Mucorales between food and infection
Dolat Abadi, S.

Citation for published version (APA):
Dolatabadi, S. (2015). Mucorales between food and infection

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Adaptation to thermotolerance in *Rhizopus* coincides with virulence as revealed by avian and invertebrate infection models, phylogeny, physiological and metabolic flexibility

Kerstin Kaerger, Volker U. Schwartze\textsuperscript{a}, Somayeh Dolatabadi\textsuperscript{a}, Ildikó Nyilasi, Stella A. Kovács, Ulrike Binder, Tamás Papp, G. Sybren de Hoog, Ilse D. Jacobsen, Kerstin Voigt

\textsuperscript{a}authors contributed equally to the manuscript

Submitted for publication
Abstract
Fungal infections caused by the ancient Mucorales (mucormycosis) are still rare, but are increasingly reported. Predisposing conditions supporting and favouring mucormycoses in humans and animals include diabetic ketoacidosis, immunosuppression, and haematological malignancies. However, comprehensive surveys to elucidate fungal virulence factors in ancient fungi are limited and so far focused on Lichtheimia and Mucor. Since causative agents of mucormycoses belong to different genera which are distantly related, differences in virulence factors could be assumed and should be studied for each genus. The presented study focused on a third important causative agent of mucormycoses, the genus Rhizopus, located in the family Rhizopodaceae. The study revealed variable virulence potential for distinct evolutionary clades, with adaptation to elevated temperatures being viewed as an important criterion for the development of human mucormycoses, although the virulence of thermotolerant vs. mesophilic species was similar in chicken egg and wax moth models. This comparability clearly revealed the existence of additional factors relevant to infection. However, neither specific adaptation to nutritional requirements nor stress resistance correlated with virulence, supporting the idea that Mucorales are predominantly saprotrophs without a specific adaptation to warm blooded hosts.

Keywords
Virulence, Rhizopodaceae, Rhizopus, Syzygites, Sporodiniella, infection model, embryonated chicken egg, Galleria, wax moth, carbon source assimilation, growth kinetics, thermotolerance

Introduction
Zygomycetes belong to one of the oldest fungal groups on earth, with known fossils from the Middle Triassic of Antarctica (Krings et al. 2012) and a diverging time calculated for their origin of around 600 mya years (Berbee et al. 2001). Contemporary descendants of these early ancestors can be found all over the world colonizing a wide range of ecological habitats, and are currently classified in several subphyla, namely Mucoromycotina, Kickxellomycotina, Zoopagomycotina, Mortierellomycotina (Hibbett et al. 2007, Hoffmann et al. 2011), and the phylum Entomophthoromycota (Humber et al. 2012). Within the Mucoromycotina, the largest order Mucorales comprises predominantly saprotrophic inhabitants of soil and organic decaying matter. Some species are also able to parasitize on plants,
insects and fungi, or they can be found as opportunistic pathogens of man and animals.

The mucoralean family Rhizopodaceae K. Schum. today encompasses three genera, namely Sporodiniella, Syzygites and Rhizopus. Although the family comprises only eleven species, saprotrophic, parasitic and pathogenic life-styles are represented within the Rhizopodaceae in a species-specific manner.

While Sporodiniella umbellata, sole species of its genus, is a facultative parasite of insect larvae (Evans et al. 1977, Chien et al. 1997), Syzygites megalocarpus, also sole species of its genus, is parasitic on members of the Dikarya (Kovacs et al. 1999). In contrast, Rhizopus species display a high variability of lifestyles and habitats. Being primarily saprotrophic fungi, several species are important plant-pathogens or spoilage agents of fresh and manufactured food e.g. soft rot caused by R. stolonifer, R. arrhizus (syn. R. oryzae) or R. microsporus (Fajola 1979, Shtienberg 1997, Lackner et al. 2009, Kwon et al. 2011). Yet, Rhizopus plays also an important role in industrial biotransformations or food processing through fermentation, especially in Asia and Africa (Nout et al. 2004 & 2009, Choudhary et al. 2013).

The most important impact on human is the pathogenic potential of some Rhizopus species causing life-threatening infections (Ribes et al. 2000, Roden et al. 2005, Skiada et al. 2011). These infections often develop rapidly, predominantly as rhinocerebral and pulmonary manifestations; and are often associated with dissemination and high mortality rates. Although mucormycoses are uncommon fungal infections compared to aspergillosis or candidiasis, their incidence is increasing in clinical settings (Ribes et al. 2000, Roden et al. 2005, Skiada et al. 2011, Gomes et al. 2011). Major risk factors for mucormycoses are diabetic ketoacidosis, immunosuppression and malignancies. In addition, infections have been found to be associated with administration of certain antifungals such as voriconazole or iron chelators like desferoxamine (Roden et al. 2005, Skiada et al. 2011, Lamaris et al. 2009).

