SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Revealing the causes of *Saccharomyces cerevisiae* var. *'boulardii'* probiotic yeast fungemia by examining fungal virulence factors

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1. INTRODUCTION

The yeast *Saccharomyces 'boulardii'*, which is in fact a subtype of *S. cerevisiae*, has been produced and marketed since the 1950s against gastrointestinal complaints (antibiotic-related or other types of diarrhea). In addition, probiotic yeast can be used successfully to prevent *Clostridioides diffile* infection, alleviate the symptoms of irritable bowel syndrome (IBS), and *Helicobacter pylori* infection. Its health benefits are supported by more than 80 randomized clinical trials based on a 2017 summary meta-analysis, and that number has been steadily rising ever since. However, its use is contraindicated in groups of patients with compromised immune system (elderly, infants, those receiving immunosuppression, HIV-infected patients) due to the high risk of fungemia. Despite the contraindication, it is common for a patient at risk to receive yeast probiotic treatment, leading to an increase in the number of yeast probiotic-induced fungaemic diseases in recent years. Such cases have been reported in the literature in the form of case studies. However, these reports are largely unrepresentative due to the lack of identification of the *S. 'boulardii'* subtype. Subtype-level identification is essential to clearly associate a *Saccharomyces* fungemia with the probiotic product itself. In addition, the number of studies investigating the mechanisms of infection and the virulence factors of probiotic yeast is negligible. The present dissertation aims to fill these gaps with the greatest possible scientific thoroughness.

Research to date has revealed that probiotic yeast, like other *S. cerevisiae*, can be manipulated relatively easily and reliably by molecular genetic methods, making it a promising candidate for a new generation of probiotics and biotherapeutic agents. It is expected that with the advent of these molecular methods, there will be an increase in research into the further development of probiotics to enhance their health effects or create new beneficial properties. However, these so-called "designer" probiotics will definitely need to be tested for safety, for which a detailed knowledge of the course of the infections they cause and the potential virulence factors of the probiotics is essential, to which the results described here can greatly contribute.

2. AIMS

The main aim of our work was to study the mechanism of *S. ‘boulardii’* probiotic yeast fungemia development using *in vitro* and *in vivo* models and the CRISPR/Cas9 genome editing method. Both commercial and clinical isolates (including blood isolates) were available in our strain collection, thus we had the opportunity to make comparisons between yeasts from different sources.

Based on these, we formulated the following objectives:

1. Development and optimization of a reliable diagnostic method to clearly distinguish clinical isolates of *S. ‘boulardii’* probiotic origin from other *Saccharomyces* or even other yeast species.

2. Investigation of virulence factors of *S. 'boulardii* commercial and clinical isolates *in vitro* (alkaline phosphatase production, protease production, haemolytic activity) and *in vivo* (*Galleria mellonella* larvae and immunosuppressed BALB/c mouse model) to assess the infectivity of the yeast strains.

3. Genomic analysis of the probiotic yeast isolates after whole-genome long-read sequencing of one of the commercial strains (Oxford Nanopore Technologies; MinION instrument), as well as assembly and annotation of its genome.

4. Based on the literature, our hypothesis was that the yeast heme oxygenase gene (*HMX1*) may be a virulence factor that promotes yeast survival in a mammalian host. Our plan was to explore the role of the *HMX1* gene in yeast survival and virulence in blood through gene deletion by CRISPR/Cas9 and characterization of the deletion strains.

4. METHODS

4.1 Isolates and strains

During the experiments, 14 Hungarian *S. 'boulardii'* isolates were used. Of these, four isolates were commercial (*S. 'boulardii'* CNCM I-745) and ten isolates were clinical. Commercial isolates were isolated at our department from two products available in Hungary. The clinical isolates were obtained from the collection of the University Clinics of Debrecen and Szeged, and detailed patient data were available on their origin. Patient data were processed in accordance with EU, state and local regulations, with the ethical approval of the Debrecen Regional and Institutional Research Ethics Council (DE RKEB/IKEB 5194-2019). Homozygous heme oxygenase-1 (*HMX1*) deletion mutant strains were generated from six isolates using CRISPR/Cas9 genome editing technology and provided with a unique strain identifier.

4.2 Genetic methods

We examined the relatedness of the strains after DNA isolation with "Multilocus Sequence Typing" [MLST; *CUP1* (complete), *HMX1* (complete), *CCA1* (788 bp fragment), *NUP116* (427 bp fragment) genes], PCR fingerprinting methods [Microsatellite-Primed PCR (MSP-PCR), interdelta fingerprinting, and microsatellite typing. Based on the results, a multiplex PCR method for the identification of probiotic yeast isolates was developed. Briefly, the method combines interdelta, two microsatellite (*YLR177w*, *YOR267c*) and ITS region primers in a single PCR reaction, resulting in products and band patterns characteristic of probiotic yeasts after gel electrophoresis.

4.3 Genomic methods

Six of the strains were selected for genomic analysis and sequenced with the Illumina NextSeq 500 system with ~50× coverage. Furthermore, in case of PY0001, the strain library was sequenced with 400× coverage in a separate run. Long-read sequencing was also used for this latter strain (MinION device, R9.4.1 Flow Cell; Oxford Nanopore, Oxford, UK). Using the resulting .FAST5 sequencing files, the genome was assembled and annotated using the LRSDAY pipeline. This assembled genome was used as a reference for mapping. SNP/INDEL analysis was performed to detect high and moderate mutations in the protein coding genes. Based on the variants, we were also able to determine allele ratios, from which the ploidy of the strains and the chromosome copy number variations (CCNV) were determined taking into account the coverage data. To confirm the results obtained by multiplex PCR and the close relationship between the isolates, a phylogenomic analysis was performed (Maximum Likelihood phylogenetics, Neighbor Net genomic network). Mapping was then performed using the entire *S. cerevisiae* pangenomic ORF .FASTA collection as a reference to determine mitochondrial DNA, Ty transposon, rDNA, and 2μ plasmid copy numbers for the haploid genome. Copy numbers of nuclear ORFs were determined using coverage data for the PY0001 reference mapping.

