Commercial strain-derived clinical *Saccharomyces cerevisiae* can evolve new phenotypes without higher pathogenicity

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Abbreviations: AmB, amphotericin B; **Flu**, fluconazole; **gDNA**, genomic DNA; **MIC**, minimal inhibitory concentration; **MSB**, menadione sodium bisulfite; **mtDNA**, mitochondrial DNA; **PBMC**, Peripheral blood mononuclear cells; **TNF-α**, Tumor necrosis factor alpha.

Keywords: baking yeast, commensal, microevolution, mycobiom, probiotic

Abstract

Scope: *Saccharomyces cerevisiae* is one of the most important microbes in food industry, but there is growing evidence on its potential pathogenicity as well. Its status as a member of human mycobiome is still not fully understood.

Methods and results: In this study, we characterise clinical *S. cerevisiae* isolates from Hungarian hospitals along with commercial baking and probiotic strains, and determine their phenotypic parameters, virulence factors, interactions with human macrophages, and pathogenicity. Four of the clinical isolates could be traced back to commercial strains based on genetic fingerprinting. Our observations indicate that the commercial-derived clinical isolates have evolved new phenotypes and show similar, or in two cases, significantly decreased pathogenicity. Furthermore, immunological experiments revealed that the variability in human primary macrophage activation after co-incubation with yeasts is largely donor- and not isolate-dependent.

Conclusion: Isolates in this study offer an interesting insight into the potential microevolution of probiotic and food strains in human hosts. These commensal yeasts display various changes in their phenotypes, indicating that the colonization of the host does not necessarily impose a selective pressure towards higher virulence/pathogenicity.

1. Introduction

The roles of *Saccharomyces cerevisiae* in producing fermented beverages and in breadbaking have made this yeast one of the most important microbes throughout human history [1, 2]. *S. cerevisiae* is a domesticated species with different clades adapted to different fermentation technologies [1, 3]. Its use in various branches of the food industry leads to frequent human exposure to *S. cerevisiae*, and besides probiotics, food-borne yeasts may also have beneficial health effects [4]. The potential of this yeast to colonize the human body has long been suspected, although its status as a transient species [5, 6] or established nature [7, 8] in the human mycobiome is still unclear. *S. cerevisiae* colonization of the gastrointestinal tract may be locally frequent [9].

Although *S. cerevisiae* is generally recognized as safe, its colonization of the human body, whether transient or long lasting, may occasionally result in infections (mycoses) [2, 10, 11]. Opportunistic *Saccharomyces* infections have become known and reported from patients from a wide range of age groups and underlying diseases [12–16], including systemic mycosis in immunocompromised patients [17]. Multiple methods are in use to characterize the traits of opportunistic pathogenic yeasts, and many of them are referred to as pathogenicity or virulence factors. However, often there is a considerable overlap in clinical and non-clinical strains in virulence traits, as recently reviewed [18]. Besides surveying virulence factors and their correlation with pathogenic potential, understanding the origin of human-related *S. cerevisiae* isolates and their relations to the different industrial and natural clades of the species can help us to shed light on how this yeast might evolve commensalism or pathogenicity.

Recently, molecular phylogenetic investigations on the global diversity of the species have identified human-adapted *S. cerevisiae* clades that have arisen from multiple opportunistic

colonization events [2, 3, 19, 20]. Most of the clinical yeasts were shown to be genetic mosaics, but several isolates were nested in the non-hybrid Wine/European clade of the species [2].

Opportunistic pathogenic *S. cerevisiae* isolates derived from baking yeasts have also been identified from patients with or without occupational exposures to baker's yeasts [21–27], although in several cases, the clinical isolates were plausibly commensal [22]. Potential risks of food yeast products were recently reviewed [28].