In addition to human predispositions, fungal prerequisites are also required for infection. Such virulence factors include pathways that facilitate adaptation to the host environment, e.g. to elevated temperatures, unfavourable pH, unbalanced osmotic conditions and nutrient limitation (Cooney et al. 2008). Furthermore, some morphological features are linked to virulence: e.g. fungal spore size is known to be related to fungal pathogenesis in Mucor circinelloides (Lee et al. 2013, Li et al. 2011). Finally, the relative burden of asexual spores in the environment might contribute to the establishment of mucormycoses. In Rhizopus the amount of spores produced differs between species and is known to be reduced for R. schipperae, a rare causative agent of mucormycosis (Ribes et al. 2000).
Although mucormycoses are seen as emerging serious fungal infections, with a large number of case reports and studies concentrating on susceptibility to antifungal drugs (Alastruey-Izquierdo et al. 2009), comprehensive evaluations of the pathogenic potential at genus- or family-level so far only exist for the Lichtheimiaceae (Schwartze et al. 2012). In addition to evaluating fungal traits potentially involved in virulence we investigated the pathogenic abilities of the Rhizopodaceae applying the embryonated chicken egg model, a model with proven suitability to assess the virulence potential of fungi, and Galleria mellonella as a second alternative infection model (Schwartze et al. 2012, Jacobsen et al. 2010).

Material and Methods

Ethics statement
All experiments were performed in compliance with the European and German animal protection law. According to this, no specific approval is needed for work performed in avian embryos before the time of hatching. The experimental protocols were reviewed and approved in regard to ethical and welfare issues by the responsible animal welfare officer. Experiments were terminated latest on developmental day 18, three days before hatching, by chilling the eggs on ice for 30-60 min.

Fungal isolates
A total of 34 isolates of the family Rhizopodaceae were included in this study (Tables 1 & 2). Strains were obtained from the Jena Microbial Resource Collection and from the Centraalbureau voor Schimmelcultures (CBS). Isolates were identified by standard microbiological procedures and by sequencing of 18S rDNA, 28S rDNA, and ITS regions. For DNA isolation strains were grown for 5-10 days on medium KK1, especially composed for Mucorales (1 % glucose, 0.44 % NaCl, 0.3 % KH₂PO₄, 0.125 % K₂HPO₄, 0.2 % yeast extract, 0.1 % KNO₃, 0.05 % MgSO₄*7H₂O, 0.05 % KCl (all Carl Roth)) at room temperature. DNA isolation, PCR and sequencing were conducted as described previously (Hoffmann et al. 2013). Primers used for amplification were: NL1 and NL4 (for 28S rDNA) (O’Donnell et al. 1993), NS1 and NS4 (for 18S rDNA) and ITS1 and ITS4 (for ITS) (White et al. 1990). Sequences generated in this study are given in Table 1.

Embryonated chicken egg model
Infections at developmental day 10 was done via the chorio-allantoic membrane as described previously (Jacobsen et al. 2010) with 10⁵ and 10⁶ spores/egg. Twenty
eggs were used for each strain. Considering ethical issues to reduce the amount of eggs needed to an inevitable number, experiments were repeated once, using FSU10059 as positive control strain and PBS as negative control. To process all strains, several individual runs were necessary, each time including positive and negative controls. Since no significant differences could be observed between three repetitions of the positive and negative controls (PBS P= 0.5629; FSU 10059 10⁵ spores/egg P= 0.2845; 10⁶ spores/egg P= 0.3398), one repetition for all thermotolerant strains was sufficient. Strains unable to grow at 37 °C were tested only once, with no significant differences between isolates of the same species (data not shown). Survival was assessed daily by candling and is summarized in Fig. 1. To assess strain dependent differences, R. microsporus was checked for 19 additional isolates. Due to reduced amount of spores, R. homothallicus was only tested with 10⁵ spores. Syzygites and Sporodiniella were not tested because they do not grow at elevated temperatures and did not produce that high amount of spores. Statistical analysis was performed with GraphPad Prism v5.03 using combined data from all individual runs.

**Galleria mellonella infection model**
In order to test whether the virulence data observed in the chicken eggs correlate to the elevated temperature used for incubation and the different abilities of the strains to grow at this temperature, a second, widely accepted, infection model was applied. Rhizopus lyococcus and R. stolonifer were chosen as representatives of the mesophilic group, R. arrhizus and R. microsporus for the thermotolerant group.

Sixth-instar larvae of Galleria mellonella (Kurt Pechmann, Langenzersdorf, Austria) were stored in the dark at 18 °C prior to use. Larvae weighing between 0.3 and 0.4 g were used, each (n=20) infected with 1×10⁶ spores. Inocula were diluted in insect physiological saline (IPS) and a volume of 20 µl was injected into the hemocoel via the hind pro-leg. Untouched larvae and larvae injected with 20 µl of IPS served as control. Larvae were incubated at 30 °C, respectively, in the dark and monitored daily up to 6 days. Significance of mortality rates was evaluated by using Kaplan-Meier survival curves with the PRISM statistics software (Mantel-Cox log rank test) using pooled data. All experiments were performed three times, each time with duplicates. Survival rates are displayed in Fig. 2. Syzygites and Sporodiniella were not tested because they did not produce enough spores.