4.4 Phenotyping experiments

All probiotic isolates were included in the phenotyping experiments. We examined colony morphology, invasiveness, senescence, killer activity and susceptibility of strains, and the frequency of petite colonies. The MAT locus typing of the isolates was performed and their sporulation ability was also tested. The virulence factors of the strains were examined by *in vitro* methods (growth at high temperature, pseudohyphal growth, extracellular protease and lipase activity, hemolytic activity). Finally, the susceptibility of the isolates to antifungal agents (fluconazole, amphotericin B, caspofungin) was determined.

4.5 Investigation of human epithelial model interactions

The interactions of the isolates with the human epithelium were examined using the Caco-2 (colon adenocarcinoma) cell line, and the adhesion and translocation of the isolates in the epithelium model were investigated. Damage assay was also perfomed. *Candida albicans* SC5314 strain was used as a positive control.

4.6 Immunology assays

Mononuclear cells from peripheral blood samples taken at the Debrecen Regional Blood Supply Center were isolated by Ficoll–Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation at the Immunology Institute of the University of Debrecen from heparinized, leucocyte-enriched buffy coats of healthy donors. The isolation was approved by the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the Faculty of General Medicine of the University of Debrecen (UD) (Debrecen, Hungary). Monocytes were purified from PBMCs by positive selection with anti-CD14 conjugated microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. Differentiation of dendritic cells (DC) was performed for 5 days at the Immunology Institute of UD. Allogeneic CD3+ pan-T cells from donors were isolated from PBMC with anti-CD3 microbeads (Miltenyi Biotec) according to the manufacturer's instructions and used for monocyte-derived dentritic cells (moDC)–T-cell coculture experiments. Phagocytosis, phenotypic and functional analysis of moDCs coincubated with *S. 'boulardii'* isolates were performed (investigation of CD40, CD80, CD83, CD86, HLA-DQ cell surface protein expression and IL-6, IL-8, IL-12, IL-1β, TNFα cytokine/chemokine secretion profile). In addition, the T-cell activating ability of moDCs coincubated with yeasts was tested by immunospot (ELISPOT) assay.

4.7 *In vivo* experiments

The virulence of the isolates was also examined in vivo using animal models [larvae of *Galleria mellonella* (wax moth), immunosuppressed BALB/c mouse]. Infection of the larvae was performed with 10^6 yeast cells (cells incubated overnight and then washed and suspended in PBS three times). Prior to the start of the experiments, the larvae were starved for 24 hours at 30°C to avoid immune activation by heat shock before inoculation. After inoculation, larval survival was monitored for 96 hours at 37°C. The mouse experiments were approved by the Animal Welfare Committee of the University of Debrecen (DEMÁB permit: 12/2014) and performed at the Department of Medical Microbiology of the University of Debrecen. Immunosuppression was induced by multiple intraperitoneal administration of cyclophosphamide [150 mg/body weight (kg) 4 days before infection, 100 mg/kg 1 day before infection, and 100 mg/kg 2 and 5 days post-infection]. Animals were infected intravenously with yeast through the lateral tail vein. Infections were performed with 1–1.5 × 10^7 yeast cells suspended in 0.2 ml of physiological saline. The number of cells in the inoculum was confirmed by serial dilutions on Sabouraud dextrose agar. The survival of the mice was followed for 6 days after infection, then mice that were still alive were euthanized, their kidneys were removed, and aseptically homogenized. After homogenization, serial dilutions with physiological saline were made and plated on YPD agar plates, and colonies were counted after 2 days of incubation at 37°C to determine the number of living cells in the kidneys [CFU/kidney weight (g)]. Infection of the mice was performed with wild-type isolates and also with *HMX1* deletion mutant *S. 'boulardii'* strains.

4.8 Investigation of the function of the heme oxygenase-1 gene (*HMX1*)

6 yeast isolates (2 commercial and 4 clinical isolates) were selected to examine the function of the heme oxygenase-1 gene (*HMX1*). Homozygous *HMX1* null mutant strains from *S. 'boulardii'* isolates were generated using the CRISPR/Cas9 system. Repair DNA (rDNA) and the plasmid

encoding the Cas9 and guide RNA (gRNA) were designed with the Benchling online software and the plasmid was assembled from the plasmids of the MoClo Yeast Toolkit (YTK, Addgene, catalog number 1000000061). The mutants were further cultured in YPD medium to lose the antimycotic resistance gene containing plasmid, so that the deletion strains became marker-free. Ultracompetent *Escherichia coli* DH5α cells were used for all cloning reactions and selected on Lysogeny Broth (LB, Miller) agar plates containing the appropriate antibiotics (ampicillin, chloramphenicol, or kanamycin) after transformation.

We examined the effect of iron starvation on the growth of six probiotic isolates and the *HMX1* deletion strains generated from them using a spot plate assay. To induce iron deficiency, synthetic defined (SD) agar medium (6.7 g/l yeast nitrogen base without amino acids, 2.2% glucose, 2% agar) was supplemented with 40, 80, or 120 mM batophenanthroline disulfonic acid disodium salt (BPS) iron chelating agent. The hemolytic activity of *HMX1* deletion strains was also determined. The virulence of the deletion strains was examined in an immunosuppressed BALB/c mouse model as described above.

