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# Fungus-associated bacteriome in charge of their host behaviour

**Running tittle:** Fungal behaviour affected by its bacteriome

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**Abbreviations:** ARE (Artificial Root Exudates), CFU (Colony Forming Units), MA (antibiotic-treated *M. hiemalis* isolate), M0 (original *Mucor hiermalis* isolate), OTU (Operational Taxonomic Unit), PDA (Potato Dextrose Agar), TSB (Tryptic Soya Broth), VOCs (Volatile Organic Compounds), WA (Water Agar), WYA (Water Yeast Agar)

## Abstract

Bacterial-fungal interactions are widespread in nature and there is a growing number of studies reporting distinct fungus-associated bacteria. However, little is known so far about how shifts in the fungus-associated bacteriome will affect the fungal host's lifestyle. In the present study, we describe for the first time the bacterial community associated with the saprotrophic fungus *Mucor hiemalis,* commonly found in soil and rhizosphere. Two broad-spectrum antibiotics that strongly altered the bacterial community associated with the fungus were applied. Our results revealed that the antibiotic treatment did not significantly reduce the amount of bacteria associated to the fungus but rather changed the community composition by shifting from initially dominating *Alpha-Proteobacteria* to dominance of *Gamma-Proteobacteria*. A novel approach was applied for the isolation of fungal-associated bacteria which also revealed differences between bacterial isolates obtained from the original and the antibiotic-treated *M. hiemalis*. The shift in the composition of the fungal-associated bacterial community led to significantly reduced fungal growth, changes in fungal morphology, behavior and secondary-metabolites production. Furthermore, our results showed that the antibiotic-treated isolate was more attractive and susceptible to mycophagous bacteria as compared to the original isolate. Overall, our study highlights the importance of the fungus-associated bacteriome for the host's lifestyle and interactions and indicate that isolation with antibacterials is not sufficient to eradicate the associated bacteria.

## 1. Introduction

Bacteria and fungi are widespread in nature and play important roles in many ecological processes. Similar to other organisms, many fungi have an associated bacteriome and there is a growing number of known endosymbionts where bacteria dwell within fungal hyphae (Bonfante and Anca, 2009; Kobayashi and Crouch, 2009). Another type of fungal-bacterial interaction is ectosymbiosis, where the bacterial partner is adhered to fungal hyphae (Stopnisek et al., 2016; Warmink et al., 2009).

The symbioses with ecto- and endofungal bacteria are often overlooked, yet they may have a profound effect on the fungus behavior and lifestyle. For example, in *Aspergillus nidulans* and *A. niger,* the ectobacteria actinomycetes and *Bacillus subtillus* respectively, were shown to affect fungal primary and secondary metabolism (Benoit et al., 2015; Schroeckh et al., 2009).

It is plausible that there is a relationship between fungi and bacteria of which both partner profit. For example, bacteria or fungi can benefit from specific compounds that are produced by the other partner if they cannot produce it themselves. Several mycorrhizal helper bacteria secrete citric and malic acids that are metabolized by *Laccaria bicolor*, promoting its growth (Duponnois and Garbaye, 1990). Conversely, ectomycorrhizal fungi may produce organic acids or sugars that can affect the composition and growth of associated bacterial communities. For example, the helper bacterial isolate *P. fluorescens* BBc6R8 can be chemoattracted by the hyphae of the ectomycorrhizal fungus *L. bicolor* S238N and the trehalose accumulated within the mycelium that promots the growth of the helper bacterium (Deveau et al., 2010). In the interaction between *S. cerevisiae* and several *Acinetobacter* species, ethanol secreted by the yeast was shown to stimulate the growth of the bacterial species and it can act as a signaling molecule, altering cell physiology (Smith et al., 2004).

Bacterial endosymbionts *Candidatus Glomeribacter gigasporarum* of arbuscular mycorrhizal fungi *Gigaspora margarita* can be involved in the vitamin B12 provision for the fungus (Ghignone et al., 2012). The co-adaptation between fungal hosts and bacterial endosymbionts

can become so tight that the genome size and the gene content of the endosymbionts is reduced. Mycorrhizal endosymbionts from Mollicutes lineages were suggested to depend metabolically on their host, and additionally have taken up regulatory eukaryotic genes horizontally (Naito et al., 2015). Clear evidence for gene transfer between Mollicute-related endobacteria and their mycorrihizal host *Dentiscutata heterogamma* was recently reported (Torres-Cortes et al., 2015). Furthermore, endobacteria can improve the fitness of their host by e.g. increasing the fungal sporulation success and raising the fungal bioenergetics capacity (Salvioli et al., 2016). Another well-studied example is the rice seedling blight pathogen *Rhizopus microsporus* (Lackner et al., 2009; Partida-Martinez and Hertweck, 2005). 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 (Gee et al., 2011; Partida-Martinez and Hertweck, 2005). Interestingly, the endobacteria enforce their vertical transmission by controlling host sporulation making use of a hrp type III secretion system (Lackner et al., 2011; Partida-Martinez et al., 2007b). 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).

Microscopic and molecular analysis showed that several nitrous oxide-producing fungal isolates of *Mortierella elongata* harboured endobacteria in their mycelia (Sato et al., 2010). The sequencing of 16S rRNA genes revealed that the $N_2O$-producing fungus *Mortierella elongate* harbored endobacteria belonging to the family *Burkholderiacea,* however, the significance of this fungal-bacterial association is unknown (Sato et al., 2010). Recently, an endophyphal bacterium living in association with *Mortierella elongate* was isolated and on the basis of phenotypic, chemotaxonomic and pylogenetic characteristics it was identified as a novel genus and species, for which the name *Mycoavidus cysteinexigens* gen. nov., sp., nov was proposed (Ohshima et al., 2016).