**Relation of growth and temperature**
Petridishes with medium KK1 were inoculated with 10 µl spore suspension containing 1000 spores. In cases of growth, the initial colony was 6 mm in
diameter. *Sporodiniella umbellata* and *Syzygites megalocarpus* were inoculated as agar slants with 6×6 mm. Plates were incubated at different temperatures. The diameter was measured two times a day across three defined lines at the bottom of the petridish (Rosenberg et al. 1975) for three technical replicates. The mean diameters of three biological replicates at 24 h and 48 h. Maximum diameter possible is 90.00 mm, equal to the size of the petridish.

**Relation of growth and stress conditions**

Petridishes with medium KK1 were supplemented with 1 M NaCl, 1.5 M NaCl, 30 µg/ml SDS, 7.5 mM caffeine, 100 µg/ml CongoRed (all Carl Roth). Petridishes were inoculated with 1000 spores in 10 µl. Plates were incubated at 30 °C (25 °C for *Sporodiniella umbellata, Syzygites megalocarpus, Rhizopus sexualis*). The relative growth [%] compared to medium without stress inducers of three replicates at 24 h (48 h for *Sporodiniella umbellata, Syzygites megalocarpus*) is given in Table 3.

**Carbon and nitrogen assimilation profiles**

Agar plates with medium MM (0.5 % (NH₄)₂SO₄, 0.05 % MgSO₄, 0.1 % KH₂PO₄, 2 % agar), supplemented with 0.2 % carbon source were inoculated with 2×10⁵ spores in 20 µl and incubated at 30 °C (25 °C for *Sporodiniella umbellata, Syzygites megalocarpus, Rhizopus sexualis*) for 3-4 days. Evaluation of growth was performed visually and categorized in: inhibition (-), growth arrest after germination (0/-), no growth (0, but this includes ‘background’ growth due to carbon traces contained in the agar), slight or no growth (0/+, difficult to distinguish from the ‘background’ growth), weak growth (+), normal growth (++), strong growth (+++, similar to the glucose containing media), stronger growth (++++). Since *Syzygites* and *Sporodiniella* did not grow in appropriate time on this medium, a different medium (10 mM KH₂PO₄/K₂HPO₄ (pH 6.6), 1.25 mM MgSO₄*7H₂O, 0.3 mM ZnSO₄*7H₂O, 0.09 mM FeCl₃*6H₂O, 0.03 mM CuSO₄*5H₂O (all Carl Roth)) was used to analyse a reduced second set of carbon sources. This time, liquid media was used in 96-well plates. Each well was supplemented with a carbon source and 500 spores. Plates were incubated for up to 6 days at optimal temperatures (37 °C, 30 °C, and room temperature). Experiment was done up to three times, each time with triplicates; except for species where no differences between isolates were observed. In those cases only two repetitions were performed. After incubation the plates were analysed visually for growth (p) or lack of growth (0). Weak growth was considered negative because of the difficulty in differentiation from background growth. Additionally chitin, pectin, citric acid and cellulose were tested as carbon sources.
The liquid medium was also used to analyse the nitrogen utilization profile. Growth was evaluated after 70-88h at appropriate growth temperatures.

**Size of sporangiospores and amount of spores**

The size of the spores for each species was determined according to standard rules after harvest from KK1 medium cultivated for 5 days under optimal growth conditions (Table 1).

*Rhizopus schipperae* is known to produce fewer spores on artificial media. To assess the relative amount of spores produced in a specific period of time *R. schipperae* was cultivated on KK1 medium (petridish with 5.5 cm diam.) for 3 days, at appropriate temperatures (room temperature, 30 °C or 37 °C). Spores were harvested by extensive washing with PBS and counted in a haemocytometer.

**Results**

**Phylogeny and clinical relevance**

The family Rhizopodaceae comprises the genera *Rhizopus*, *Syzygites* and *Sporodiniella* with few, closely related species. Only species of the genus *Rhizopus* have clinical relevance, with *R. arrhizus* and *R. microsporus* predominantly described as potential agents of severe mucormycoses (Ribes et al. 2000, Roden et al. 2005). The species *R. schipperae*, *R. caespitosus* and *R. homothallicus* are less frequently observed in human infections (Chakrabarti et al. 2010, Weitzman et al. 1996).