Growing evidence on the pathogenic potential of probiotic-derived *S. cerevisiae* opportunistic pathogens is also available. *S. cerevisiae* probiotics and biotherapeutic agents are marketed as '*S. boulardii*' and advised for patients with dysentery [29]. These strains are asporogenous natural isolates, with moderate [30] or often considerable virulence factors [31, 32]. Fungaemia related to a commercial probiotic strain was reported in an intensive care unit and in the case of premature neonates [33, 34]. Consequently, caution was proposed for immunosuppressed patients regarding the use of such probiotics [14, 31]. In the case of immunocompetent hosts, intravenous inoculation with probiotics caused septic shock that was spontaneously cured, indicating that these strains are not highly virulent pathogens [35, 36].

Regardless of whether different *Saccharomyces* isolates of the human mycobiome are exogenous opportunists or true commensals in the gut, studying their adaptations related to virulence, possible antimycotic resistance or their interactions with the immune system is important. In our study, we characterized clinical *S. cerevisiae* isolates from Hungary along with dietary and probiotic strains available in the country with genotyping methods and assessed their various phenotypic features and immunostimulatory activity, in order to determine whether the clinical isolates can be traced back to the commercial strains and if so, whether their phenotypes show consistent differences. Using the characterization of

commercial strains and four genetically related isolates, we show that these commercial-derived clinical *S. cerevisiae* are apparently subjected to microevolution in the human host.

2. Materials and Methods

Strains, isolates and identification. Isolates were obtained as single-cell colonies from patients in the university clinics in Debrecen and Szeged (Hungary) (isolation data is listed in Supplementary File S1). Strains of baker's, wine and probiotic yeasts were obtained commercially and were given individual identifiers (Supplementary File S1).

Genetic characterization. Interdelta fingerprinting [37] was used for strain typing and applied to establish genetic relatedness of strains and isolates. The strain-isolate pairs with identical or near identical patterns were considered having a very recent common (commercial) ancestor. These yeasts were further subjected to karyotyping, RAPD-PCR, microsatellite PCR, and mitochondrial DNA-RFLP to confirm their genetic similarity and close relatedness. Methods for typing are listed in Supplementary File S1.

Phenotypic characterization. The sporulation capability, colony morphology, pseudohyphal and invasive growth, susceptibility to antifungal and oxidative stress generating agents, growth under osmotic, chemical and high temperature stress conditions, extracellular hydrolytic enzyme production, and hemolytic activity were assessed for all yeast isolates and strains to provide a broad overview of their phenotypic characteristics, with methods described in Supplementary File S1 in detail.

116 *Damage assay* was conducted using a Cytotoxicity Detection Kit - LDH (Hoffmann-La
117 Roche, Basel, Switzerland) with Caco-2 cell monolayer in 96-well plates following the
118 manufacturer's protocol as described in Supplementary File S1.

119 *Insect pathogenicity model.* The *Galleria mellonella* larva pathogenicity model [38] was used
120 to assess the pathogenicity of the strains and isolates *in vivo*. This model enables the rapid
121 assessment of the pathogenicity of opportunistic fungi (e.g. *Candida* spp) on a large number
122 of infected hosts and is comparable to murine models [39]. Details of the experiments are
123 listed in Supplementary File S1.

124 *Immunological experiments.* Phagocytosis assay using flow cytometry and phenotypic and
125 functional analysis of yeast-exposed human primary macrophages differentiated from four
126 healthy donors' PBMCs were used to assess immune activation by the individual strains and
127 isolates. Experimental procedures and PBMC isolation and macrophage differentiation are
128 described in Supplementary File S1.

129 *Statistics.* Measurements carried out in triplicates (hemolysis, extracellular enzyme activity,
130 damage and phagocytosis assays) and quadruplicates (macrophage phenotype) allowed
131 statistical comparisons among different yeast strains and isolates, as described in
132 Supplementary File S1.

133

134 **3. Results**

135

136 *Fingerprinting and relations among the strains and isolates*

For strain typing, the resolution of interdelta fingerprinting and microsatellite typing for two loci were compared. All three combinations of interdelta PCR resulted in distinct fingerprints for all commercial yeasts, while both microsatellite analyses were unable to distinguish between two of the baking strains (BY2 and BY3) (Supplementary File S2). We thus relied primarily on interdelta fingerprinting to assess the genetic relatedness among the commercial strains and the clinical isolates, similarly to previous works [26-27]. The strains and isolates with identical or highly similar fingerprinting patterns (Figure 1 a-c) were further characterized by microsatellite typing, RAPD-PCR, mtDNA-RFLP, and karyotyping (Figure 1 d-h).