4.9 Statistics and data visualization

To compare the results obtained for the yeast isolate groups (C: commercial, M: mycosis-causing and NM: non-mycosis-causing groups, and ΔΔC: commercial, ΔΔM: mycosis-causing and ΔΔNM: mycosis-causing groups) and yeast strains, VassartStats (http://vassarstats.net/), Astatsa (https://astatsa.com/) and Statistics Kingdom (https://www.statskingdom.com/) online statistics applications were used. When comparing two data sets, two-sample T-test (for equal variances) or Welch-test (for non-equal variances) was used if the data sets followed a normal distribution. For non-normal distributions, the Mann-Whitney test was used. ANOVA was used to compare more than two data sets in case of normal distribution, then Tukey HSD test was used to determine which data sets differed significantly from the others. For more than two non-normally distributed data sets, the Kruskal-Wallis test was used and the differences between the data sets were determined using the Dunn post-test (corrected for Benjamini-Hochberg FDR). To analyze mouse and larval survival, the Log-rank (Mantel-Cox) test was performed and the results were illustrated with Kaplan-Meier survival curve. For the statistical evaluation of the spot plate assay, the “gray-value” measurement function of ImageJ was used.

5. RESULTS

5.1 Species identification and MLST

A total of 16 yeast isolates were identified as *S. cerevisiae* by the MALDI-TOF method at the Department of Medical Microbiology of the DE, all of which were isolated at the Clinical Center of the University of Debrecen. ITS analysis revealed that one of these isolates (DE22293) was incorrectly identified as *S. cerevisiae*, because it was in fact an isolate of *P. kudriavzevii*. Therefore, this isolate was excluded from further analyzes. The clinical isolates from Szeged mentioned in the list of used strains were not yet available for this experiment, so they were not analyzed.

In addition to the ITS region, sequencing of four nuclear genes on different chromosomes was performed and commercial isolates were included in the study. For three isolates, the *HMX1* gene was unreadable or only partially readable, and for six isolates, the ITS region was unreadable due to different alleles containing INDELs. Sequencing of all four genes and the ITS region in the other isolates yielded readable sequences in which numerous single nucleotide polymorphisms (SNPs) were found. The sequences were concatenated for each and every strain (3854 nucleotides in total) and phylogenetic analysis was performed, which identified eight clades. Eight isolates, together with the commercial yeasts, formed a single clade. However, heterozygous SNPs were found in isolate DE10397 for the *CCA1*, *CYT1*, *HMX1*, and *NUP116* genes (ITS was unreadable), that were separated by cloning into a vector. Sequencing revealed that this isolate was identical to probiotics and carries both identical and different haplotypes in 1–7 SNPs as probiotics. The gene sequences of the remaining seven isolates in the clade (DE6507, DE27020, DE35762, DE3912, DE42533, DE42807, DE45866) did not differ from the probiotics and even contained a common heterozygous position within the ITS1 region (A/G, position 121). The other sequenced regions were homozygous in these probiotic related isolates.

5.2 PCR-Fingerprinting and microsatellite typing

The resolution of the MSP-PCR method was low, only two groups could be distinguished when the isolates were analyzed. The commercial probiotics and the seven isolates of probiotic origin mentioned above were uniform but at the same time indistinguishable from the other isolates. The interdelta fingerprinting method was able to classify the isolates into nine groups, and the commercial probiotics as well as the seven potentially probiotic-derived clinical isolates formed a uniform clade with this method as well. Microsatellite typing, performed by my colleagues at the Department of Molecular Biotechnology and Microbiology [Co-author result (microsatellite typing): Hanna Rácz

Lovászné, Dr. Valter P. Pfliegler, Department of Molecular Biotechnology and Microbiology], identified homozygous alleles for all probiotics and clinical yeast for *YLR177w* (chromosome XII) which contains 4–12 CAG repeats. The *YOR267c* microsatellite was homo, di-, and trizigote, with 11–41 CAA repeats. All commercial probiotics and clinical isolates of potential probiotic origin were clearly separated from the other isolates with this method, and each was homozygous for the two microsatellites. The close relationship of these 11 isolates was supported by the fact that none of them was able to sporulate during our experiments.

5.3 New multiplex PCR protocol for *S. 'boulardii'* identification

By combining PCR-based methods, we have developed and optimized a rapid multiplex PCR method that is suitable for strain level identification, possess high diagnostic value and does not require extensive sample preparation. Based on MLST, MSP PCR, interdelta, and microsatellite typing results, as well as other work on interdelta and microsatellite typing, we combined the primers required to amplify the interdelta, *YLR177w*, *YOR267c*, and ITS regions in a single multiplex PCR reaction. Primer concentrations were optimized to avoid large differences in intensity between bands after gel electrophoresis. For probiotics and clinical isolates of probiotic origin, this method yielded a well-recognizable band pattern of three groups (600–850 bp, 210–270 bp, and 100–140 bp) and 7 + 1 (<75 bp) bands. For proper resolution, 2% TBE agarose gel and 100 V were used for 1 hour for gel electrophoresis. A different band pattern was observed for the other non-probiotic clinical isolates, which allowed us to distinguish seven groups. These patterns were clearly distinct from the band pattern of probiotic and probiotic-derived isolates.

In addition, no *Saccharomyces* specific sequence was present for the *P. kudriavzevii* isolate used as a control, but an ITS band was present. The method was tested with both purified and colony DNA and another enzyme (DreamTaq; Thermo Fischer Scientific, Waltham, MA, USA). Using Dream taq polymerase, we got essentially the same results as using GoTaq. The size of two bands changed and the band intensities differed when the results obtained with GoTaq and DreamTaq were compared, but this did not affect the evaluation of the patterns. In addition, *S. cerevisiae* wine, food, bioethanol and laboratory strains were tested with the multiplex method. The band patterns obtained for these strains were very different from those obtained for probiotics, so the method can be reliably applied to the identification of the *S. 'boulardii'* subtype. We were able to distinguish *S. 'boulardii'* and *S. cerevisiae* isolates in our strain collection by this multiplex PCR.