The virulence potential of the different *Rhizopus* species was determined in chicken embryos. The strain *R. microsporus* FSU10059 was used to establish the model and to show the reproducibility of the experiments. Eggs were infected with $10^5$ or $10^6$ spores per egg. Mortality of the chicken embryos was dependent on the concentration of spores (Fig. 1) and highly reproducible in three independent experiments ($10^5$ spores/egg $P=0.2845$; $10^6$ spores/egg $P=0.3398$). The non-clinically species *R. lyococcus* was found to be less virulent with survival rates between 75-90 % even at high infection doses ($10^6$ spores/egg) (Fig. 1). Infections with *Rhizopus stolonifer* led to 80-85 % survival of the embryos at the low infection dose ($10^5$ spores/egg) and 60-70 % survival at the high infection dose.

In contrast, mortality was higher in chicken embryos infected with the two most-common pathogenic species, *R. arrhizus* (42.5-52.5 % survival) and *R. microsporus* (<40 % survival), at both infection doses. Whereas *R. microsporus* could be considered moderately to highly virulent, *R. arrhizus* together with
<table>
<thead>
<tr>
<th>Species</th>
<th>FSU number</th>
<th>CBS or alternative number</th>
<th>Mating type</th>
<th>Geography</th>
<th>Source</th>
<th>Spore size [μm]$^3$</th>
<th>28S</th>
<th>18S</th>
<th>ITS</th>
</tr>
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<tbody>
<tr>
<td><em>R. arrhizus var. arrhizus</em></td>
<td>FSU5857</td>
<td>CBS 112.07</td>
<td>Minus</td>
<td>Netherlands</td>
<td>NA</td>
<td>47.13 ± 21.99</td>
<td>KJ408556</td>
<td>KJ408539</td>
<td>KJ408568</td>
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<tr>
<td><em>R. arrhizus var. deleterar</em></td>
<td>FSU8743</td>
<td>RA 99-880</td>
<td>Plus</td>
<td>Texas</td>
<td>Human</td>
<td>50.95 ± 20.62</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>R. caespitiosus</em></td>
<td>FSU11356</td>
<td>CBS 427.87</td>
<td>Minus</td>
<td>India</td>
<td>NA</td>
<td>49.89 ± 26.58</td>
<td>KJ408564</td>
<td>KJ408548</td>
<td>NA</td>
</tr>
<tr>
<td><em>R. homothallicus</em></td>
<td>FSU2530</td>
<td>CBS 336.62</td>
<td>Homothallic</td>
<td>Guatemala</td>
<td>Desert soil</td>
<td>63.48 ± 14.85</td>
<td>KJ408554</td>
<td>KJ408537</td>
<td>KJ408567</td>
</tr>
<tr>
<td><em>R. lyococcus</em></td>
<td>FSU10053</td>
<td>CBS 398.95</td>
<td>NA</td>
<td>Unknown</td>
<td>NA</td>
<td>72.24 ± 27.58</td>
<td>KJ408562</td>
<td>KJ408545</td>
<td>NA</td>
</tr>
<tr>
<td><em>R. lyococcus</em></td>
<td>FSU9996</td>
<td>CBS 117.43</td>
<td>NA</td>
<td>Netherlands</td>
<td>Grain</td>
<td>88.39 ± 34.27</td>
<td>KJ408559</td>
<td>KJ408542</td>
<td>NA</td>
</tr>
<tr>
<td><em>R. microsporus</em></td>
<td>FSU10059</td>
<td>CBS 102277</td>
<td>Plus</td>
<td>Unknown</td>
<td>Human</td>
<td>NA</td>
<td>KJ408561</td>
<td>KJ408543</td>
<td>KJ408571</td>
</tr>
<tr>
<td><em>R. microsporus</em></td>
<td>FSU10049</td>
<td>CBS 308.87</td>
<td>NA</td>
<td>Australia</td>
<td>Human</td>
<td>27.98 ± 5.36</td>
<td>KJ408560</td>
<td>KJ408544</td>
<td>KJ408570</td>
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<tr>
<td><em>R. microsporus</em></td>
<td>FSU9932</td>
<td>CBS 294.31</td>
<td>Plus</td>
<td>France</td>
<td>Cow</td>
<td>55.55 ± 11.89</td>
<td>KJ408558</td>
<td>KJ408541</td>
<td>KJ408569</td>
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<tr>
<td><em>R. spiciferae</em></td>
<td>FSU10234</td>