It is a common practice prior performing experiments with soil-borne fungi to pre-culture them on a media supplemented with antibiotics (Ballhausen et al., 2016; Singh et al., 2015). In our lab working with the saprotrophic fungus *Mucor hiemalis* (Zygomycota) isolated from the rhizosphere of *Carex arenaria* (sand sedge) (De Rooij-van der Goes et al., 1995), we observed that treatment with broad-spectrum antibiotics strongly altered the fungal morphology and hyphal extension. Based on this observation, we aimed first to determine the bacterial community associated with *Mucor hiemalis* and to test if the antibiotic treatment resulted in bacteria-free fungus. Furthermore, we aimed to reveal how changes in the bacterial community affect fungal fitness, behavior, metabolites production and interactions.

## 2. Material and Methods

### 2.1 Fungal strains and growth conditions

The *Mucor hiemalis* isolate M0 obtained from the rhizosphere of *Carex arenaria* (Sand sedge) collected from sandy dune soil in the Netherlands was originally isolated on malt extract agar supplemented with 50 ppm oxytetracycline (De Rooij-van der Goes et al., 1995). Here, the antibiotic-treated isolate MA derived from the isolate M0, after plating on King's B agar with antibiotics (rifampicin and kanamycin 50 mg/ml, final concentration), and transferred to Water Yeast Agar (WYA) (Garbeva et al., 2011) and Oatmeal agar OA (24 g/L Difco, France) supplemented with rifampicin and kanamycin (50 mg/ml final concentration). The spores were washed in rifampicin and kanamycin solution (50 mg/ml final concentration) and collected over glass wool with sterile deionized water and stored at -80°C. Spores of the M0 and MA isolates were grown on nutrient-rich 0.5 strength Potato dextrose agar (PDA, Oxoid, England; pH 6), and nutrient-poor Water agar + $(NH_4)_2SO_4$, pH 6.7 (Garbeva et al., 2011).

### 2.2 Fungal identification

The identity of the isolate M0 and MA 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 DNA MiniPrep (Zymo Research, USA) according to the manufacturer's instructions. Extracted DNA was used for PCR amplification with a master mix containing 1x FastStart High Fidelity Reaction Buffer (Roche) with 18 mM $MgCl_2$ (Roche), 0.04 U FastStart High Fidelity Enzyme Blend (Roche), 200 µM of each dNTP, 0.6 µM ITS1 and ITS4 primer. The thermal protocol was as followes: initial denaturation at 95°C for 5 minutes, and 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and a final elongation at 72°C for 10 minutes. The PCR product was cleaned using a PCR purification kit (QIAGEN Benelux B.V., The Netherlands) and sent for Sanger sequencing to Macrogen Europe (Amsterdam,

The Netherlands). Obtained sequences were checked for quality using BioEdit (Hall, 1999) and identified using NCBI nucleotide database BLASTn (Altschul et al., 1990).

### 2.3 Hyphal extension of fungal isolates M0 and MA

For measuring hyphal extension, 6-mm-diameter agar disks taken from the edge of the fungal hyphae of M0 or MA (pre-grown on 0.5 strength PDA) were plated in the middle of nutrient-rich 0.5 strength PDA or nutrient-poor WA plates (8.5 cm diameter). Per fungal isolate and agar type (i.e. PDA and WA) three plates were set-up. The plates were sealed with parafilm and incubated at 20 °C for 5 days. On the fifth day, for each plate the extension of fungal hyphae was measured with a ruler in three coordinates and the average extension was calculates.

### 2.4 Competition assay of fungal isolates M0 and MA

Two plugs taken from the edge of the (on 0.5 strength PDA) pre-grown fungal hyphae of M0 or MA were placed with 3 cm distance from each other on a new 0.5 strength PDA plate (8.5 cm diameter) and incubated at 20°C. As control, both fungal isolates were incubated separately on 0.5 strength PDA at the same time. For each treatment three replicates were set-up. The hyphal extension was monitored at day 7, 14 and 21 after inoculation. Pictures were taken with a Panasonic DMC-FZ200 digital camera. The area of M0 and MA hyphal extension was measured with AxioVision V 4.9.1.0 (Carl Zeiss Microscopy GmbH, Germany). A relative area for the hyphal extension per replicate was calculated by dividing the area obtained in the competition experiment per fungal isolate by the average area obtained for each control.

### 2.5 Bioassay for testing the fungal growth inhibition by bacterial secondary metabolites

158 The bacterial strains *Burkholderia sp.* AD024 (De Ridder-Duine et al., 2005, Schulz-Bohm et
159 al., 2015) and *Collimonas fungivorans* Ter 331 (De Boer et al., 2004) were incubated overnight
160 at 22°C in 0.1 Tryptic Soya Broth (TSB) (Tyc et al., 2014), washed with phosphate buffer
161 (10mM $KH_2PO_4$, pH 6.5), and the $OD_{600}$ was adjusted to $10^8$ cells per ml. 50 µl of the bacterial
162 suspension was spread horizontally in the middle of a Petri-dish containing 1.5 % (v/v) water
163 agar (Schmidt et al., 2015) supplied with artificial root exudates (WA + ARE) as described
164 previously by Schulz-Bohm et al. (2015). Per liter WA, 18 ml ARE stock solution was added,
165 implying that 134 µg carbon per ml agar was additionally supplied. Bacteria were incubated
166 for three days at 20°C and a plug of 8 mm diameter with fungal hyphae (pre-grown on 0.5
167 strength PDA) was transferred to the top of the Petri-dish. Agar plates were wrapped with two
168 layer of parafilm and incubated at 20°C. After four days, the distances between the edge of
169 the fungal hyphae and bacterial biofilm (refer to as inhibition zone) were measured. Pictures
170 of agar plates were taken with a Panasonic DMC-FZ200 digital camera. Pictures of fungal
171 hyphae were taken and processed with a Olympus SZX12-ILLK200 stereo microscope
172 (Olympus Cooperations, Japan) in combination with AxioVision V 4.9.1.0 (Carl Zeiss
173 Microscopy GmbH, Germany).

