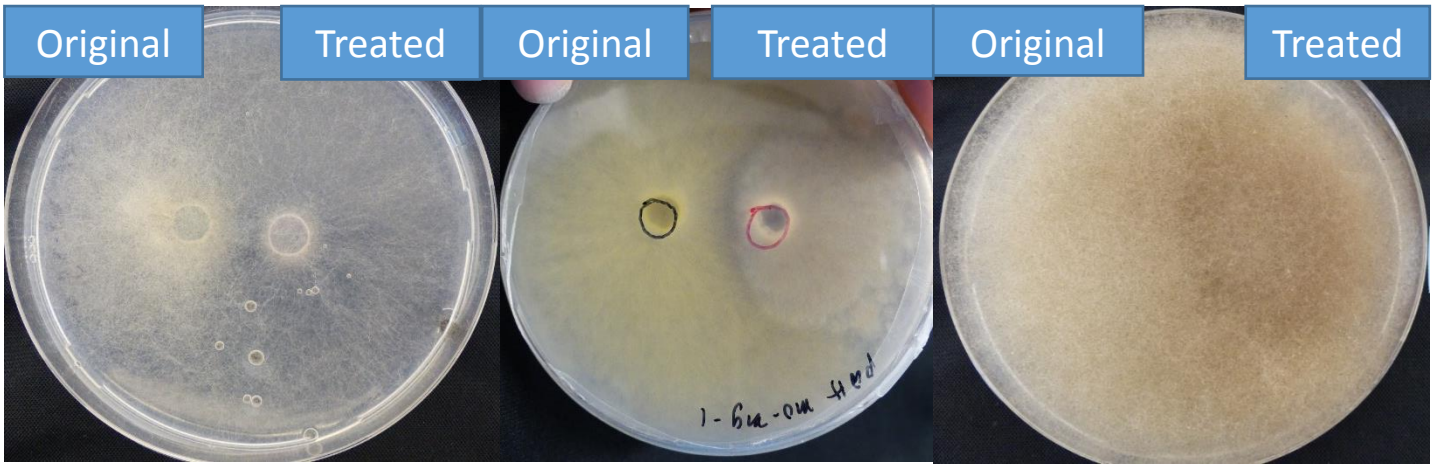
The identity of the previously appearing colonies, as determined by the 16S rRNA sequence, were *Staphylococcus epidermidis, Staphylococcus warneri* and *Kocuria sp.*. These identities were not observed in the clone library. More isolates are currently in preparation for sequencing.

Bacterial quantification by qPCR

Bacterial quantification by using qPCR yielded no significant differences between the isolates or nutrient conditions (not shown). The primers used for qPCR were co-amplifying fungal DNA and hence cannot be used for quantification.

Anastamosis assay

Plating the M0 and M9 isolates together showed again the morphological differences described above (fig. 11). No phenotype transfer has been observed under nutrient poor conditions, the



morphological differences did not merge. Under nutrient rich conditions expansion of the pigmentation phenotype was observed, however with time the differences described above slowly

Figure 11 Pictures of the original and treated isolate plated together on WA after 27 days (left) and PDA after 26 (middle) and 27 days (right).

started to disappear.

VOCs profiles

Volatile profiles of the M0 and M9 isolates were significantly different (fig. 12). Both profiles were

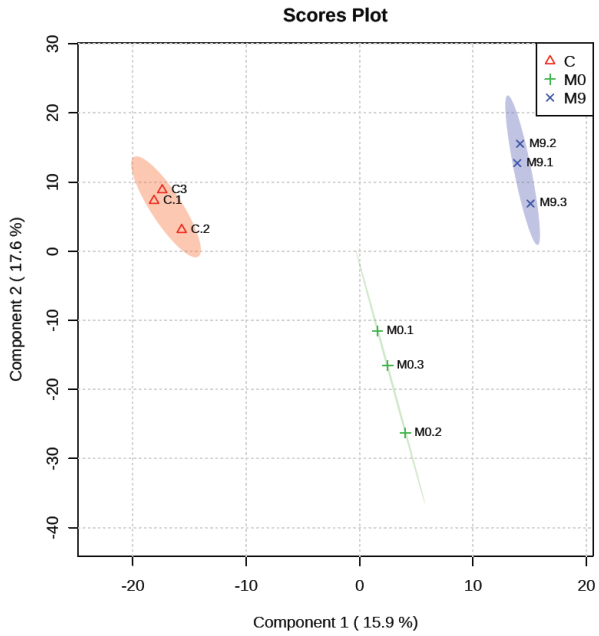


Figure 10: Scores plot between the selected PCs. The explained variances are shown in brackets.

Figure 12 PCA plot of the volatile profiles produced by the original isolate (M0), treated isolate (M9) and medium controls (C)

also significantly different from the medium controls.