<td>CBS 138.95</td>
<td>NA</td>
<td>Texas</td>
<td>Human</td>
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<td>KJ408563</td>
<td>KJ408546</td>
<td>KJ408572</td>
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<tr>
<td><em>R. sexualis</em></td>
<td>FSU11355</td>
<td>CBS 102880</td>
<td>Homothallic</td>
<td>Italy</td>
<td>Leaf litter</td>
<td>230.87 ± 142.20</td>
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<td>KJ408549</td>
<td>KJ408573</td>
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<tr>
<td><em>R. stolomifer</em></td>
<td>FSU9872</td>
<td>NA</td>
<td>NA</td>
<td>Germany</td>
<td>Human</td>
<td>286.85 ± 146.22</td>
<td>KJ408557</td>
<td>KJ408540</td>
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</tr>
<tr>
<td><em>R. stolomifer</em></td>
<td>FSU763</td>
<td>DSM 63011</td>
<td>NA</td>
<td>Germany</td>
<td>Bread</td>
<td>555.41 ± 246.60</td>
<td>KJ408551</td>
<td>KJ408534</td>
<td>NA</td>
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<tr>
<td><em>Sporodiniella umbellata</em></td>
<td>FSU11407</td>
<td>CBS 195.77</td>
<td>NA</td>
<td>Ecuador</td>
<td>Membracidae</td>
<td>NA</td>
<td>KJ408566</td>
<td>KJ408547</td>
<td>NA</td>
</tr>
<tr>
<td><em>Syzygites megalocarpus</em></td>
<td>FSU728</td>
<td>NA</td>
<td>Homothallic</td>
<td>Germany</td>
<td>Mushroom</td>
<td>2412.29 ± 1712.94</td>
<td>KJ408550</td>
<td>KJ408533</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 1: List of species used in this study. Strain numbers, origin, mating type, spore size and sequences generated for identification are given. NA= not available.
<table>
<thead>
<tr>
<th>CBS number</th>
<th>Mating type</th>
<th>Geography</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS 339.62</td>
<td>Plus</td>
<td>Indonesia</td>
<td>Tempe</td>
</tr>
<tr>
<td>CBS 337.62</td>
<td>NA</td>
<td>Indonesia</td>
<td>Tempe ?</td>
</tr>
<tr>
<td>CBS 130971</td>
<td>Plus</td>
<td>Netherlands</td>
<td>Wood chips pile</td>
</tr>
<tr>
<td>CBS 699.68</td>
<td>Plus</td>
<td>Ukraine</td>
<td>Soil</td>
</tr>
<tr>
<td>CBS 130967</td>
<td>NA</td>
<td>Indonesia</td>
<td>Tempe</td>
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<td>CBS 130968</td>
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<td>Tempe</td>
</tr>
<tr>
<td>CBS 700.68</td>
<td>Minus</td>
<td>Georgia</td>
<td>Forest soil</td>
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<td>CBS 289.71</td>
<td>Plus</td>
<td>Italy</td>
<td>Starch-containing material</td>
</tr>
<tr>
<td>CBS 112588</td>
<td>Plus</td>
<td>Indonesia</td>
<td>Tempe</td>
</tr>
<tr>
<td>CBS 112586</td>
<td>Plus</td>
<td>Indonesia</td>
<td>Tempe</td>
</tr>
<tr>
<td>CBS 346.49</td>
<td>NA</td>
<td>NA</td>
<td>Eleusine coracana</td>
</tr>
<tr>
<td>CBS 631.82</td>
<td>Minus</td>
<td>China</td>
<td>Bread</td>
</tr>
<tr>
<td>CBS 537.80</td>
<td>Plus</td>
<td>South Africa</td>
<td>Sorghum malt</td>
</tr>
<tr>
<td>CBS 357.93</td>
<td>Plus</td>
<td>Java, Indonesia</td>
<td>Tempe</td>
</tr>
<tr>
<td>CBS 124669</td>
<td>Plus</td>
<td>Greece</td>
<td>Human</td>
</tr>
<tr>
<td>CBS 536.80</td>
<td>Plus</td>
<td>South Africa</td>
<td>Sorghum malt</td>
</tr>
<tr>
<td>CBS 359.92</td>
<td>Minus</td>
<td>Australia</td>
<td>Human</td>
</tr>
<tr>
<td>CBS 228.95</td>
<td>NA</td>
<td>Java</td>
<td>Tempe</td>
</tr>
<tr>
<td>CBS 343.29</td>
<td>Plus</td>
<td>USSR</td>
<td>Air</td>
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</table>