174

175 **2.6 Bacterial and yeast isolation and identification**

176 Mycelium of M0 and MA (pre-grown on 0.5 strenght PDA) was collected in 7 ml sterile
177 phosphate buffer (10 mM $KH_2PO_4$, pH 6.5). The mycelium was sheared using a MICRA D-9
178 homogenizer (MICCRA GmbH, Germany) for 5 minutes. The resulting suspension was
179 shaken for 45 minutes at 22°C and filtrated using membrane pore sizes of 10µM (Millipore,
180 The Netherlands) as well as 3µM (Schleicher and Schuell, Germany). 350 µL aliquots of the
181 filtrate were plated on R2A medium (Difco, France), WYA and 0.1 TSB (Tyc et al., 2014)
182 containing 100 mg/L filter sterilized cycloheximide (Sigma-Aldrich, The Netherlands) and 50
183 mg/L thiabendazole (Sigma-Aldrich, The Netherlands) to inhibit the growth of M0 and MA.
184 Three volatile compounds: dimethyldisulfide, dimethyltrisulfide and benzonitrile (Sigma-

185 Aldrich, The Netherlands) with known antifungal but no antibacterial activity (Garbeva et al.,
186 2014a), were added in concentrations of 160 µmol to a sterile filter paper positioned at the
187 edge of the plate as described previously by Garbeva et al. (2014a). Plates supplemented
188 with volatiles were kept in a protective cabinet at room temperature (± 20°C).

189 PCR amplification of 16S rRNA genes from the isolates was either performed directly with
190 colony material diluted in sterile deionized water or with isolated DNA using the QIAamp DNA
191 Mini Kit (Qiagen, The Netherlands) according to the manufacturer's protocol. The PCR mix
192 contained 0.17 mg/ml BSA, 0.33 µM of each primer (27F or 515F and 1492r; Lane, 1991), 1.5-
193 2 µl template, and 1x PCR Mastermix (Thermo Scientific, The Netherlands) containing
194 0.05U/µl Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$,and 0.4 mM of each dNTP. The
195 thermal protocol was as followed: initial denaturation at 95°C for 8 min, 5 cycles at 95°C for
196 60 sec, at 40°C for 60 sec, and at 72°C for 90 sec, and 35 subsequent cycles at 95°C for 60
197 sec, at 53°C for 30 sec, and 72°C for 70 sec. The final elongation was at 72°C for 5 min. The
198 16S rRNA PCR product of each (bacterial) isolate was purified and sent for sequencing to
199 LCG genomics (Berlin, Germany). Sequences were identified using NCBI nucleotide database
200 and BLASTn algorithm (Altschul et al., 1990). Microscopic pictures to describe the cell
201 morphology were taken and processed with Axio Imager M1 (Carl Zeiss Microscopy GmbH,
202 Germany) in combination with AxioVision V 4.9.1.0 (Carl Zeiss Microscopy GmbH, Germany).
203 All isolates were tested on the resistence against kanamycin and rifampicin by plating them
204 on 0.1 TSB containing 50 µg/ml (final concentration) of each antibiotic. The agar plates were
205 incubated for at least three weeks at 20°C and regularly checked for potential growth.

206

207 **2.7 DNA extraction from fungal hyphae**

208 Hyphae (pre-grown on 0.5 strength PDA) were transferred into Lysin-Matrix E tubes
209 (Biomedicals, The Netherlands), weighted (refer to as fresh-weight), and freeze-dried in liquid
210 nitrogen. Subsequently, fungal-bacterial DNA was extracted with phenol-chloroform as

described by Schulz-Bohm et al. (2015). Nucleic acid extracts were stored at -80°C or were immediately used for PCR amplification.

### 2.8 Quantitative PCR of bacterial *rpoB* genes

Primers to quantify the gene of the β subunit of the bacterial RNA polymerase (*rpoB*) were designed based on an alignment of 118 *rpoB* sequences of phylogenetic different bacteria available at NCBI (latest visit: 30/06/15, Table S3). The designed primers RpoB-fw1 (5'-GAAGGTCCGAACATCGGTCT-3') and RpoB-r1 (5'-TGCATGTTCGAGCCCATCA-3') amplify a fragment of 370 bp in the conserved region of *rpoB*. Quantitative PCR (qPCR) of the *rpoB* gene was performed with a BioRad C1000 TouchTM Thermal Cycler (Bio-Rad, The Netherlands) whereas each template was quantified in triplicates resulting in a total of nine reactions per fungal isolate (3 technical x 3 biological replicates). The 20-µl reaction mixture consisted of 1-fold SensiFAST™ SYBR® No-ROX Kit (Bioline GmbH, The Netherlands), BSA (0.5 µg µl-1), 500 nM RpoB-fw1, 500 nM RpoB-r1, and 5 µl of diluted template DNA (2-5 ng/ µl) or nuclease-free water in case of the negative control. Conditions for the quantification were as followes: 5 min initial denaturation at 95°C , ensued by 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 63°C, elongation for 30 sec at 72°C, and fluorescence signal detection for 15 sec at 82°C. Immediately after the 40th PCR cycle, a melting curve analyses (Fig. S5) from 63°C to 95°C with increments of 0.5°C was followed. Agarose gel electrophoreses of qPCR products displayed single bands of the expected size. Furthermore, sequencing of cloned *rpoB* qPCR products revealed that solely bacterial *rpoB* genes were targeted by RpoB-fw1/RpoB-r1 despite a huge background of fungal DNA (Fig. S2). Gene copy numbers were calculated according to a standard curve (Fig. S4) and corrected for potential inhibition (Zaprasis et al., 2010). A standard curve was set up by serially diluting of a pGEM-T vector containing an *rpoB* gene fragment of the strain *Burkholderia sp.* AD024.