Discussion

Soil is a highly competitive habitat where fungi and bacteria both compete and cooperate to sustain themselves (De Boer et al. 2005, Kobayashi and Crouch 2009). Recent studies have shown that cooperation via endosymbiosis is widespread in fungi (Hoffman and Arnold 2010, Stopnisek et al. 2015), while the ability to adhere to fungal hyphae is a widespread trait among bacteria (Scheublin et al. 2010, Rudnick et al. 2015). These symbiotic bacteria have been shown to affect pathogenesis (Partida-Martinez and Hertweck 2005, Minerdi et al. 2008), mycorrhization (Bonfante and Anca 2009) and primary or secondary metabolism (Schroeckh et al. 2009, Benoit et al. 2014, Stopnisek et al. 2015). Many of these interactions occur via volatile organic compounds (VOCs) as inhibitory, signaling or growth promoting agents (Effmert et al. 2012, Schmidt et al. 2015). Transcriptomic analysis revealed that *Laccaria bicolor* can distinguish between a helper, neutral or antagonistic bacterium and respond accordingly (Deveau et al. 2015).

The aim of this project was demonstrating the migration of endobacteria out of the fungal host under nutrient limiting conditions. Bacterial migration was hypothesized because a slime layer appeared around the hyphae of the saprotrophic mucormycotina *Mucor hiemalis*. Two *M. hiemalis* isolates were compared: one original isolate (M0) and one antibiotic treated isolate (M9). Both isolates were associated with a diverse bacterial community with distinct composition for M0 and M9. The antibiotic treatment caused associated community shift affected morphology, pigmentation, hyphal extension and volatile emission profile.

Bacterial consortium

The *M. hiemalis* isolates M0 and M9 were associated with a diverse bacterial consortium. The consortium differed between the isolates based on an antibiotic and washing treatment. Clone library identified more bacterial taxa in the treated isolate compared to the original isolate, with dominance of *Dermacoccus* sp and *Sphingomonas* sp. The identified genera *Sphingomonas* (Boersma et al. 2009, Hoffman and Arnold 2010)*, Pseudomonas* (De Boer et al. 2005, Frey-Klett et al. 2011)*, Acidovorax* (Giordano et al. 2013) and *Acinetobacter* (Hoffman and Arnold 2010) are described to be associated with fungi. Staphylococci have been shown to interact with *Candida albicans* in medical systems (Peleg et al. 2010), which is not a representative environment for soils.

Both *Pseudomonas* and *Sphingomonas* adhere to the same isolate of *M. hiemalis* out of a collection of rhizosphere bacteria from *Carex arenaria* (Rudnick 2015). Despite the same origin of M0 and tested bacteria, the other genera in the clone library were not found to adhere to fungi by Rudnick (2015). For their study, the same original isolate was used and plated with oxytetracicline and streptomycin and found to be free of bacteria by PCR. This difference may exemplify difficulties in detecting endobacteria in fungi. For the other genera (*Dermacoccus, Kytococcus* and *Phenylobacterium*) this may be the first report of a close relationship with fungi.

It is most likely that the bacterial taxa found in the treated isolate are also present in the original isolate, but were not sampled in the relatively small clone library. These bacteria could have survived the antibiotic treatment by resistance to the applied antibiotics or protection by the host. Host protection may depend on the bacteria occurring inside or outside the cell, but bacterial location remains to be confirmed. Also, a new trial of cleaning the fungus of bacteria did not result in a bacteria free isolate similarly to the earlier treatment. The antibiotic treatment may have affected bacterial quantity, but no differences in bacterial quantity were observed. However, the bacteria remain to be quantified because the qPCR primers were not specific and also amplifyed eukaryotic fungal DNA.

Bacterial colonies have been successfully isolated from M0 and M9 using three antifungal volatiles: benzonitrile, dimethuldisulfide and dimethyltrisulfide. The sensitivity of fungi to volatiles compared with resistance or even positive effects for bacteria made the used volatiles ideal agents for isolation of fungal associated bacteria (Garbeva et al. 2014a, Schmidt et al. 2015). The use of volatiles is therefore a promising new method for the isolation of fungal associated bacteria. The isolation of bacteria confirms their presence in M0 and M9, however their identities remain to be determined.

Role of the bacteria for the fungus

The title of this thesis contains the question whether these bacteria are friends or foes for the fungus. As noted earlier bacteria can be mutualists, pathogens, predators and competitors of fungi (De Boer et al. 2005, Kobayashi and Crouch 2009). The original isolate grew faster compared to the antibiotic treated isolate under both nutrient rich and nutrient poor conditions. Final biomass was higher for treated isolate M9, but not significant. The original isolates invested more in hyphal extension, while the treated isolate invested more in hyphal density. Benefits and costs associated with these different morphologies would need further research. Faster growth of M0 would suggest a positive effect of the bacteria lost during the treatment. Bacteria inducing different morphologies has also been observed on *Gigaspora margarita* (Lumini et al. 2007) and on the member of the mucorales *Rhizopus microsporus* (Partida-Martinez et al. 2007b).

Bacteria have been shown to affect secondary metabolism of fungi (Schroeckh et al. 2009, Benoit et al. 2015) including volatiles (Minerdi et al. 2009). Here, M0 and the antibiotic treated M9 were shown to have a different volatile emission profile. The role of the differently produced volatiles remains to be elucidated.

