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Food preparation with mucoralean fungi: A potential biosafety issue?

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ABSTRACT

Mucorales have been used for production of fermented food in Asia and Africa since time immemorial. Particularly *Rhizopus* species are rapidly growing, active producers of lipases and proteases and occur naturally during the first stages of soybean fermentation. Two biosafety issues have been raised in recent literature: (1) pathogenicity, *Rhizopus* species being prevalent opportunists causing erosive infections in severely compromised patients, and (2) toxicity, strains harbouring endosymbiotic *Burkholderia* producing toxic secondary metabolites. At the molecular level, based on different gene markers, species identity was found between strains used for food processing and clinical strains. In this study, we screened for bacterial symbionts in 64 *Rhizopus* strains by light microscopy, 16S rRNA sequencing, and HPLC. Seven strains (11 %) carried bacteria identified as *Burkholderia rhizoxinica* and *Burkholderia endofungorum*, and an unknown *Burkholderia* species. The *Burkholderia* isolates proved to be able to produce toxic rhizoxins. Strains with endosymbionts originated from food, soil, and a clinical source, and thus their presence could not be linked to particular habitats. The presence of *Burkholderia* in *Rhizopus* producing toxins could not be excluded as a potential risk for human health. In contrast, given the type of diseases caused by *Rhizopus* species, we regard the practical risk of infection via the food industry as negligible.

Introduction

Since ancient times fungi belonging to the order Mucorales have been used in the preparation of Asian and African traditional foods and condiments that are based on ground soybeans, with the aim to enhance microbial pre-digestion. For example, the natural flora of Indonesian tempe comprises lipase-producing *Rhizopus* species (Nout & Rombouts 1990).

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Chinese sufu is spontaneously mould-fermented by *Actinomucor* and *Mucor* species (Han *et al.* 2004), and *Mucor circinelloides* is used for starter cultures in Asian food (Hesseltine 1983; Nout & Aidoo 2010). Mucoralean species are involved in the natural colonization of Korean meju which is stored in open air during wintertime (Hong *et al.* 2012) and also play a role in the production of several kinds of cheese (Hermet *et al.* 2012). *Rhizopus* strains are commonly isolated from alcoholic beverages in Indonesia, China, and Japan (Schipper & Stalpers 1984). They are used at industrial scale for the production of hydrolytic enzymes and other metabolites (Hesseltine 1965; Rabie *et al.* 1985).

On the other hand, over the last few decades, we have seen a steady increase in prevalence of Mucorales fungi from the same taxonomic order in severe, often fatal, human infections (Skiada *et al.* 2011). In the past, such infections were regarded as highly coincidental, whereas today the Mucorales are listed as third most important opportunistic infectious agents after candidiasis and aspergillosis (Kontoyiannis *et al.* 2010). Their incidence is increasing in hosts with severe immune or metabolic impairment, e.g. due to hemomalignancy, haematopoietic stem cell transplantation or uncontrolled ketoacidotic diabetes mellitus, with an estimated incidence of 0.43 cases/10^6 persons per year in Spain (Torres-Narbona *et al.* 2007) and 1.7 cases/10^6 persons in the U.S.A. (Rees *et al.* 1998). Among Mucorales, *Rhizopus* species are the most common cause of mucormycosis (Ibrahim *et al.* 2012). Autopsy studies showed that *Aspergillus* and *Candida* infections were 10–50 fold more common in this patient population (Yamazaki *et al.* 1999), but mortality of mucormycosis is much higher (>50 %) (Quan & Spellberg, 2010) due to the acute clinical course of mucoralean infections. This makes mucormycosis one of the most important entities in clinical mycology.

It has often been claimed that clinical and foodborne strains belong to different species or variants (Jennessen *et al.* 2005), or at least that strains applied in food production could be regarded as specialized, domesticated industrial mutants. Six varieties were distinguished in *Rhizopus microsporus* [viz. var. *microsporus*, var. *azygosporus*, var. *chinensis*, var. *oligosporus*, var. *rhizopodiformis*, and var. *tuberosus* (Liu *et al.* 2007)] and two in *Rhizopus arrhizus* (var. *delemar* and var. *arrhizus*). Among these, var. *oligosporus* and var. *delemar* were considered to be the ones used in food preparation, whereas particularly var. *rhizopodiformis* was thought to be involved in human infection. However, Dolatabadi *et al.* (2014a,b, 2015a) showed that *R. microsporus* is genetically homogeneous, and that also in *R. arrhizus* clinical strains were identical to strains used in the preparation of food and condiments.