## 2.9 Cloning of *rpoB* genes and sequence analysis

The *rpoB* qPCR products obtained for DNA extracted from the original fungal isolate M0 or the antibiotic-treated fungal isolate MA were pooled and purified with 20 % polyethylene glycol 6000 (AppliChem, Germany) and 2.5M sodium chloride after following protocol. One volume of PCR product was mixed with one volume of the polyethylene glycol solution, incubated for 15 min at 37°C, and centrifuged for 10 min at 10,000 x g. The resulting pellet was washed with 70 % ethanol and resuspended in DNAse/RNAse-free water. Purified qPCR products were ligated into a pGEM-T vector plasmid (Promega, USA) and transformed in *Escherichia coli* JM109 competent cells (Promega, USA) according to the manual instructions. Clones were picked and checked for the correct insert by M13 PCR (Messing, 1993). In total 70 M13 PCR products with the correct length (35 per fungal isolate) were purified and sequenced by LCG genomics (Berlin, Germany).

Sequences were analyzed with MEGA 6 (Tamura et al., 2011) and BLASTn. Based on a pairwise distance matrix of aligned *rpoB* nucleotide sequences (Kimura 2-parameter model, substitution includes transitions and transversions, pairwise deletion for gaps/missing data treatment), sequences were assigned to different Operational Taxonomic Units (OTUs) and the diversity index CHAO was calculated with DOTUR (Schloss and Handelsman, 2005). A threshold value of 97 % was used to define species-level OTUs (Adékambi et al., 2009). The coverage of the gene libraries was calculated according to Schloss et al. (2004) and rarefaction curves were constructed after the method of Hurlbert (Heck et al., 1975; Hurlbert, 1971).

## 2.10 Phylogenetic analyses

All phylogenetic trees were constructed with MEGA 6. The algorithms Maximum-Likelihood (Kimura 2-parameter model, partial deletion, 10,000 bootstraps), Neighbour-Joining (Saitou and Nei, 1987; Kimura 2-parameter model, complete deletion, 1000 bootstraps), and

263 Maximum-Parsimony (Subtree-pruning-regrafting search method, complete deletion, 1000
264 bootstraps) were applied. Trees for 16S rRNA gene sequences from bacterial isolates and
265 *rpoB* clone sequences were based on an alignment of 800 and 398 nucleotide positions,
266 respectively.

267

268 **2.11 Nucleotide sequence accession numbers**

269 Sequences were submitted to GenBank. Accession numbers: KX057404 - KX057471 (*rpoB*
270 clones sequences) and KX057472 - KX057478 (16S rRNA gene sequences of bacterial and
271 yeast isolates associated to M0 and MA).

272

273 **2.12 Volatile trapping and measurement**

274 For the collection of volatiles, glass petri dishes were used with lids with an exit to which a
275 steel trap with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd, United
276 Kingdom) was fixed (Garbeva et al., 2014b). Volatiles were collected for 20 hours at days 3
277 and 6. The traps were closed and stored at 4°C until analysis. Incubations were done in
278 triplicates, including medium controls.

279 Trapped volatiles were desorbed using an automated thermodesorption unit (model UnityTD-
280 100, Markes International Ltd., United Kingdom) at 210°C for 12 min (Helium flow 50 ml/min)
281 and trapped on a cold trap at -10°C. The trapped volatiles were introduced into the GC-QTOF
282 (model Agilent 7890B GC and the Agilent 7200A QTOF, USA) by heating the cold trap for 3
283 min to 280°C. Split ratio was set to 1:20, and the column used was a 30 × 0.25 mm ID RXI-
284 5MS, film thickness 0.25 µm (Restek 13424-6850, USA). Temperature program used was as
285 follows: 39°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
286 15°C/min and finally to 300°C at 40°C/min, hold 20 min. The VOCs were detected by the MS
287 operating at 70 eV in EI mode. Mass-spectra were extracted with MassHunter Qualitative
288 Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, USA) using the GC-Q-

TOF qualitative analysis module. The obtained mass spectra were exported as mzData files for further processing in MZmine. The files were imported to MZmine V2.14.2 (Pluskal et al., 2010) and compounds were identified via their mass spectra using deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA http://www.nist.gov) and Wiley 9th edition mass spectral libraries and by their linear retention indexes (LRI). The LRI values were calculated using an alkane calibration mix before the measurements in combination with AMDIS 2.72 (National Institute of Standards and Technology, USA). The calculated LRI were compared with those found in the NIST and in the in-house NIOO-KNAW LRI database. After deconvolution and mass identification peak lists containing the mass features of each treatment (MZ-value/Retention time and the peak intensity) were created and exported as CSV files for statistical processing via MetaboAnalyst V3.0 (www.metaboanalyst.ca; Xia et al., 2015; Xia et al., 2012).

### 2.13 Test on the effect of fungal VOCs on bacterial growth and motility

A double plate-within-a-plate system (Schmidt et al., 2015b) was used to test the effect of fungal volatiles emitted by the M0 and MA isolates on bacterial growth and motility. A 6 mm plug with fungal hyphae (pre-grown on 0.5 strength PDA) was transferred on 1.5 % WA or 0.5 strength PDA (Schmidt et al., 2015b) on a small Petri-dish (35 mm diameter) located in one half of a bipartite Petri-dish (Fig. S3). The other half of the bipartite Petri-dish was filled with 12 ml 1.5 % WA + ARE (described above) or with 0.3 % WA + ARE for the motility assay. After the fungus was incubated for three days at 20°C, 10 µl droplets of bacterial suspensions of different cell concentration ($10^3$ to $10^6$ CFU per ml) were added on WA + ARE, i.e. per replicate four droplets (from lower to higher cell concentration) were placed horizontally to the border of the two compartments (Fig. S3A). In case of the motility assay, one droplet of 10 µl bacterial suspension ($10^7$ CFU per ml) was placed in the middle of the second compartment filled with 0.3 % motility agar (Fig. S3B). The bacterial suspension consisted either of *Burkholderia sp.*

AD024 or *Collimonas fungivorans* Ter331 which were grown overnight at 22°C in 0.1 TSB and washed with phosphate buffer (10mM $KH_2PO_4$, pH 6.5). Bipartite Petri-dishes were sealed two-times with parafilm and incubated for six days at 20°C.

