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Typing and virulence factors of food-borne *Candida* spp. isolates

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ABSTRACT

Food-borne yeasts, excluding yeasts used as starter cultures, are commonly considered as food spoilage microorganisms. However, the incidence of non-*C. albicans Candida* (NCAC) infections has increased considerably over the past two decades. Although 15 *Candida* species are frequently identified as pathogens, a threat to human from food-borne *Candida* is poorly recognized. In the present study food-borne NCAC were characterized for the virulence factors, known to be associated with yeast pathogenicity. All food-borne strains in planktonic forms and 89% in biofilm structures represented biotypes established for *C. albicans*, and 61% demonstrated hemolytic activity. 56–94% of food-borne isolates formed biofilms on glass and biomaterials at a level comparable to clinical *C. albicans*. Nine out of eighteen tested food-borne NCAC strains (*C. krusei*, *C. lusitaniae*, *C. famata*, *C. colliculosa*, *C. parapsilosis*, *C. tropicalis*) showed similarity to clinical *C. albicans* in terms of their biotypes and the tested virulence factors, allocating them in a group of risk of potential pathogens. However, their capacity to grow at 37 °C seems to be the preliminary criterion in the study of potential virulence of food-borne yeasts.

1. Introduction

Among food-borne pathogens, which are responsible for food intoxication, toxicoinfection or infection, bacteria, viruses, parasites and mycotoxin-producing fungi are listed (Bhunia, 2008). Yeasts isolated from food, excluding yeasts used as starter cultures in food production, are considered as food spoilage microorganisms, but not food-borne pathogens (Perricone et al., 2017).

On the other hand, yeast from the genus *Candida* are associated with invasive fungal infections among at-risk groups and are recognized as a major cause of morbidity and mortality in the healthcare environment (Nur, 2014; Pappas et al., 2016). *Candida* species can colonize and cause disease at several anatomically distinct sites including skin, oral cavity, vagina, vascular system, liver, spleen, heart, valves or eye (Silva et al., 2012; Strollo et al., 2017).

Candida sp. represents normal microbial flora of human gastrointestinal (GI) tract or could be implemented to digestive system by the oral route with food. However, these opportunistic pathogens are considered as a possible reservoir of disseminated candidiasis, especially in compromised patients (Segal, 1994; Strollo et al., 2017). The main reservoirs of *Candida* sp. is the gastrointestinal tract and systemic infections predominantly originate from this niche (Dalle et al., 2010). The risk of deep-seated and systemic candidiasis originating from GI tract increases during antibacterial treatment, which changes the natural balance of microbiota, enabling fungal overgrowth and persorption even through undamaged mucosa (Segal, 1994). Cancer and anticancer treatment can damage GI mucosa, allowing the yeasts passage across the membrane into the bloodstream and their dissemination into the viscera. Furthermore, *C. albicans* is capable of adhering to, invading and damaging human oral epithelial cells, becoming a source of infection already in the mouth (Dalle et al., 2010).

Although, at least 15 *Candida* species are associated with human diseases, more than 90% of invasive candidiasis is caused by 5 most common pathogens, i.e. *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (Nur, 2014). However, other non-*C. albicans Candida* (NCAC) species such as *C. guilliermondii*, *C. lusitaniae*, *C. kefyr*, *C. famata*, *C. inconspicua*, *C. rugosa*, *C. dubliniensis*, *C. norvegensis* have shown gradual emergence as a cause of invasive candidiasis, as their isolation rate from medical specimen has increased up to 10-fold in the last two decades (Pfaller et al., 2014).

The pathogenicity of *Candida* species is mediated by a number of virulence factors, including adherence and biofilm formation, the ability to evade host defenses and the production of tissue-damaging hydrolytic enzymes, e.g. proteases, phospholipases and hemolysins (Nur, 2014). Filamentous growth of yeast is also believed to play an important role in tissue invasion. However, although the correlation between morphological forms and virulence has been demonstrated for *C. albicans* and some NCAC species, it has been found that hyphal transition is not requisite to invade host tissue by *C. glabrata* or *C. parapsilosis* (Silva et al., 2012).

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Among the factors known to contribute to the pathogenicity of yeast, enzymes play a significant role as they provide access to nutrients or affect the function and viability of the host cells (Chaffin et al., 1998). In this context, enzymatic biotyping based on the activity of 5 hydrolases: esterase, valine arylamine, naphtol-AS-BI-phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase seems to be a useful tool for epidemiological purposes (Brajer et al., 2005; Kurnatowska and Kurnatowski, 1998; Williamson et al., 1987).

Despite significant research concerning the virulence of *C. albicans* and NCAC species isolated from medical specimens, relatively little is known about the risk factors of food-borne *Candida* species. The potential threat posed by food-borne yeast, similarly to endogenous strains, resulted both from the virulent capabilities of the strain and host-related factors. In the case of yeasts present in food products, the amount of yeast ingested with food is also crucial. Although minimum infectious doses, useful in assessing the risk of consuming certain foods contaminated with pathogens, are known for bacterial and viral pathogens, these values have not been determined for food-borne yeasts.

Previously, we demonstrated a high homology of clinical and food-borne *Candida* isolates in relation to their biochemical profiles, susceptibility to anticandidal agents and hydrophobic properties (Maroszyńska et al., 2013; Rajkowska et al., 2017) that justifies the hypothesis of a potential threat to humans from this little recognized group of yeasts.