The combined use of three primer combinations for interdelta-PCR resulted in a dendrogram that showed the following clusterings of commercial and clinical yeasts: the isolate Sz1 (from cervix) with the baking strain BY1; the isolates Sz7 and Sz9 (from stomach and throat, respectively) with BY2; and isolate DE27020 (from bronchus) with PY1 (Supplementary File S1-2). Pattern identity (isolates Sz7, Sz9, DE27020) or high similarity (isolates Sz1) between commercial strains and certain clinical isolates strongly implies common ancestry, meaning that these clinical isolates are derived from commercial yeasts and share very recent common ancestors with the given batches of commercial yeasts analyzed in this study. The common ancestors of the commercial-clinical counterparts are plausibly batches of the same commercial yeasts manufactured earlier (especially in the case of yeasts with pattern identity), or closely related yeasts commercialized under different trade marks. Furthermore, the isolates Sz8 and DE27290 were also found to group together with baking strains BY1, BY2 and BY3 (Supplementary File S2), implying some level of genetic relatedness.

Microsatellite, mtDNA-RFLP and RAPD-PCR band patterns were identical in the case of all commercial-clinical counterparts identified by interdelta analysis. However, mtDNA-RFLP patterns were also indistinguishable between BY1 and BY2. Microsatellite analysis identified

tri- and tetraallelic strains and isolates (Supplementary File S2), suggesting poly- or aneuploidy. Karyotyping produced distinct chromosomal band patterns for the two baking and the probiotic yeast (Figure 1). All baking yeasts and their clinical counterparts possessed supernumerary bands in the region of small chromosomes (chromosomes I, VI and III): two extra bands in the case of BY1 and one in Sz1, and two bands in the case of BY2 and its counterparts (identical among these yeasts) (Supplementary File S2). Chromosome II showed size polymorphism when BY1 and Sz1 were compared. An additional supernumerary band was observed for BY2 and its counterparts between chromosomes V and XI (Supplementary File S2). Thus, BY2 and its counterparts Sz7 and Sz9, and PY1 and its counterpart DE27020 showed identical karypotypes, whereas BY1 and Sz1 showed occasional polymorphism in chromosome sizes.

Characteristics of clinical isolates related to baking yeast strains.

Data on phenotypic features and virulence factors enabled us to compare clinical isolates and their commercial counterparts (the clinical isolates and the commercial strains that showed the highest genetic similarity, in some cases identity, with these, viz. PY1 and DE27020; BY1 and Sz1; BY2 and Sz7, Sz9). Table 1 highlights the phenotypic differences between these strain/isolate pairs.

The isolate Sz1, as compared to the baking yeast BY1 (Table 1), showed decreased sporulation rate (40% *vs.* 90%). The pseudohyphae of Sz1 were short, while BY1 possessed long chain-like pseudohyphae. Sz1 showed reduced growth at 39°C compared to the baker's strain (Supplementary File S3). Tolerance of sulfite and pH8 stress was higher in Sz1. Phospholipase activity was not significantly different at 30°C, but at 37°C the baker's strain showed a significantly higher activity ($p<0.01$). Higher protease activity ($p<0.05$) and β

186 hemolytic activity (p<0.01) were found in the clinical isolate. Pathogenicity in the insect
187 model, as well as macrophage phenotypes and phagocytosis after exposure to BY1 and Sz1
188 were highly similar (Table 1, Supplementary Files S3-S5).