Colonies at the spot where the lowest dilution of bacterial cells per ml was added were used for the analysis of bacterial growth. In case of bacterial motility, the diameter of the bacterial colony was measured at four different spots (Fig. S3B) and a mean value was calculated (refer to as colony extension) for further analyses.

### 2.14 Statistical analysis

Statistical analysis on volatolomic data was performed using MetaboAnalyst V3.0 (Xia et al., 2012; Xia et al., 2015). To identify significant mass features, One-way-ANOVA with post-hoc Tukey test (HSD- test) were performed. To identify important mass features, PLS-D analysis was performed. Mass features were considered to be statistical relevant if p- and FDR- values were ≤ 0.05. Statistical relevant mass features were further used for compound identification.

The statistical analysis on hyphal extension of *M. hiemalis* as well as on the number of bacterial colonies and on colony extension when they were grown in presence of fungal VOCs was performed with R 3.1.1 (http://www.r-project.org/) using One-way-ANOVA Turkey's HSD test (De Mendiburu, 2014). Student's t-tests on data obtained by qPCR as well as for bioassays on fungal growth competition and on fungal growth inhibition by bacterial secondary metabolites were conducted with SigmaPlot 12.5 (Systat Software). The 5% level was taken as threshold for significance.

# 3. Results

## 3.1 Composition and abundance of the bacterial community associated with the fungus *M. hiemalis* based on *rpoB* gene

The composition of bacterial community associated with the fungus *M. hiemalies* was determined by targeting the gene of the β subunit of the bacterial RNA polymerase (*rpoB*). For this purpose, primers were newly designed with the aim to amplify a broad spectrum of phylogenetic different bacteria but not fungal DNA. In total 68 *rpoB* clone sequences were retrieved from both the original M0 isolated and antibiotic-treted MA isolate (Fig. 1A and Fig. S2). The total coverage was 94 % and rarefaction analysis showed an out-plateauing curve (Fig. 1B) indicating that sequencing was sufficient for the coverage of fungus-associated bacterial species. For M0, the coverage of *rpoB* sequences was lower than for MA (91 % and 97 %, respectively). This is reflected by a stronger out-plateauing rarefaction curve (Fig. 1C) and a lower CHAO diversity index for MA compared to M0 (6 and 19, respectively). In general, *rpoB* clone sequences could be assigned to the phyla *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (classes: *Alpha-, Beta-,* and *Gamma-Proteobacteria*) (Fig. S2). Clone sequences retrieved from M0 were affiliated to *Actinobacteria* (3 %) and *Proteobacteria* (97 %, dominated by *Alpha-Proteobacteria* [53 %], followed by *Gamma-* and *Beta-Proteobacteria* [29 % and 15 %, respectively]). *RpoB* clone sequences from MA were affiliated with *Bacteroidetes* (3%), and *Proteobacteria* (97 %, dominated by *Gamma-Proteobacteria* [65 %], followed by *Alpha-* and *Beta-Proteobacteria* [24 % and 9 %, respectively]). Within the *rpoB* clone library of M0, most sequences could be assigned to *Bradyrhizobium oligotrophicum* (41 %) and for MA, sequences were mostly related to *Serratia marcescens subsp. sakuensis* (47 %) (Fig. 1A).

In total, the bacterial community between M0 and MA appeared to be distinct. More diverse bacteria at the species-level were associated to M0. The bacterial community of the original M0 isolate was dominated by *Alpha-Proteobacteria* (*Bradyrhizobium* and *Brevundimonas*), the community associated to the antibiotic-treated isolate MA appeared to be dominated by *Gamma-Proteobacteria* (*Serratia, Pseudomonas* and *Rhodanobacter*) (Fig. 1A). After the

antibiotic treatment sequnces of *Mycobacterium* spp., *Stenothrophomonas maltophylia*, *Bradyrhizobium oligotrophicum* and *Bradyrhizobium liaoningense* were not detected.

The PCR quantification revealed that the *rpoB* gene copy number per gram fresh fungal hyphae for M0 ($3.47 \times 10^4 \pm 1.09 \times 10^4$) was about 1.4 fold higher compared to MA ($2.64 \times 10^4 \pm 8.53 \times 10^3$). However, the difference was not significant, indicating that the treatment with broad-spectrum antibiotics reduced the number of associated bacteria to the fungus *M. hiemalis* only slightly.

### 3.2 Isolation of *M. hiemalis* associated bacteria and yeast

Fungus-associated bacteria were isolated from both the original M0 and the antibiotic-treated MA isolate by using a combination of three different volatile organic compounds (VOCs) with known antifungal activity (Garbeva et al., 2014a). Only by applying a combination of all three volatiles the growth of *Mucor* hyphae could be successfully inhibited while the growth of fungus-associated bacteria was enhanced. In total, seven isolate types with different colony and cell morphologies were obtained (Fig. 2). Sequences of those isolates were related to different bacteria and to one yeast. Isolates retrieved from M0 were related to the *Alpha-Proteobacterium Ochrobactrum intermedium* strain AG1 (100% identity), the *Gamma-Proteobacterium Stenotrophomonas maltophilia* strain Zunyi-F (99 % identity), and the *Firmicute Bacillus sp.* A-3-15 (100 % identity). Most isolates were related to *S. maltophilia* (Fig. 3). Sequences related to this species as well as sequences related to *Alpha-Proteobacteria* were also found for the *rpoB* clone library of M0 (Fig. 1 and Fig. S1). Isolates obtained from MA were related to the *Actinobacterium Kocuria kristinae* strain VTT E-82147 (99 % identity) and *Micrococcus sp.* O-1 (99 % ident), the *Firmicute Staphylococcus sp.* ccc_1 (99 % identity) as well as to the yeast *Meyerozyma guilliermondii* strain Nc49HB-1 (99 % identity). Sequences related to *Staphylococcus* and *Kocuria* were also exclusively detected for MA by next generation sequencing of 16S rRNA (Table S2) but not by cloning of *rpoB* qPCR products. All isolates were tested on resistance to rifampicin and kanamycin –

antibiotics previously applied to *M. hiemalis*. Only *S. maltophila* and the yeast *Meyerozyma guilliermondii* (obtained from M0 and MA, respectively) grew on TSB with rifampicin and kanamycin (Fig. 2).