Another possible threat to food safety related to *Rhizopus* is the occasional presence of two types of toxins, viz. the cytostatic and antimitotic polyketide macrolide rhizoxin and the hepatotoxic cyclopeptide rhizonin (Jennessen *et al.* 2005). Rhizoxin and congeners are a family of 16-membered macrolactones first isolated from a plant-borne isolate of *R. microsporus* by Iwasaki *et al.* (1984), and which are potent anticancer drugs. The plant disease caused by the fungus is known as rice seedling blight, for which the characteristic symptom, i.e. abnormal swelling of seedling roots, is thought to be due to inhibition of cell division (Hong & White 2004). Rhizonin A is acutely toxic for ducklings and rats (Wilson *et al.* 1984) and affects mainly the liver and kidneys causing 100 % mortality (Rabie *et al.* 1985). The toxins were initially reported as the first "mycotoxins" from lower fungi, but they are actually biosynthesized by the endosymbiotic bacteria *Burkholderia rhizoxinica* or *Burkholderia endofungorum* (Partida-Martinez *et al.* 2007a) residing within the cytosol (Partida-Martinez & Hertweck 2005; Scherlach *et al.* 2006; Partida-Martinez & Hertweck 2007b; Partida-Martinez *et al.* 2007c; Scherlach *et al.* 2012). The toxin-producing bacteria are transmitted both vertically and horizontally in the *Burkholderia*–*Rhizopus* symbiosis (Lackner *et al.* 2009a), and host fungus and symbiont have likely evolved through co-speciation. Bacterial endosymbionts enter fungal spores during vegetative reproduction. Intriguingly, the reproduction of the host seems to be hijacked by the symbionts, with the host being unable to sporulate when endosymbiont has been removed (Partida-Martinez *et al.* 2007d). Endobacteria are able to infect compatible host organisms under laboratory conditions (Moebius *et al.* 2014).

The bacterial–fungal interaction has likely undergone a shift from parasitism to mutualism (Partida-Martinez *et al.* 2007c; Schmitt *et al.* 2008). The fungal host has gained insensitivity towards the antimitotic agent produced by the bacteria, whereas bacteria enhance their dispersal via fungal spores (Schmitt *et al.* 2008). On the basis of the endobacterial genome sequence, various factors have been identified that warrant the persistence of the symbiosis (Leone, *et al.* 2010; Lackner *et al.* 2011b, Lackner *et al.* 2011c). Bacteria may act as virulence factors for the fungus inside the host cell, eventually weakening or killing the tissue, and both fungus and symbiont benefit from nutrients acquired from the decaying material (Lackner *et al.* 2009b). The bacterial compounds are active antimitotic agents in the femto-to pico-molar range (Scherlach *et al.* 2006) and should be avoided for human consumption (Rohm *et al.* 2010).

The aim of the present paper is to evaluate possible health implications of the use of potentially invasive and toxin-producing fungi in the food industry. Species diversities and rates of differentiation of clinical, foodborne, and endosymbiotic strains were investigated, and the distribution of toxin-producing strains over all strains of the species was quantified. The presence of the symbiosis was measured in an *in vitro* study which comprised spontaneous *vs.* domesticated strains involved in food production.

Materials and methods

Strains

Sixty-four strains (*Rhizopus arrhizus* and *Rhizopus microsporus*) from the reference collection of the Centraalbureau voor Schimmelcultures (CBS-KNAW Fungal Biodiversity Centre) and the ARS Culture Collection (NRRL) were considered for bacterial symbiosis and toxin detection (Table 1). For preservation and serial transfer, 5 % Malt Extract Agar (MEA, Oxoid, Basingstoke, U.K.) in 8 cm culture plates were used and incubation was done at 30 °C for 2 d. Strains originated from different sources and from different continents.