189 Differences among the clinical isolates Sz7 and Sz9 and the baking strain BY2 included
190 colony morphology, sporulation ability, cell size, invasive growth into the agar medium, and
191 tolerance of high temperature (Table 1, Supplementary File S3). Tolerance of sulfite and NaCl
192 stress was elevated in the case of both clinical isolates. Copper stress tolerance was decreased
193 in Sz9. Both clinical isolates were unable to grow at pH 8, while BY2 displayed weak growth
194 (Supplementary File S3). Phosholipase activity of the baker's strain at 37°C was significantly
195 higher than that of Sz7 (p<0.05). At 30°C, BY2 did not display lipolytic activity, while both
196 clinical isolates were lipolytic. Proteolytic activity of Sz9 was significantly elevated (p<0.05).
197 The α-hemolytic activity was elevated in the case of Sz9 and lower in the case of Sz7 after 1 d
198 (p<0.05), and elevated in the case of Sz7 after 2 d (p<0.05). β-hemolytic activity was
199 significantly higher in the case of Sz7 (p<0.01) and Sz9 (p<0.001) (Table 1). Pathogenicity in
200 the *Galleria* larvae showed remarkable differences between these yeasts. The strain BY2
201 killed 30 % of the inoculated larvae and the survival rates of Sz7 and Sz9 were significantly
202 higher: Sz7 killed only 5 % of the larvae (p<0.05) and all larvae inoculated with Sz9 survived
203 (p<0.01) (Supplementary File S5). Significant differences were not found in the case of
204 macrophage phenotypes and phagocytosis after exposure to BY2 and the two isolates
205 (Supplementary Files S3-4).

206

207 *Characteristics of the clinical isolate related to the probiotic yeast.*

208 The isolate DE27020 showed minor differences in phenotypes when compared to the
209 probiotic strain PY1 (Table 1). At 39°C, DE27020 showed stronger growth than PY1

(Supplementary File S3). Lack of sporulation, colony and pseudohyphal morphology, and cell size were remarkably similar (Supplementary File S3). The clinical isolate's tolerance to sulfite and lithium stress was elevated compared to PY1, while other tested stress factors did not show such differences. Extracellular hydrolase production was highly similar for both yeasts. Damage assay conducted on Caco-2 epithelial cells revealed an elevated cell damage (p<0.01) in the monolayer when exposed to DE27020. However, both yeasts caused relatively low cell death when compared to the high control (Table 1, Supplementary File S3). Pathogenicity in the larva model was similarly very low in the case of both yeasts (Supplementary File S5). No significant difference was found when the extent of phagocytosis or macrophage phenotypes were compared (Table 1, Supplementary Files S3-4).

4. Discussion

To determine if any of the clinical isolates can be traced back to commercial *S. cerevisiae* strains used in the food industry, we applied interdelta genotyping. This method has been applied to identify commercial derived clinical isolates before [26-27] and our results showed that it has a better resolution as microsatellite typing, mtDNA-RFLP or RAPD also applied in this study. We identified three clinical isolates as baker's yeast- and one as probiotic-derived, and the similarity between the genotyping profiles enabled us to propose very recent common ancestry for these clinical yeasts and three of the commercial yeast strain batches used in this study (common ancestors are plausably batches of the same commercial yeasts manufactured earlier, especially in the case of identical genotyping markers, or genetically highly similar commercial yeasts). This genetic relationship among certain isolates and baker's or probiotic strains was further backed by identical microsatellite alleles, identical RAPD-patterns,

identical mtDNA-RFLP profiles and identical or highly similar karyotypes. The presence of two to four microsatellite alleles, occasional changes in interdelta fingerprints, and the small variations in karyotypes in the case of the baker's yeast BY1 and its clinical counterpart Sz1 suggests that these yeasts possess an polyploid/aneuploid genome that is more prone to changes than that of the PY1 probiotic yeast's and its clinical counterpart's genome. This observation of occasional variability that is well known among yeasts subjected to microevolution in different environments [40,41 and references therein] is also in concordance with the fact that the baker's yeasts and their counterparts are capable of sporulation, while PY1 and DE27020 are asporogenous. Additionally, some closely related clinical isolates of undetermined affilities (*viz.* Sz2, Sz3, Sz4 and Sz5) also showed no sporulation (or <1 % sporulation) and these isolates also displayed some differences in phenotypes but had identical interdelta profiles (Supplementary File S2-5).