5.4 Genomics: whole-genome assembly and annotation

Using the long and short sequence readings, a complete genome of 16 nuclear chromosomes and two mitochondrial contigs was assembled using the LRSDAY pipeline for the commercial isolate PY0001 (*S. ‘boulardii’* CNCM I-745). The mitochondrial genome was complemented with the mitochondrial genome of the *S. ’boulardii’* isolate named CIM (using the genome assemblies of Peter *et al*.). Based on short reads the strain CIM contained only one SNP compared to the strain PY0001. The pipeline was not optimized for the assembly of small circular genome constructs, hence the 2μ plasmid was not assembled. The genome assembly was highly synthenic with the *S. cerevisiae* S288C reference genome, and the total genome size including mitochondria was 11,868,337 bp, compared to the 12,157,105 bp S288c reference. Based on the annotation of the genes, telomeric regions, and centromers the difference between the two strains is mostly due to the absence of some genes present in S288c and the absence of Ty1-3-4 retrotransposons. However, the genome of PY0001 contains ORFs not present in S288c. It should be noted that the mitochondrial genome of the CIM strain is longer than the mitochondrial genome of S288c reference (87,321 bp and 85,779 bp, respectively).

During the identification of the genes, 3 ETS sequences (“external transcribed spacer”) were erroneously marked as ORFs. These were identified by SGD BLAST, and then deleted from the annotation list (.GFF3 file). Thus, 5717 nuclear protein coding genes were identified in PY0001. Later, this assembled genome was used as a reference for genomic analysis of six isolates used for *HMX1* gene knockout.

Based on the mapping on the eight known *Saccharomyces* species, the genomes of the probiotic yeasts did not contain any significant introgression, so further analysis of these was omitted.

5.5 Phenotypic differences between commercial, mycosis and non-mycosis *S. ‘boulardii’* isolates

To determine phenotypic differences between probiotic isolates, we first examined colony morphology. After ten days of incubation, the colonies were smooth and white in case of all isolates. However, different phenotypes appeared in colony senecence studies, and a complex colony morphology was observed in the isolates causing fungemia. The proportion of mitochondrial “petite” mutants was typically low, ranging from 0 to 1.22%, and there was no significant difference between the isolate groups. None of the isolates sporulated at the temperatures tested (25 °C and 37 °C), but MAT locus typing confirmed that all of the isolates were homotallic. Furthermore, all samples were non-killer and sensitive to killer toxins (types 1 and 2) [Co-author results (petite-test, MAT locus typing, killer test): Dr. Valter P. Pfliegler, Department of Molecular Biotechnology and Microbiology]. Non of the colonies were invasive regardless of the type of medium or the temperature applied during incubation we did not observe flocculation, and the pseudohyphal morphology was medium-dependent. Based on the results of the spot-plate assay, the growth of the isolates was identical at 37 °C and 39 °C, while at 42 °C, the isolates PY0001 and PY0002 showed a more intensive growth compared to the other samples, and the isolate 2251/2018 did not show growth.

5.6 Extracellular virulence factors of commercial, mycosis and non-mycosis *S. 'boulardii'* isolates (extracellular phospholipase and aspartate protease production, haemolytic activity)

Based on phenotypic clustering, we were able to distinguish two main groups: One had lower protease production and β-hemolysis, and higher phospholipase production, and the other was characterized by the opposite. However, when virulence factors were evaluated separately, statistically significant differences were found between the isolate groups. According to the Tukey HSD test, non-mycosis clinical isolates had significantly ($p < 0.05$) lower *Prz* (protease zone) values than commercial isolates (*i.e.* they had higher protease production). For the same isolates, the mean *Pz* (phospholipase zone) values were significantly higher ($p < 0.01$), indicating that this group had the lowest rate of lipase production. There was no significant difference between groups for α-hemolysis, but β-hemolytic activity was significantly higher in the mycosis causing clinical isolates ($p < 0.01$) compared to commercial and non-mycosis isolates.

5.7 Sensitivity to antifungal agents

When tested for susceptibility to antifungal agents, commercial isolates showed higher caspofungin MIC values compared to the non-mycosis isolates. Based on the MIC values, the samples were divided into two clusters. The values obtained for amphotericin B ranged from 0.125 to 0.25 μg/mL (the higher value was true for all isolates except PY0003). In case of fluconazole, the values ranged from 2 to 8 μg/mL (half of the isolates had an MIC of 8 μg/mL), while for caspofungin the values were 0.25 or 0.5 [Co-author results (antifungal sensitivity test): Dr. Renátó Kovács, Department of Medical Microbiology].

5.8 Interaction with epithelium (adhesion, damage, transmigration), morphology

During the experiments, we found that none of the isolates adhered to the epithelium model [Co-author result (adhesion experiment): Dr. Valter P. Pfliegler, Department of Molecular Biotechnology and Microbiology) and the MTT assay showed no greater than 17% damage to the Caco-2 cells [Co-author result (MTT assay): Dr. Dániel Nemes, Department of Pharmaceutical

Technology). Transmigration was not observable of *S. 'boulardii'* isolates, with the exception of the non-mycosis isolates DE3912 and 551/2018, that crossed the epithelium after four and six days, respectively. Biofilm formation was not observed even after six days. DMEM medium was used in the experiments, in which the isolates showed yeast morphology and formed short pseudohyphae. The positive control *C. albicans* strain, in contrast to the probiotic yeasts, showed adhesion, damage, and rapid transmigration (with hyphal growth) as well in this model.