Table 2: List of isolates of *R. microsporus* used for extension of the virulence test in chicken eggs to survey isolate specificity. NA= not available.

*R. homothallicus* (mortality rate 65 %) and *R. caespitosus* (mortality rate dose-dependent 40-70 %) were moderately virulent. The most virulent strain tested here was *R. schipperae* with 97.5-100 % mortality as early as three days after infection.

To assess the variability of the virulence potential within a species, 19 additional strains of *R. microsporus* isolated from the environment, food and human patients (Table 2) were tested in chicken embryos. No significant difference was found regarding their origin and their potential to cause lethal infections. While some clinical isolates showed higher virulence than food isolates (e.g. CBS 124669 [human] vs. CBS 228.95 [tempeh] P= 0.5721), there were also isolates from tempeh with higher virulence compared to clinical isolates (e.g. CBS 339.62...
Adaptation to thermotolerance in *Rhizopus* [tempeh] vs. CBS 124669 [human] P=0.0428. Overall mortalities range between 60 to 100 % (average 80 %) for all strains.

<table>
<thead>
<tr>
<th>Relative growth [%]</th>
</tr>
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<tbody>
<tr>
<td>Species</td>
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<tr>
<td><em>R. microsporus</em></td>
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<tr>
<td><em>R. microsporus</em></td>
</tr>
<tr>
<td><em>R. microsporus</em></td>
</tr>
<tr>
<td><em>R. caespitosus</em></td>
</tr>
<tr>
<td><em>R. homothallicus</em></td>
</tr>
<tr>
<td><em>R. schipperae</em></td>
</tr>
<tr>
<td><em>R. arrhizus</em></td>
</tr>
<tr>
<td><em>R. arrhizus</em></td>
</tr>
<tr>
<td><em>R. lyococcus</em></td>
</tr>
<tr>
<td><em>R. stolonifer</em></td>
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<tr>
<td><em>R. lyococcus</em></td>
</tr>
<tr>
<td><em>R. sexualis</em>*</td>
</tr>
<tr>
<td><em>Syzgites megalocarpus</em></td>
</tr>
<tr>
<td><em>Sporodiniella umbellata</em></td>
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Table 3: Comparison of relative growth [%] under stress conditions compared to 100% growth without stressor. Cultures were incubated at 30 °C (*, **25 °C) and analyzed after 24 h (*48 h) after inoculation.

* temperature for growth was 25 °C. Time-point for measurement was 48 h because of the delayed growth at 24 h.

**… temperature for growth was 25 °C.

**Role of temperature adaptation**

Growth at elevated temperatures is known to be an important virulence factor in several fungal pathogens. To investigate if thermotolerance of the different species correlated with virulence in the embryonated egg model, growth at different temperatures was determined. A clear shift in the temperature profiles between the virulent and attenuated species was found. While the attenuated species grew well between 25 °C and 30 °C, the growth optimum for the virulent clade including *R. microsporus*, *R. arrhizus*, *R. caespitosus*, *R. homothallicus* and *R. schipperae* was 37 °C or higher. The mesophilic *R. stolonifer* and *R. lyococcus* were able to germinate at 37 °C, but did not grow well.
While thermotolerance is a prerequisite for a pathogen to cause infections in warm-blooded animals, additional virulence factors have been found to be involved in the infection process of fungal pathogens. To investigate whether the observed reduced virulence of the mesophilic *Rhizopus* species was caused only by their reduced thermotolerance, infection experiments were carried out using wax moth larvae. In contrast to the chicken embryos the larvae could be incubated at 30 °C, a temperature at which the growth rate of the mesophilic species was comparable to or even higher than for the thermotolerant species. *Rhizopus arrhizus* and *R. microsporus* as representatives of the thermotolerant species induced high mortality rates in *Galleria* (86-100 %) with *R. arrhizus* being significantly more virulent than *R. microsporus* (P<0.0001; Fig. 2). Yet, *R. arrhizus* was faster growing at this temperature than *R. microsporus*, eventually supporting faster spreading within the larvae. The tested isolates of *R. stolonifer* and *R. lyococcus* were significantly less virulent than *R. arrhizus* and *R. microsporus* (P<0.0001; Fig. 2). Despite the lower incubation temperature, the results from the *Galleria* experiments resemble those from the chicken model (Fig. 1), indicating additional adaptations supporting virulence of the thermotolerant *Rhizopus* species.

**Stress resistance and metabolic flexibility**

In addition to adaptation to temperature, coping with arising stress conditions in the changing host environment is an important feature affecting virulence in fungal pathogens. Therefore, resistance towards osmotic stress and cell wall stress was tested. Thermotolerant and mesophilic species showed comparable susceptibility to the different stressors and no correlation was found between stress resistance and virulence of the species.

In order to survive in the host, pathogens have to be able to acquire nutrients from the resources within the host. Thus, metabolic flexibility might influence virulence. Therefore, we analyzed the utilization of different carbon- and nitrogen sources by *Rhizopus* species. As primary soil inhabiting fungi, all species tested were able to utilize carbon sources originating from living or decaying plant material like xylose, xylitol, pectin, cellobiose and common sugars or sugar alcohols like glucose, fructose, galactose, mannose, mannitol and sorbitol. Maltose and starch could not be utilized by *R. stolonifer*, *R. sexualis* and *Syzygites*. *Sporodiniella* was unable to use soluble starch. None of the tested species could use the complex polysaccharides xylan or cellulose. *Rhizopus caespitosus* and *Syzygites* are the only fungal species tested capable to utilize citric acid, a common organic acid in mushrooms (Valentao et al. 2005).
Within animal hosts fermentable sugars like glucose, fructose or galactose have often limited availability. All of them can be assimilated by all *Rhizopus* species. For alternative carbon sources only the amino acids arginine, tyrosine and partially phenylalanine were exclusively metabolized by the virulent species. Most of the other amino acids could not be metabolized by any *Rhizopus* species.