### 3.3 Comparison of the hyphal extension

The comparison of hyphal extension revealed differences between the original M0 isolate and the antibiotic-treated MA isolate on both media, with significantly higher hyphal extension of M0 (Fig. 3). Besides hyphal extension, morphological differences in pigmentation were observed between the two isolates under both nutrient conditions (Fig.S 4 and 5). On the nutrient-rich media, M0 had yellow pigmentation and MA had brown pigmentation (Fig. 4).

### 3.4 Competition between the fungal isolates M0 and MA

By incubating both fungal isolates M0 and MA together in Petri-dishes, the area of hyphal extension as compared to the monoculture was significantly lower for MA (57 ± 6 % [day7], 35 ± 4 % [day14]; n= 3) than for M0 (74 ± 4 % [day7], 65 ± 3 % [day14]; n = 3). After seven days of incubation, the hyphal extension of both competing isolates did not further increase. Only the hyphal network became denser, and a clear separation zone between both fungal isolates was visible (Fig. 4). For the control (i.e. the monocultures of both fungal isolates), the Petri-dishes were fully covered with mycelium after 14 days of incubation (Fig. 4).

### 3.5 Growth of M0 and MA in presence of rhizobacteria

In the presence of the rhizobacterium *Burkholderia sp*. AD024, the hyphal extension of MA towards the bacterial biofilm was more inhibited as compared to M0 (Fig. 5C and I). The inhibition zone,i.e. the distance between bacterial biofilm and the fungal hyphae, was significantly bigger for MA (73 ± 6 mm) than for M0 (43 ± 12 mm).

In case of *Collimonas fungivorans* Ter 331, there was no significant difference between M0 and MA. However, the fungal hyphae of MA were visibly affected by the presence of *Collimonas* compared to M0 (Fig. 5B, F, H, and. L). Such difference was not observed for incubations with *Burkholderia sp.* AD024 (Fig. 5B, D, H, and J).

### 3.6 Volatiles produced by *M. hiemalis* isolate M0 and MA

Headspace volatiles produced by *M. hiemalis* M0 and MA on nutrient-rich PDA and nutrient-poor media WA were collected at day 6 after inoculation and subsequently analysed using GC/MS. Different blends of volatiles were produced by the two isolates under both nutrient conditions (Fig. 6) with more volatile compounds produced on the nutrient-rich PDA media. Interestingly the volatile blend of the MA isolate on nutrient-poor WA media did not differ from the control without fungus (Fig. 6). Several compounds, such as trifluorbenzene, 1 butanole and 1-butanol-2methyl, were detected only in the headspace of M0 isolate, while heptane-2,4-dimethyl was produced only by the MA isolate (Table 1). Numerous compounds could not be assigned with certainty to a volatile organic compound and remained unknown. Some of these unknown compounds were also discriminative between M0 and MA.

### 3.7 Effect of fungal volatiles on rhizobacteria

By exposure to fungal volatiles emitted by MA on PDA, the numbers of colonies of rhizobacteria *Burkholderia sp*. AD024 and *Collimonas fungivorans* Ter 331 were significantly increased in comparison to M0. Such significant difference was not observed when the fungal isolates were incubated on WA (Fig. 7A and B). In addition, the number of *C. fungivorans* Ter 331 colonies was significantly reduced by exposure to volatiles of M0 growing on PDA (Fig. 7B).

The motility of both bacteria (reflected in colony extension) was differently affected by the volatiles of M0 and MA (Fig. 7C and D). The swimming motility of *C. fungivorans* Ter 331 was

442 significantly higher in presence of volatiles produced by MA growing on PDA as compared to
443 M0 (Fig. 7D).

444 The swimming motility of the bacterial strain *Burkholderia* sp. AD24 was significantly increased
445 by exposure to volatiles of both fungal isolates whereas volatiles of M0 seemed to stimulate
446 more the motility of this strain compared to volatiles of MA (Fig. 7C).

447

## 4. Discussion

The fates of bacteria and fungi are ecologically intimately connected in soil and rhizosphere. Close association of bacteria with fungi, both endo- and ectosymbionts is well known (Frey-Klett et al., 2011). Most methods for fungal isolation and purification are involving application of broad-spectrum antibiotics (Singh et al., 2015; Oliveira et al., 2013). The model soil-borne fungus *Mucor hiemalis* used in our study was originally isolated by plating on media supplemented with oxytertracycline (De Rooij-van der Goes et al., 1995). To ensure that we are using bacteria-free fungus, spores of *Mucor hiemalis* were washed with antibiotics and plated on a media supplemented with rifampicin and kanamycin. The treatment with these broad-spectrum antibiotics strongly altered the fungal morphology and hyphal extension. Hence, the purpose of our study was to determine the bacterial community associated with the fungus *M. hiemalis* and to examine the effect of the two broad-spectrum antibiotics on the fungus-associated bacteriome and, consequently, on fungal behavior and interactions.