5.9 Immunological interactions (investigation of phagocytosis and cell surface molecules of moDCs, cytokine/chemokine levels and T-cell activation)

There were no significant donor-dependent differences in the phagocytosis activity of moDCs, nor was there a significant difference in the phagocytosis activity of moDCs between commercial and mycosis isolates (results obtained in collaboration with the Institute of Immunology).

After co-incubation with yeasts, the changes in the surface of moDCs were examined for the following cell surface molecules: costimulatory proteins (CD40, CD80, and CD86), CD83 maturation marker, and HLA-DQ antigen presenting protein. We examined the donor-dependent differences in this case as well and compared the changes in fluorescence obtained from commercial and mycosis-causing isolates [results obtained together with colleagues (Dr. Kitti Pázmándi, Dr. Tünde Fekete) of the Institute of Immunology]. With the exception of HLA-DQ, we found that DCs from different donors responded differently to coincubation with yeasts, indicating that the amount of cell surface molecules was highly dependent on the donor. When comparing the two groups of isolates, no significant difference was found for any of the DC cell surface markers. Changes in the concentration of proinflammatory cytokines (IL-6, IL-12, IL-1β, and TNF-α) and the chemokine IL-8 in the supernatant of the cells were also determined. We found that there were significant differences between DCs from different donors, however, no significant differences were found when comparing commercial and mycosis-causing isolate groups.

T-cell activation and polarization assays revealed not only significant donor-dependent differences, but also that mycosis-inducing isolates significantly induced (two-sample T-test, $p < 0.05$) the production of IL-17 compared to commercial isolates [Co-author results (cytokine and T-cell activation experiments): Dr. Kitti Pázmándi, Dr. Tünde Fekete, Institute of Immunology].

Overall, based on immunological studies, phenotypic clustering clearly distinguished the control *C. albicans* strain from *S. 'boulardii'* isolates and divided the latter into two clusters. The cluster that resulted in higher immune activation included two commercial isolates and the two isolates that caused mycosis.

5.10 *G. mellonella* larvae infection

Using the *G. mellonella* larval model, we compared the pathogenicity of 14 probiotic isolates and isolate groups (commercial, non-mycosis, mycosis) with PBS and pathogenic *C. albicans* controls. Non-mycosis isolates showed significantly lower pathogenicity than the mycosis or the commercial isolates. Intra-group comparisons among non-mycosis isolates showed statistically significant differences in two cases both involving the non-mycosis 2251/2018 sample, which was characterized by very high larval survival.

5.11 Virulence and survival in immunosuppressed BALB/c mouse model

During mouse experiments there was no significant difference between commercial isolates ($p > 0.05$), but significant differences were found when clinical isolates were compared ($p < 0.01$). Some isolates had remarkably high virulence: 465/2018 (28% survival), DE45866, DE42533, and 2251/2018 (42% survival).

Regarding kidney burden, the commercial isolates did not differ significantly from each other ($p > 0.05$), while in the case of clinical isolates the CFU values of the DE27020, DE6507 and DE35762 isolates were significantly [$p < 0.01$ (DE27020); $p < 0.01$ (DE6507); $p < 0.01$ (DE35762)] lower than the CFU values of isolate 2251/2018. The commercial and clinical strains did not differ significantly from each other, however, the isolate groups did. Non-mycosis isolates showed significantly [$p < 0.01$ (C); $p < 0.001$ (M)] higher kideny burden compared to the commercial and the non-mycosis isolates. [Co-author result (mice infections): Dr. Renátó Kovács, Department of Medical Microbiology].

5.12 Comparison of the genomic background of probiotic yeasts involved in gene knockout experiments

. Both the phylogenomic dendrogram and the SNP network showed that *S. 'boulardii'* isolates are highly similar to each other, and to the *S. 'boulardii'* CIM strain of Peter *et al*. The isolates contain approximately 3000 variant positions (compared to the PY0001 reference) in the genome of more than 11 million bp, most of which were heterozygous SNPs. It should be noted that analysis of short reads of PY0001 also revealed SNPs when mapping to the PY0001 reference, which were all heterozygous positions (they appeared as SNPs after mapping the diploid yeast due to the use of haploid genome assembly). The two commercial isolates appeared to be the most similar among the isolates [Co-author result (Maximum Likelihood Phylogeny): Dr. Valter P. Pfliegler, Department of Molecular Biotechnology and Microbiology].

Based on the coverage plots generated from the mapping files and the allele ratios plotted for the chromosomes, we determined that all six isolates have diploid, euploid genomes, and do not show segmental duplications or large deletions. Based on the median coverage of each of the genes in the PY0001 reference, the copy number of the genes in all 6 isolates was determined. Copy number variations (1 or 2 copies per haploid genome) were found in only 10 genes located on eight different chromosomes, and 1–5 such genes were present in the genome of every isolate. Seven of the genes involved in CNV were either dubious ORFs or uncharacterized genes. In addition, there were copy number differences in the case of the *YDL069C* (*CBS*; mitochondrial respiratory chain complex III assembly), *YLR211C* (*ATG38*; macroautophagia-associated gene), and *YPR166C* (*MRP2*; involved in mitochondrial translation) genes. *HMX1* was a single copy gene (for haploid genome) in each yeast.