A similar effect was observed when amino acids were used as sole nitrogen source. Thermotolerant species were generally able to utilize all 20 tested amino acids while mesophilic *Rhizopus* species lacked the ability to grow on several amino acids, including lysine, cysteine, histidine, isoleucine, threonine and valine. All other nitrogen sources tested revealed no obvious differences.

**Infection-related morphological features**

Since infections with *Rhizopus* species occur mainly in the respiratory tract, the small size of fungal spores may contribute to the success of fungal infections. In addition, fungal spore size is known to be related to fungal pathogenesis in *Mucor circinelloides* with larger spores being more virulent (Li et al. 2011). For the genus *Rhizopus*, spore size differs largely between species ranging from average volume of 28 µm³ to 555 µm³. Spores from thermotolerant species were in general smaller compared to spores from mesophilic species (Table 1). However, there was no correlation between spore size and virulence in the thermotolerant species.

In addition to spore size, the burden of fungal spores in the environment can be important for the development of mucormycoses as a high spore burden increases the likelihood that spores are inhaled in sufficient numbers to establish infection. In our artificial setting the relative amount of spores produced in a specific period of time differed considerably between *Rhizopus* species. Within the thermotolerant species *R. schipperae* and the homothallic *R. homothallicus* produced the lowest number of spores. Generally, homothallic species (also *R. sexualis*) produced less asexual spores than heterothallic species.
Figure 1: Virulence of different *Rhizopus* species in embryoated eggs. Chicken eggs were infected at developmental day 10 with $10^5$ (A) and $10^6$ (B) spores ($n=20$) of thermotolerant species (left) and mesophilic species (right). Survival was assessed daily over a period of six days post infection. Pooled results are shown as Kaplan-Meier-curves.

Figure 2: Kaplan-Meier-curves of age dependent survival of *Galleria mellonella* larvae infected with different *Rhizopus* spp.. 20 sixth-instar larvae per group were infected each with $10^6$ spores of thermotolerant species (left panel) and mesophilic species (right panel).
Discussion
All recent phylogenetic analyses strongly support separation of mesophilic and thermotolerant species of the Rhizopodaceae, although the relationship between the species in each supported group is not finally solved (Abe et al. 2006, Hoffmann et al. 2013, Walther et al. 2013). The mesophilic group contains species able to grow around 25 °C to 30 °C but displaying reduced growth rates at higher temperatures. However, the ability to grow at elevated temperatures of 37 °C or above, as seen for the thermotolerant Rhizopus species, is a prerequisite for colonization of warm-blooded hosts.

The genus Rhizopus exhibits the largest impact on mankind, being important in agriculture and industry and is furthermore the main causing agent of mucormycoses, followed by Lichtheimia and Mucor. The three genera are responsible for 70 to 80 % of the reported infections, predominantly as rhinocerebral, pulmonary or disseminated manifestations, and associated with high mortality rates (Skiada et al. 2011, Gomes et al. 2011). From the mesophilic species of the genus Rhizopus, only R. stolonifer can be found in clinical settings, but is seen rarely; mostly as agents of allergic alveolitis or superficial infections but being predominantly non-invasive (Ribes et al. 2000). The thermotolerant species R. arrhizus and R. microsporus are reported more frequently in severe infections than any other species from the Rhizopodaceae (Wimander et al. 1980, Scholer et al. 1983). In our study, R. schipperae, R. caespitosus and R. homothallicus displays a virulence potential comparable to that of R. arrhizus and R. microsporus (Fig. 1), yet they were only isolated rarely from human infections (Chakrabarti et al. 2010, Weitzman et al. 1996). This suggests that additional factors might be required for infection of humans. One of those aspects could be the abundance of species and the burden of fungal spores in the human environment. Although all species of Rhizopus are distributed worldwide, and the natural habitats are similar for nearly all species, like soil, or decaying organic matter including wood, and especially sugar-rich fruits, they are isolated from environmental samples with different frequencies. Rhizopus arrhizus is found most frequently, followed by R. stolonifer and less frequently by R. microsporus (Ribes et al. 2000). While R. arrhizus and R. stolonifer are found to similar extends, the majority of infections is caused by R. arrhizus (50 %) and R. microsporus (15-25 %) (Roden et al. 2005, Alvarez et al. 2009). This could be explained by the lower virulence potential of R. stolonifer observed in this study. In contrast, R. schipperae, R. caespitosus and R. homothallicus appear to be less abundant in the environment, judging from the few available specimens from public culture collections, or from the fact that R. schipperae is only known from two reported cases with no obvious natural substrate presented (Weitzman et al. 1996). Furthermore, R. schipperae fails to
sporulate on most artificial media, which was confirmed in this study. If sporulation is also low in natural habitats, this could explain the few known isolates. Furthermore, low numbers of spores in the environment would likely result in very limited exposure of humans to *R. schipperae*, thereby explaining the limited number of reported human infections despite the significant virulence potential.