Our results revealed that a high diversity of bacteria is associated to *M. hiemalis*. Interestingly, the antibiotic treatment did not significantly reduce the amount of bacteria associated to the fungus but rather changed the community composition by shifting from initially dominating *Alpha-Proteobacteria* to dominance by *Gamma-Proteobacteria*. The antibiotic-treated *M. hiemalis* isolate revealed less diverse bacterial community as compared to the original isolate. Interestingly, several OTUs (*Rhodanobacter* ssp., *Pseudomonas* sp., and *Bacteroides* spp) not detected in the original M0 isolate were detected in the antibiotic treated MA isolates. It is plausible that by affecting some of the antibiotic sensitive strains other “silent” bacteria carried by the fungus became more dominant. The composition of the non-culturable bacterial community associated with the fungus *M. hiemalis* is determined based on housekeeping *rpoB gene* which allowed to target bacteria and, in addition, to avoid co-amplification of fungal DNA. Our attempt to determine the composition of the bacterial community based on 16S rRNA next generation sequencing demonstrated also differences between the bacterial communities

474 (supplementary material Table S2 and Fig. S1). However, the majority (more than 90%) of
475 OTUs were assigned to fungal mitochondrial DNA (Fig. S1A and B).

476 Plausible explanation for bacterial survival after antibiotic treatment is the resistance to the
477 applied antibiotic or protection by the fungal host. This is in line with another study that failed
478 to obtain bacteria-free fungi (Sharma et al., 2008). Analogous, it is also challenging to isolate
479 bacteria that are free from fungal mycelium as often both partners are dependent on each
480 other (Sato et al., 2010). Here, we applied a novel method for the isolation of fungal-associated
481 bacteria by the application of three volatile compounds (namely benzonitril, dimethyldisulfide
482 and dimethyltrisulfide) with strong antifungal activity and stimulating effect on bacterial growth
483 (Garbeva et al., 2014a). The high fungal sensitivity to volatile compounds (Effmert et al., 2012;
484 Garbeva et al., 2014a) compared to high resistance or even growth promotion for bacteria
485 (Garbeva et al., 2014b; Schmid et al., 2015a) make volatiles suitable compounds for isolation
486 of fungal-associated bacteria. However, difference in fungal susceptibility to volatiles was
487 observed and reported in several independent studies (Effmert et al., 2012 Schmidt et al.,
488 2015). The yeast *Meyerozyma guilliermondii* was not affected by the volatile treatment
489 indicating that yeast may be less susceptible to volatiles. The results of the bacterial isolation
490 likewise revealed differences in the communities between the original and antibiotic-treated
491 *M. hiemalis*. While isolates obtained from the original M0 isolate belonged to *Alpha*- and
492 *Gamma-Proteobacterium* and *Firmicutes*, the isolates obtained from the antibiotic-treated MA
493 belonged to the phyla *Actinobacteria*, *Firmicutes* and yeast. Interestingly, the test for
494 resistance to antibiotics revealed that only *Stenotrophomonas* and the yeast were resistant to
495 rifampicin and kanamycin, indicating that the fungal host may indeed play a major role in
496 protecting their associated bacteria. Several sequences related to the bacterial isolates such
497 as for *Stenotrophomonas, Staphylococcus* and *Kocuria* were also detected in the *rpoB* clone
498 library and in the next generation 16S rRNA sequencing approach.

499 The shifts in fungal-associated bacterial community led to clear changes in morphology and
500 behaviour of *M. hiemalis*. For instance, the hyphal extension of the original isolate was

501 significantly faster as compared to the antibiotic-treated isolate. The direct competition
502 experiment between the two isolates revealed an advantage in plate colonization for the
503 original M0 isolates indicating that the fast hyphal extension can be beneficial for *M. hiemalis*
504 in terms of resource competition. Whereas bacterial motility in water-unsaturated soil is
505 assumed to be highly restricted, mycelia spread efficiently in the soil, penetrate air-water
506 interfaces and cross over air-filled pores (Jimenez-Sanchez et al., 2015; Warmink et al., 2011).
507 Hence, fast hyphal extension may be beneficial for bacteria and, therefore, may be stimulated
508 by the *M. hiemalis*-associated bacterial community. Similar stimulation of hyphal growth by
509 bacteria was reported for the interaction of *Amanita muscaria* and *Streptomyces sp*. AcH505
510 where bacteria showed an enhanced production of the secondary metabolite auxofuran, which
511 promotes the extension of the fungal mycelium (Frey-Klett et al., 2011).

512 Besides hyphal extension, susceptibility to antifungal compounds produced by bacteria was
513 also significantly affected by changes in the *M. hiemalis* associated bacterial community,
514 thereby the antibiotic-treated isolate appeared to be more sensitive to antifungal compounds
515 as compared to the original isolate. This may point at role of the fungus-associated bacteriome
516 in the protection of its host.

517 Fungal-associated bacteria have been shown to affect secondary metabolism of the fungi
518 including the production of volatiles (Minerdi *et al.*, 2008; Splivallo et al., 2015; Vahdatzadeh
519 et al., 2015). Due to the shifts in bacterial community, we observed changes in fungal
520 pigmentation and volatile emission. In the last years, it has become evident that microbial
521 volatiles can play major roles in long-distance interactions within soil microbial communities
522 acting as infochemicals or antimicrobial compounds (Effmert et al., 2012; Schmidt et al.,
523 2015a; Schmidt et al., 2015b; Schulz-Bohm et al., 2015). The shifts in fungal-associated
524 bacterial community led to shifts in volatile production of the original and the antibiotic-treated
525 *M. hiemalis* isolates which, consequently, affected their interactions. The volatiles emitted by
526 the original M0 isolate on the nutrient-rich PDA inhibited the growth of *C. fungivorans* Ter 331,
527 whereas the antibiotic-treated MA isolate stimulated the growth of this strain. Bacteria from

528 the genus *Collimonas*, were previously shown to colonize and grow on living fungal hyphae,
529 a phenomenon called mycophagy (De Boer et al., 2004; Leveau et al., 2010). Interestingly,
530 the motility of *C. fungivorans* Ter 331 was significantly stimulated by the volatiles emitted by
531 the antibiotic-treated *M. hiemalis* implying that volatiles might play a role as long-distance
532 signals for attracting such mycophagous bacteria. Both, volatile-mediated interactions and
533 direct interactions may indicate that the antibiotic-treated isolate is more attractive and
534 susceptible to mycophagous bacterium as compared to the original isolate.