Each of the commercial-derived clinical isolates in this study were sampled from different patients, from anatomical parts such as cervix, stomach, throat, and bronchus, on different dates and thus represent individual colonization events. None of the patients were diagnosed with *S. cerevisiae* fungaemia, thus their isolates may have been either (i) pathogens not causing fungaemia at the time of sampling; (ii) non-pathogenic yeasts with commensal adaptations; or (iii) recently ingested commercial strains transiently associated with the patients. The latter scenario is unlikely as all isolates displayed various phenotypes and often some level of genetic variability when compared to the genetically most similar commercial yeasts, thus these isolates have apparently already been subjected to microevolution after the colonization of the hosts. Additionally, the *Galleria* pathogenicity model did not indicate high pathogenicity for the isolates, thus the most likely scenario is a commensal lifestyle.

Phenotypic characterization not only revealed differences between the commercial strains and their clinical counterparts but also among the clinical isolates, including differences between

Sz7 and Sz9, two isolates genetically indistinguishable from BY2 (Table 1). Elevated or impaired growth at higher temperatures, a possible virulence factor of the species [42,43] and other opportunistic fungi as well, was both found in the case of the isolates, without changed invasive growth. Similarly to clinical isolates tested previously [43], none of the yeasts in this study were able to grow at 42°C. As noted before [18], growth and phenotype at temperatures higher than 37°C may be important, but not crucial in virulence. Sulfite tolerance, interestingly, was uniformly elevated in the clinical isolates. Only one isolate displayed changes in lithium and and one in copper tolerance. It is unclear how these traits are related to pathogenicity but higher lithium and copper tolerance have been found among pathogenic yeasts [44, 45]. Susceptibility testing to antimycotics or MSB showed only minor differences among the strains and their clinical counterparts.

Differences in the production of extracellular hydrolytic enzymes were commonly observed: isolates either showed reduced or unaltered phospolipase activities and elevated or unaltered protease activities at the host's physiological temperature. Hemolytic activity was more pronounced in the case of all baker's yeast derived isolates (Table 1). Damage assay with Caco-2 epithelial cells revealed significant differences only in one strain–isolate pair (PY1 and DE27020).

Pathogenicity of the yeasts in this study was assessed using the *Galleria* larva model, which is capable of providing generalized information on the pathogenicity of a wide range of microorganisms [39, 46]. In our study, only few yeasts were able to significantly decrease larval survival (Supplementary File S5). PY1 and DE27020, BY1 and Sz1 had no significant effect on the larvae's survival. Significant pathogenicity was found in the case of the baker's strain BY2, while it's clinical counterparts were non-pathogenic (isolates Sz7 and Sz9).

Phagocytosis experiments and characterization of the macrophage activation were carried out using primary macrophages from three and four (respectively) different healthy donors. Macrophages are known for their phagocytic activity and antigene presentation in peripheral tissues, and understanding how they form the first line of defence during yeast infections is crucial [47, 48]. Our experimental design allowed us to evaluate if the macrophage responses to yeasts are mostly dependent on the individual strain/isolate or the individual donor. The latter scenario is backed by our data, as high variances between individual donors were found (Supplementary Files S3-4). The macrophages of four different donors not only showed remarkable differences in their activation and phenotypes after co-incubation with a given yeast, but many yeasts induced relatively weak activation in the case of some donors while inducing very high relative activation in others. This variability was marked for most of the tested macrophage features when all 19 yeasts were compared (Supplementary File S4). These donor-dependent differences (that apparently mask strain and even strain-group level differences in macrophage activation) have previously not been uncovered, as experiments are usually conducted using one donor – one yeast strain setups [49, 50]. As proinflammatory processes have been associated with increased virulence of the species [46], donor dependent differences in phagocyte activation may be important in future studies on virulent and avirulent *S. cerevisiae*.