Based on pangenome ORF mapping, we determined the copy numbers of mitochondrial DNA, 2μ plasmid, rDNA, and transposonscalculated for the haploid genome.The number of mitochondrial genomes was 17–25 per haploid genome in the isolates, while the plasmid number varyied on a larger scale, from 31 to 58. Copies of rDNA ranged from 60 to 90 in most cases. 15–16 copies of Ty2 retrotransposons were detected, and 2 copies of Ty5 were determined in all isolates. Thus, the 6 isolates showed high degree of similarity in copy number of genes and other traits.

Changes in allele coverage rates on chromosomes were plotted based on the AD (allelic depth) values of the alleles identified during variant analysis and the genotypes identified by the software. In all cases, the clustering of heterozygous positions was observed mainly on chromosomes I–VI and also on X, XII, and XIII. Chromosome VIII and XIV were completely homozygous.

Based on the genotype values, we determined where there are sites in the genome of the other 5 isolates relative to the PY0001 isolate that indicate longer LOH or GOH events. The heterozygous positions of the sequenced and assembled PY0001 isolate were largely grouped on chromosomes I-VI (for the left arm of chromosome IV only), X, XII, and XIII. Overall, the proportion of heterozygous positions on the chromosomes was low and large homozygous regions were essentially characteristic of the genome (e.g., chromosomes VIII and XIV were almost completely homozygous). GOH and LOH regions on 7 different chromosomes were found in each of the other 5 isolates. In the case of PY0002, this was represented by only a short region, but we found significantly more and longer allele ratio changes in the other four clinical isolates. Overall, the results for heterozygosity differed between the 6 isolates [Co-author result (investigation of allele proportions): Dr. Valter P. Pfliegler, Department of Molecular Biotechnology and Microbiology].

Finally, for all six sequenced strains, we determined which variants cause significant changes in the protein coding genes (e.g., early stop codon, stop codon loss, frameshift) in homo- or heterozygous form. High-effect mutations affected a total of 90 genes, taking all isolates into account. In PY0001, the genes involved were those containing heterozygous mutations (note that the reference genome used for mapping was PY0001, so only heterozygous variants are expected for this yeast genome). Of these 90 genes, 43 were not involved in all six isolates simultaneously and thus show differences in genetic background. A well-defined function can be assigned to a total of 30 of these based on homology to the S288c reference sequence. Of the genes affected, *GLT1* and *SLX5* interact with *HMX1* based on *Saccharomyces* Genome Database (SGD). In the former gene, the mutation was in heterozygous form in all isolates, while *SLX5* was not affected in PY0001 and it was homozygous in the other five genomes.

Analysis of mutational effects on gene function identified 497 genes (of which 30 were doubious ORFs) that contained moderate effect mutations (e.g., a single amino acid substitution). As in case of high-effect mutations, most of the identified variants were found in all isolates (heterozygous only for PY0001 and most often heterozygous for the other isolates). There were a total of 73 genes that were affected in only some, but not all, isolates. Genes with moderate mutations include *CHK1*, *ESS1*, *GLT1*, *IME1*, *KCH1*, *LGE1*, *MXR1*, *PPR1*, *SPB4*, and *CST6*, which interact with *HMX1* based on SGD. The genes containing medium effect mutations were in heterozygous form in most of the isolates, but DE35762 was homozygous for *CHK1* and DE6507 for the *LGE1* mutation. Finally, *HMX1* and its immediate upstream region were not affected by any modifying, moderate, or high-effect mutations in any of the isolates according to the variant annotation.

Of the altogether 11 genes in interaction with *HMX1* affected by large- or medium-effect mutations, GO Term analysis revealed enrichment in nucleus-residing proteins ($p < 0.05$) (but found no enrichment in process or in function). *HMX1*-interacting genes were not more or less likely to be affected by mutations than genes that were not in interaction with it (Fischer’s exact test, $p > 0.05$).

5.13 Successful deletion of *HMX1* with CRISPR/Cas9 system

After transformation described in the Materials and Methods section, colonies were isolated from the transformed yeasts involved in the experiment. The success of the *HMX1* gene deletion was confirmed by PCR. On the one hand the entire ORF was amplified (to prove the fact of gene deletion), on the other hand the correct incorporation or the repair DNA was also verified by using a cassette-specific primer. Based on gel electrophoresis, we confirmed that the *HMX1* gene was found only in the wild-type isolates, indicated by a 1553 bp band. For deletion mutants, a much shorter band of 682 bp was seen. The reason is that the primers used for the verification are located “upstream” and “downstream” of the *HMX1* ORF, so a PCR product will show up in case of the deletion strains as well, but the shorter band size obtained by gel electrophoresis indicates the success of the gene deletion. To check the insertion of the deletion cassette primers amplifying the “upstream” region of the cassette were used that (the “forward” primer was –417 bp from the insertion cassette and the “reverse” primer was bound to the cassette). When this primer pair was used, no band appeared on the gel for wild-type isolates, in contrast to the deletion strains, when a band of 437 bp was observable. Thus, gel electrophoresis confirmed that gene knockout was successful in all six isolates.

5.14 Deletion of *HMX1* resulted in decreased growth under iron deprivation

Previous studies have shown that deletion of *HMX1* interferes with the iron metabolism of *S. cerevisiae*, making it impossible for cells to use intracellular heme-containing molecules as an iron source during iron starvation. Therefore, we examined the growth of the deletion mutants under iron starvation.The results described below are referring to the second spot on the "spot-plate", containing 5×10^3 cells.