535 The composition and abundance of volatiles was affected by the nutrient conditions with more
536 volatiles produced on the nutrient-rich PDA media. Several independent studies have reported
537 that the volatile profiles of bacteria and fungi are strongly dependent on growth conditions,
538 interactions and nutrient availability (Garbeva et al., 2014b; Schmidt et al., 2015b; Schulz-
539 Bohm et al., 2015; Tyc et al., 2015; Weikl et al., 2016). Hence, it is questionable whether the
540 same volatile-mediated interactions will occur in nature under nutrient-limited conditions. In
541 soil, the rhizosphere is a “hot-spot” of microbial activity, where approximately 20 to 40% of the
542 phytosynthetic carbon fixed by a plant is released as root exudates (Jones et al., 2009;
543 Philippot et al., 2013). For a long time, it has been assumed that the rhizosphere is mainly
544 dominated by bacteria, however, recent studies revealed significant utilization of root exudates
545 by saprotrophic fungi (Hannula et al., 2012). Therefore fungal-bacterial interactions as the one
546 described in this study may take place in the rhizosphere and have an effect on the
547 rhizosphere microbiome, which consequently, may play an important role for plant growth and
548 health.

549 Overall, in the present study we described diverse bacterial community associated with the
550 saprotrophic fungus *M. hiemalis* most probably carried as a contaminant from the natural
551 environment. Our results revealed that antibiotic treatments can cause shifts in this bacterial
552 community that consequently affect the host in terms of morphology, behavior, secondary
553 metabolite production and interactions.

554 Evidence has emerged over the past years that endo- and ectosymbiotic bacteria are
555 widespread in fungi. Hence, similar to animals and plants, fungi are never alone as they
556 constantly carry their bacteriome. It remains questionable if the fungal isolates in the pure
557 culture collections are really free of bacteria, as the isolation with antibacterials is clearly not
558 sufficient to eradicate the associated bacteria. Future research should be directed to study
559 fungal bacteriome, bacterial localization, the mechanisms of interactions, and its significance
560 for evolution, trophic interactions and ecosystem functioning.

561

562

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713

## 7. Figures and Tables

**Figure 1.** Phylogenetic distribution (A) and rarefaction analysis (B and C) of *rpoB* gene sequences from bacteria associated to *M. hiemalis*. Sequences retrieved from *M. hiemalis*, original isolate (M0) and the isolate treated with antibiotics (MA), were assigned to species-level OTUs based on a threshold value of 97% identity. Data corresponding to M0 and MA are indicated in black and grey, respectively. Rarefaction analysis was performed for all sequences obtained from both isolates (B) as well as for the subset per fungal isolate (C). Solid lines in panel B and C represent 95% confidence intervals.

**Figure 2.** Phylogenetic association and characteristics of bacterial and yeast isolates associated to *M. hiemalis*. Bacteria and yeast were isolated from *M. hiemalis,* original isolate (M0) and isolate treated with antibiotics (MA). A Maximum-Likelihood tree of representative 16S rRNA gene sequences was calculated. Percentages of replicate trees (> 50%) in which the associated taxa clustered together in the bootstrap test (10,000 bootstraps) are shown next the branches. Filled squares indicate confirmed tree topology with Maximum-Parsimony and Neighbor Joining (bootstraps > 70 %) calculations with the same dataset. Accession numbers are included in parentheses. Table next to the tree represents general characteristics of the bacterial isolates such as resistance to rifampicin (Rif) and kanamycin (Kan). Scale bar in microcscopic pictures represent 2 µm.

**Figure 3.** Hyphal extension of *M. hiemalis* isolates. Both, the original (M0) and antibiotic-treated isolated (MA) were incubated on 0.5 strength Potato dextrose agar (PDA) or water-agar (WA).

**Figure 4.** Growth of the *M. hiemalis* isolates M0 and MA (original isolate and antibiotic- treated, respectively) on 0.5 strength Potato dextrose agar for 7 (A, D, G), 14 (B, E, H) and 21 days

741 (C, F, I). Fungal isolates were incubated either alone (M0: G-I; MA: D-F) or together (left: MA;
742 right: M0) on one Petri-dish (A-C).
743
744 **Figure 5.** Effect of two soil bacteria strains on hyphal extension of the *M. hiemalis* isolates M0
745 (A-F) and MA (G-L). The bacteria were pre-incubated for three days without fungus followed
746 by a four days incubation with the fungus. M0 represents the original and MA the antibiotic-
747 treated fungal isolate. Overviews of the assays are presented in panels A,C, E, G, I and K and
748 detailed views of mycelia growth in panel B, D, F, H, J and L.
749
750 **Figure 6.** PLS-DA score plot of volatile compounds produced by the original (M0) and
751 antibiotic-treated (MA) *M. hiemalis* isolates. A multivariate analysis was performed on mass
752 features of detected compounds which were emitted by the fungi on water-agar (WA; panel
753 A) or Potato dextrose agar (PDA; panel B) Controls, volatiles released by the agar media
754 without *M.hiemalis*, are included in the analysis..
755
756 **Figure 7.** Effect of fungal volatiles on the development (A and B) and on swimming motility (C
757 and D) of two rhizobacterial strains. The rhizobacteria were exposed to volatiles produced by
758 original (M0) and antibiotic-treated M. *hiemalis* isolate (MA) growing on 0.5 Potato dextrose
759 agar (PDA) or water-agar (WA). Bacterial growth and motility were assessed by counting of
760 colonies appearing at the highest dilution and measuring of the colony extension, respectively.
761 Different letters represent statistical different values tested by One-way-ANOVA.
762
763 **Table 1.** Volatile organic compounds produced by *Mucor hiemalis* on potato dextrose agar.