Relatively rapid microevolutionary changes that influence the biology of opportunistic fungi have been found previously [51] in the case of other species. To the best of our knowledge, our results are the first to imply that commercial-derived *S. cerevisiae* baker's yeasts may evolve under an apparently commensal lifestyle with altered phenotypes but without elevated pathogenicity (often clearly decreased pathogenicity). As humans may come into contact with industrial strains of fungi on a daily basis, assessing the phenotypes, epidemiology and the potential evolution towards a pathogenic or commensal lifestyle of these fungi is relevant

from the aspects of clinical as well as evolutionary microbiology. Yeasts are often excreted/cleared from the human body but their fate is apparently more complex [36, 52, 53]. Yeasts may also become commensal or opportunistic. These possibilities are summarized in Figure 2. The phenotypic features including virulence factors observed in the cases of the commercial-derived isolates in this study might represent responses to adaptive pressures inside the human host (*e.g.* changed stress tolerance) or, on the contrary, a relaxed selection compared to the circumstances under industrial strain selection (*e.g.* decreased phospholipase activity).

Yeasts, especially *S. cerevisiae* are species of clinical interest and simultaneously model organisms in this field. Here, we have found indication that commercial food strains of the species are capable of persisting as commensals in different niches of the human body for periods long enough to allow microevolutionary changes, resulting in isolates possessing genetic markers identical to, or very similar to commercial strains, but having distinct phenotypes. The diverse phenotypes of commercial-derived isolates suggest no selective pressure towards higher virulence and pathogenicity during this process. Further studies involving phenotypic analysis and genomics of different *Saccharomyces* strains and isolates may provide an unprecedented insight into how our most widely used domesticated microbe evolves in human hosts and help us to better understand its pathogenic potential.

Author contributions

W.P.P. and I.P. designed project and experiments. E.U., R.K. & L.M. collected and identified isolates, and collected patient data. W.P.P., E.B., Á.J. & I.Zs conducted phenotypic experiments. K.P., A.B., W.P.P. & E.B. conducted immunological experiments. W.P.P., E.B.,

Z.A., & M.S. conducted and evaluated genetic experiments. W.P.P., I.Zs. & E.B. compiled literature data. W.P.P., E.B. & I.P. wrote the article.

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Conflict of Interest

The authors declare no conflict of interest.

5. References

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Figure 1.

Comparison of commercial yeasts' and their clinical counterparts' genotyping profiles. a: interdelta fingerprinting with primers delta1-2. b: interdelta fingerprinting with primers delta12-2. c: interdelta fingerprinting with primers delta12-21. d: RAPD pattern with primer 24. e: microsatellite typing for locus YPL009c. f: microsatellite typing for locus YOR267c. g: mtDNA-RFLP with enzyme RsaI. h: electrophoretic karyotype. Lane 1: BY1. Lane 2: Sz1. Lane 3: BY2. Lane 4: Sz7. Lane 5: Sz9. Lane 6: PY1. Lane 7: DE27020.

Figure 2.

Possible outcomes of the intake of *S. cerevisiae* probiotic and baker's strains: clearence, commensal, or pathogenic persistence with studies describing these highlighted. Question mark represents inconclusive records (*e.g.* the pathogenicity of isolates was not estimated).

Table 1.

Phenotypic differences in commercial–clinical yeast pairs. Each clinical isolate is compared to the commercial counterpart. Significant changes in the obtained continuous values, >twofold differences in MIC values, and differences in stress tolerance, pseudohyphal type, etc. are indicated.

Supplementary File S1.

Supplementary File 1: Supplementary materials and methods.

Supplementary File S2.

Supplemtary File S2. Comparison of commercial strains using interdelta and microsatellite typing; interdelta genotyping and dendrogram of the strains and isolates; chromosomal band patterns and chromosome polymorphisms of the strains and their clinical counterparts.

Supplementary File S3.

Supplementary file 3. Phenotypic features and macrophage interactions of the commercial strains and clinical isolates (measurements).

Supplementary File S4.

Macrophage activation and extent of phagocytosis of the commercial strains and clinical isolates. Donor-dependent differences are visualized as web graphs of obtained fold-change values. Phagocytosis visualized with confocal laser scanning microscopy is presented for the strain PY1.

Supplementary File S5.

Survival data and statistics of larvae inoculated with different commercial yeast strains and clinical isolates.