When 40 μM BPS was applied, the majority of mutant strains (AI0001, AI0003, AI0005, and AI0009) showed significantly lower growth ($p < 0.001$; $p < 0.001$; $p < 0.05$ and $p < 0.001$; respectively) compared to the wild-type isolates. Strain AI0007 was less viable than it parental isolate 2251/2018, however, this difference was not significant ($p > 0.05$). Interestingly, the AI0011 deletion mutant showed significantly stronger growth than the wild-type isolate DE35762 from which it was derived. Among the mutant strains, the growth of AI0001, AI0003, AI0005, and AI0007 was significantly ($p < 0.01$; $p < 0.01$; $p < 0.001$; $p < 0.001$, respectively) lower compared to wild-type isolates when the medium was supplemented with 80 μM BPS. The difference in growth was not significant ($p > 0.05$) between strains DE6507 and AI0009, but the latter showed weaker growth. As in previous conditions, AI0011 showed a significantly ($p < 0.001$) stronger growth compared to the original strain (DE35762). Applying 120 μM BPS, most strains were unable to grow or their growth was negligible. Isolate 465/2018 was an exception, since it was able to grow. The *HMX1* deletion mutant derived from this isolate (AI0005) did not grow, so these two isolates showed a significant ($p < 0.05$) difference in growth. These results suggest that the deletion of the gene *HMX1* resulted in lower growth in most of the strains under iron starvation. This was not true for strain AI0011, as its growth was significantly higher than the growth of the wild-type isolate DE35762. The reason for this may be the different genetic background.In addition, the isolate groups were compared. When comparing wild-type isolates, we found that non-mycosis isolates showed significantly stronger growth compared to the commercial and mycosis groups at each BPS concentration (40 μM: $p < 0.01$; 80 μM: $p < 0.01$; 120 μM: $p < 0.05$). For *ΔΔHMX1* mutant strains, significant ($p < 0.05$; ΔΔC < ΔΔNM) differences were found between the ΔΔcommercial and ΔΔnon-mycosis groups at a concentration of 40 μM BPS. When 80 or 120 μM BPS was applied, there was no significant difference between the deletion groups.

The wild-type isolate groups were compared with their corresponding deletion groups, and the same growth trend was observed as when the strains were compared in pairs: the deletion groups showed a lower increase compared to the wild-type isolate groups. Significant differences were found in the comparisons at all BPS concentrations used, which were as follows: 40 μM BPS: $p < 0.001$, C > ΔΔC; $p < 0.01$, NM > ΔΔNM; 80 μM BPS: $p < 0.001$, C > ΔΔC; $p < 0.01$, NM > ΔΔNM; and 120 μM BPS: $p < 0.05$, NM > ΔΔNM. Similarly, when all wild-type isolates were compared to all deletion mutants, the latter group showed a weaker increase, and this difference was significant at 80 μM ($p < 0.001$) and 120 μM ($p < 0.001$) BPS. Thus, based on a comparison of isolate groups, the knockout of the *HMX1* gene reduced the viability of most of the strains under iron starvation, although these differences were not always significant.

In addition to BPS, the growth of wild-type and *HMX1* deletion mutants was also compared in liquid medium containing 50% RPMI-1640 and 50% human serum, which did not contain free iron, that could be used by yeast. Significant growth differences were observed for the PY0001, PY0002, 465/2018, and DE35762 isolates compared to their knockout mutant counterparts ($p < 0.001$, $p < 0.01$, $p < 0.05$, and $p < 0.0001$, respectively). Interestingly, in three of these isolates (465/2018, 2251/2018 and DE35762), the mutant strains showed stronger growth after 24 h, while in the other cases, the deletion strains showed weaker growth. The 2251/2018 isolate also showed weaker growth, but the difference was not statistically significant compared to the growth of the AI0007 mutant strain. The AI0009 strain showed weaker growth in all replicates than DE6507, however, the difference was not significant [Co-author result (growth in human serum): Zoltán Tóth, Department of Medical Microbiology].

5.15 Hemolytic activity was not affected by *HMX1* deletion

We wanted to examine whether the virulent isolates could be identified based on α- and β-hemolysis, and also the effect of the absence of the *HMX1* gene on hemolytic activity. When the deletion mutant strains were compared in pairs with the wild-type ones, the non-mycosis 2251/2018 ($p < 0.01$; 2251/2018 > AI0007; α-hemolysis), the mycosis DE6507 ($p < 0.05$; DE6507 < AI0009; α-hemolysis) and the commercial PY0001 ($p < 0.01$; PY0001 > AI0001; β-hemolysis) isolates showed a significant difference in hemolytic activity. Thus, strain-level comparisons did not reveal a clear effect of the absence of *HMX1*. There was no significant difference in either α- or β-hemolysis values when comparing the isolate groups.

5.16 Deletion of *HMX1* caused significantly higher kidney burden in immunosuppressed BALB/c mice

Wild-type isolates showed different virulence in mouse experiments, which was significant ($p < 0.0001$). The highest mortality (28% survival) was observed in the case of 465/2018, but 2251/2018 also caused high mortality (<45% survival). Interestingly, the knockout of *HMX1* abolished these significant differences ($p > 0.05$). When comparing the wild-type and deletion strains pairwise, we found that the gene knock-out only caused a significant change (an increase in both cases) in virulence only in the case of two isolates (PY0001: $p < 0.05$; DE35762: $p < 0.01$).

The number of live yeast cells in the kidneys [kidney burden, CFU/kidney weight (g)] was also determined. Based on the statistical analysis the CFU of all but one (465/2018) wild-type isolates was significantly lower than that of the deletion mutants. Of the mutant strains, strain AI0003 showed the highest mean CFU [Co-author result (infection with wild-type isolates): Dr. Renátó Kovács, Department of Medical Microbiology]. I performed the infection of the deletion strains together with the colleauges (Dr. Renátó Kovács, Dr. Frucsina Nagy, Zoltán Tóth, Lajos Forgács) of the Department of Medical Microbiology.