Further indications for the effect of the bacteria on the fungus could be acquired by a transcriptomic (Deveau et al. 2015) or proteomic (Moretti et al. 2010) approach to get more insight in the fungal response towards associated bacteria. However, for these approaches the genome of the fungus and bacteria needs to be known.

Bacterial migration

An attempt was made to transfer the bacterial consortia via anastomosis and migration to provide indications for their role. It was expected that endobacteria would transfer via anastomosis throughout the coenocytic hyphae, whereas ectobacteria may be transferred by exploiting the hyphae for dispersal (Warmink et al. 2011). Plating the isolates M0 and M9 together did not result in clear effects of bacterial transfer, however under nutrient rich conditions pigmentation differences started to disappear with time.

Similarly, the slime layer initially proposed to consist of endobacteria leaving the fungus did not transfer by plating the isolates together. This slime appeared around the hyphae after each of 3-5 times re-plating on M0 but not on the antibiotic treated M9. Previous reports revealed descriptions of *Mucorales* as producers of extracellular polysaccharides (De Ruiter et al. 1992), which may explain the presence of the slime layer. These results suggest that the slime layer produced is most probably a morphological feature of the fungus affected by bacteria, instead of endobacteria leaving the fungus.

Further research

Follow up studies may aim to localize the bacteria. Species specific probes may be used to see if the fungus indeed hosts endobacteria testing the hypothesis that the ectobacteria were most sensitive

to the treatment. Another major remaining question is the amount of bacteria in both isolates, which may be achieved by a different bacterial housekeeping gene than 16S rRNA. Another major question which needs confirmation is the role of the bacteria for the fungus. The data presented in this thesis did not enable assigning a clear positive or negative fitness effect for the fungus. Molecular approaches may reveal a change in stress factors associated with the presence of bacteria as done by Deveau et al. (2015) and Stopnisek et al. (2015), providing indications for the role of the bacteria. Volatile production profile was shown to differ between the isolates, it will be interesting to elucidate the role of the differently produced volatiles. At this point it is not possible to indicate if the volatiles are of fungal or bacterial origin.

This study is focused only on the mucormycotina *Mucor hiemalis*. It may be interesting to see if more fungal culture collections have bacterial symbionts and their influence on the fungus. This may occur more often considering the widespread presence of endosymbiotic bacteria in fungi (Hoffman and Arnold 2010) and the widespread ability of fungal adherence among bacteria (Scheublin et al. 2010, Rudnick et al. 2015). Intimate bacterial-fungal interactions are common and can change the fungal behavior. The ecological relevance of fungal pure cultures may therefore be questioned.

Bacteria adhere to fungal hyphae for migration across soil pores allowing the colonization of different environments (Warmink et al. 2011). In this thesis, it was shown that bacteria present in the original isolate M0 increased fungal extension but not the final biomass. This may be the first indication of bacteria actively encouraging fungi to grow to increase migration distance. However, this hypothesis will need to be confirmed. A second positive effect for the bacteria may be protection from stressor suggested by bacterial survival of the treatment.

Fungi are major consumers of root exudates in the rhizosphere, while bacteria are proposed to obtain that energy from the fungus (Hannula et al. 2012, Rudnick 2015). It may therefore be an advantage for bacteria to grow adhering to or inside fungi. If more bacteria are able to change the behavior of the fungus as shown in this thesis or in the examples given, it may have a large influence on soil biological functioning for instance plant growth promotion, disease promotion or suppression and biogeochemical cycles (Frey-Klett et al. 2011, Mendes et al. 2013).

The advantages for bacteria are evident, but the advantages for fungi remain to be determined. Recently, it was noted that the microbiome functioning of animals and plants shows similarities (Mendes and Raaijmakers 2015), which may even be extended to fungi. The high abundance of intimate fungal-bacterial interactions may be surprising considering the low amount of diseases observed in fungi (Kobayashi and Crouch 2009), especially when considering the easy invasion of fungi (Moebius et al. 2014).

Conclusion

Both an original and an antibiotic treated isolate of the mucormycotina *Mucor hiemalis* was shown to be associated with a diverse bacterial consortium. The community of this consortium differed between the original isolate M0 and the antibiotic treated M9. The treatment and change in community affected morphology, pigmentation, hyphal extension and volatile emission profile. These bacteria have been identified and some of these bacteria have been isolated by using antifungal volatiles. The original fungal isolate M0 grew faster than the antibiotic treated M9, but final biomass was not significantly different reflecting the differences in morphology. The differences between M0 and M9 are evident, but the fitness effects of these remain to be determined. Indications may be profided by elucidating the exact role of these bacteria for the fungus for instance via molecular methods. Positive effects for bacteria are more evident. Fungi can be an important food source for bacteria and may therefore benefit by intimate interactions. Fungi may also relieve

stressors for bacteria. Finally, bacteria may benefit by increasing their migration capacity via promoting hyphal extension.

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