A quantitative measure of the pathogenicity as the likehood of causing disease was evaluated in in vivo models for different microorganisms (Auerbuch et al., 2001; de Emery et al., 2017; Pilatti et al., 2016; Yoon et al., 2011). To minimize an animal testing the attempts of correlation of microorganism virulence factors and the pathogenicity have been undertaken. As a result quantitative PCR-based competitive index for *Salmonella* (Yoon et al., 2011), competitive index assay to evaluate the virulence of *Listeria monocytogenes* (Auerbuch et al., 2001) or molecular pathogenicity indexes for *Pasteurella multocida* (Furian et al., 2016) have been proposed. To express the strength of virulence factors of *C. albicans* and NCAC species in vitro some indexes are used with a proteinase index and a hemolysin index as the most common (Deorukhkar and Saini, 2015; Seneviratne et al., 2016). In a variety of research on *Candida* the tendency of numerical evaluation or calculating the specific values characterizing virulence features (e.g. phospholipase activity, esterase activity, adherence to buccal epithelial cells, biofilm formation) can be observed (Deepa et al., 2015; Deorukhkar and Saini, 2015; Rossoni et al., 2013; Seneviratne et al., 2016). However the numerical graduation of *Candida* virulence factors is disputable due to the differences in the answer of the host cells, their comparative analysis in a set may serve a useful tool in the screening procedures.

The aim of the study was to assess virulence factors associated with genetically characterized food-borne NCAC species in terms of their enzymatic biotypes, hemolytic activities and biofilm formation. In the present study the comparative assessment of the chosen *Candida* virulence features was carried out in relation to clinical *C. albicans* and by the use of statistical analysis.

2. Material and methods

2.1. Yeast

In the present study, 18 food-borne *Candida* isolates (Table 1) were tested in comparison to two clinical isolates: *Candida albicans* ATCC 10231 isolated from a man with bronchomycosis (American Type Culture Collection) and *C. albicans* cl/MP/01 isolated from faeces of a patient with symptoms of candidiasis (Department of Laboratory Diagnostics of Polish Mother's Memorial Hospital - Research Institute in Lodz, Poland).

All yeast isolates were previously characterized according to their biochemical profiles (Maroszyńska et al., 2013). Subcultivation of yeasts for tests was carried out on Sabouraud dextrose chloramphenicol agar (SDA, Merck KGaA, Germany) at 30 °C for 24 h.

Table 1
Origin of tested food-borne isolates.

Species	Origin	Isolate
C. lusitaniae	Fruit yoghurt	fo/82/1, fo/79/1
	Feed	LOCK 0004, LOCK 0006
C. krusei	Pickled cucumber	fo/BM/02, fo/MP/02
	Bakery	LOCK 0008, LOCK 0009
C. boidinii	Pickled cucumber	fo/BM/03, fo/MP/01, fo/MP/03
C. famata	Fruit yoghurt	fo/82/2
	Herring salad	fo/LI/02
C. colliculosa	Fruit yoghurt	fo/KO/02
C. parapsilosis	Fruit yoghurt	fo/82/3
C. tropicalis	Pickled cucumber	fo/BM/01
C. pelliculosa	Feed	LOCK 0007
C. rugosa	Sauerkraut	fo/BG/05

2.2. Chromosomal DNA isolation and analysis

Chromosomal DNA isolation was conducted using a CHEF Genomic DNA Plug kit (Bio-Rad, Poland) according to the method described by Schwartz and Cantor (Schwartz and Cantor, 1984). Chromosomes were separated by pulsed field gel electrophoresis in 0.8% agarose gel with 0.5 × TBE running buffer (45 mM Tris, 45 mM boric acid, and 10 mM EDTA; pH 8.2), at 12 °C, using a CHEF-DR II system (Bio-Rad, Poland). The program parameters were the following: 120 s for 24 h, and 240 to 360 s for 24 h, all at 4,5 V/cm. Separated chromosomes were post-stained with 50 μg/ml ethidium bromide (Sigma-Aldrich, USA).

2.3. Hemolysis

Hemolytic activity was determined on Sabouraud dextrose agar supplemented with 3% glucose and containing 7 ml of fresh sheep blood (GrasoBiotech, Poland) per 100 ml of medium (pH 5.6), according to Luo et al. (2001). The isolates' suspensions were prepared in sterile saline (0.85% NaCl) to a turbidity corresponding to 2 in the McFarland scale (10^8 cells/ml). Ten microliters of these suspensions were spotted on the sugar-enriched sheep blood agar medium so as to yield a circular inoculation site of about 10 mm in diameter. The plates were incubated at 37 °C in 5% CO_2 for 48 h. In the case of beta hemolysis, hemolytic activity was evidenced by the translucent halo around growing yeasts, and expressed by a hemolytic index, calculated by dividing a diameter of the colony plus the hemolysis zone by the colony diameter.

2.4. Growth at different temperatures

For the viability measurements, ten microliters of the yeasts suspensions were prepared and inoculated on SDA plates as described above for hemolysis assay. The plates were incubated at 37 or 42 °C for 24–72 h. The appearance of yeast colonies indicated their ability to grow at the tested temperatures.

2.5. Biofilm formation

The biofilm-forming ability of yeasts on different surfaces (glass, polypropylene, polyethylene terephthalate) was analysed according to Baillie and Douglas (1999). Briefly, *Candida* strains were grown in liquid Sabouraud medium (Merck KGaA, Germany) over 24 h at 30 °C. Biomass was then washed twice with PBS buffer (NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.144%, KH_2PO_4 0.024%; pH 7.4) and adjusted to a cell density of 10^6 cells/ml. A 2 ml aliquot of the suspensions was inoculated onto the material slices (20 mm × 20 mm) placed in 6-well tissue plates (Greiner Bio-One GmbH, Germany) and left for 90 min at 30 °C in an orbital shaker (75 rpm) to adhere. The plates were rinsed