No clear correlation between virulence and special nutritional requirements or differences in the ability to cope with stress was observed in our study. Whether the observed small differences in the profiles of C- and N-sources contribute to virulence remains to be determined. A recent study of pathogenic *Lichtheimia* species likewise identified only few differences in nutritional requirements between strains (Schwartze et al. 2012). For *Lichtheimia* and *Rhizopus* the carbon utilization profiles differ for raffinose, lactose, melibiose, inosine, glycine and pyruvate which could be utilized by *Lichtheimia* spp. but not by any *Rhizopus* species. On the other hand, *Rhizopus* species are able to use glycerol and ethanol, whereas species of *Lichtheimia* do not. Yet there are few amino acids which were exclusively used by thermotolerant *Rhizopus* species, a feature which could contribute to the survival within the host, but needs further studies.

Beside adaption to temperature or available nutrients, coping with arising stress conditions in the changing host environment affects virulence, as demonstrated for e.g. *Candida albicans* and *Aspergillus fumigatus* (Bates et al. 2005, Nakagawa et al. 2003, Duran et al. 2010). In our study, mesophilic species showed a trend towards higher tolerance to osmotic stress due to excess of sodium chloride, but without species-specific differences (Table 3). A similar concordance was observed between virulent and attenuated species of *Lichtheimia* (Schwartze et al. 2012). No obvious differences could be observed for cell wall stresses, although the applied stress conditions generally led to reduced growth compared to normal conditions in *Rhizopus*. Yet, this is less pronounced than in other mucoralean pathogens (Table 3). Nevertheless, no correlation between tolerance to stress and the observed virulence could be made, in contrast to virulence of evolutionary derived fungi like *Candida* (Bates et al. 2005, Nakagawa et al. 2003, Duran et al. 2010). Yet, pathogens of the derived fungi are often adapted to their hosts, whereas mucoralean fungi seem to be not, which could explain why there is not obvious difference in stress tolerance between potential pathogenic and non-pathogenic species.

An additional factor that could affect virulence of fungi is the ability to produce hydrolytic enzymes aiding in the degradation of host tissue, such as glycosidases, lipases and proteases. Previous comprehensive tests for the presence
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of gelatinase, urease, lipase, amylase, cellulase, laccase and tyrosinase within different isolates of *R. microsporus* sampled from various substrates (environment, food, clinical) revealed no differences in hydrolytic activity. In contrast, significant difference was observed in the production of the iron chelating compounds, the siderophores, by strains of food and clinical origin (Dolatabadi et al. 2014). As siderophores are important for iron acquisition of some pathogens within the host (Symeonidis et al. 2009), this observation suggests a link between siderophore production and clinical relevance of strains. However, no correlation between the presence of siderophores or the origin of the isolate and the observed virulence of *R. microsporus* strains was observed in this study (Dolatabadi et al. 2014).

Another interesting observation in recent studies on the virulence of Mucorales is the relation between differences in spore size and virulence, where larger spores produced by the minus mating type of *Mucor circinelloides* are more virulent than smaller spores produced by the plus mating type (Lee et al. 2013, Li et al. 2011). A second observation is that hyphal-stage of a fungus is more virulent than yeast-stage. Lee et al. (2013) demonstrated for the first time, that morphogenesis is also linked to virulence in zygomycetes. Comparing spore size with the genus *Rhizopus*, no correlation to virulence could be made. Further studies on species level will reveal if spore size in mucoralean fungi is related to virulence as demonstrated for *Mucor circinelloides*. Avian infection model-mediated virulence analysis yields objective results that overcome the disadvantages of mammalian infection models being time consuming, laborious and conflicting with ethic aspects. Therefore, it can be expected that the assessment of virulence of *Rhizopus* spp. applied to the embryonated chicken egg infection model will play a crucial role in future investigations of host-pathogen interactions by the utilization of knock-out mutant-based identification of virulence factors. Future experiments should also include various, distinctly related zygomycetes to elucidate comparability of virulence factors with the background of long time speciation of microorganisms not specifically adapted to warm blooded hosts.

**Acknowledgements**

We thank Birgit Weber (HKI, Jena) for excellent technical assistance in the performance of the virulence tests in the embryonated hen egg model and Caroline Hörttagl (Medical University Innsbruck) for valuable help with the *Galleria* infection model. We thank Domenica Schnabelrauch (MPI Chemical Ecology, Jena) for technical support in DNA sequencing. Research of INy and TP was supported by the grants OTKA PD101613 and OTKA NN106394, respectively.
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