6. DISCUSSION

6.1 Identification of *S. 'boulardii'* by a new PCR-based method

Both PCR-based species identification and PCR fingerprinting methods used for subtyping can be applied in food and clinical microbiology to replace or complement time-consuming diagnostic tests. Intra-species diversity of food and clinical yeasts has received particular attention recently. The microbe of *S. cerevisiae* is widely used in the food industry, but is also considered an opportunistic pathogen. Subtyping methods are rarely used in clinical mycological diagnostics, although this would be a prerequisite for understanding how and why yeast-based probiotics cause infections.

The new multiplex PCR method described is suitable for the accurate and rapid identification of yeast infections of probiotic origin, thus it can be used even in everyday diagnostics. The use of different genotyping methods may lead to different results, so it is worth to combine them into a single, multiplex reaction for a more accurate result. Our method was optimized using the isolate collection of the University Clinic of Debrecen by sequencing five nuclear genes, analyzing interdelta regions, and performing MSP fingerprinting as well as microsatellite typing. Of these, the interdelta, two microsatellite, and ITS regions were amplified in a single multiplex PCR reaction. The interdelta primers are specific for the LTR (long terminal repeat) regions of the Ty1 and Ty2 transposons. The former transposon group is missing from the probiotic yeast based on literature data and our genome annotation as well, resulting in a unique band pattern during fingerprinting. The microsatellite alleles used in our studies are specific for *S. 'boulardii'*. It is worth noting that MLST, which is otherwise a time-consuming method, have low-resolution and in case of heterozygous INDEL mutations it has limitied applicability. Probiotic yeasts are known to be heterozygous for the MAT locus, however they are not able to sporulate, and this was also true for our *S. 'boulardii'* isolates.

Using our multiplex method, probiotic isolates show a characteristic band pattern after gel electrophoresis, allowing rapid visual evaluation without the use of an automated gel analysis pipeline. The method can be quickly applied to samples that already have been found to be *Saccharomyces* yeast. MALDI-TOF is the most common species identification method in clinical microbiological diagnostics. Because live microbial colonies are used for this method as well, our multiplex method can be used directly for species identification and subtyping after a MALDI-TOF analysis by performing colony PCR followed by gel electrophoresis. It is estimated that the entire workflow can be completed within 3 hours after the MALDI-TOF has identified the sample as *S. cerevisae*. Fungal ITS-specific primers serve as a positive control in the multiplex PCR reaction, in case the sample has previously been incorrectly identified as *Saccharomyces*.

Using our optimized multiplex PCR, we have shown that clinical yeasts of probiotic origin are common among *S. cerevisiae* isolates. These isolates originated from various human body sites and from several patients: 16 yeasts (DE342, DE722, DE3912, DE6507, DE10397, DE11595, DE22293, DE27020, DE27290, DE29607, DE35762, DE42533, DE42651, DE42807, DE43763, DE45866) isolated from 16 patients were identified as *S. cerevisiae* by MALDI-TOF at the University Clinic of Debrecen during the studied time-period, one of which was misidentified by the evaluation software. The seven probiotic isolates were from three female and four male patients. Patients varied significantly in age, ranging from infant to elderly.Only three fungaemia cases occurred during the course of our project, two of which were of probiotic origin and the other probiotic-derived isolates were likely to be colonizing.

Research on the effects and safety of probiotics is still lagging behind. With our multiplex PCR method, it can be determined quickly and easily whether a yeast infection is of probiotic origin or not. This may, in general, facilitate the safety evaluation of yeast probiotics. Using this method, we found that a significant proportion of *Saccharomyces* isolates were of probiotic origin. This observation may be important not only for clinical mycologists, but also for researchers who study the mycobiome of hospitalized patients, the phenomenon of microbial dysbiosis, or who are researching and developing new therapeutic applications of probiotic yeasts.

6.2 Interactions of the probiotic yeast with the human host

The occurrence of probiotic-derived infections prompted us to investigate the virulence factors and host-microbe interactions of *S 'boulardii'* applying the complex methodology proposed by Anoop *et al* (2015). During the experiments, classical virulence factors (phospholipase secretion, aspartate protease secretion, and hemolytic activity), high temperature tolerance, immune activation, as well as the interactions of yeasts with the Caco-2 epithelial model (adhesion, damage, transmigration) were examined. In addition, other phenotypic traits were examined as well as the *G. mellonella* larval infection model and an immunosuppressed mouse infection model were used to assess pathogenicity *in vivo*. We used 14 isolates (both commercial and clinical) for the experiments, which is an exceptionally large number compared to similar studies. The examination of merely 1–2 probiotic isolates is typical. Our goal was to look for potential adaptations that could promote the survival, virulence, and thus pathogenic behavior of *S. 'boulardii'* in the human body. The phenotype and interactions of this yeast with human epithelial and immune cells, pathogenic bacteria and yeasts, as well as experimental animals have been studied in detail in previous studies. Additionally, dozens of case studies have been written describing probiotic yeast as a pathogenic agent. However, to the best of our knowledge, there has been no study to date that not only characterizes the commercial strain, but compares it to other isolates that have potentially evolved in the human body as well (apart from one previous departmental work in which a probiotic isolate was characterized). The phenotypic properties of fungi not only determine their ability to colonize and infect, but are also subject to selection in certain anatomical niches of the host. There have been few studies that have examined *S. cerevisiae* in this regard.

The commercial and clinical isolates we used came from a single geographical region, namely Eastern Hungary. In addition to the yeasts involved in the multiplex method development, three additional clinical isolates from Szeged were used in these studies, thus ten clinical and four commercial yeasts were examined. During strain maintenance, the number of passages was minimized (only two passages were carried out before the experiments) in order to avoid the accumulation of mutations and genomic structure variations during culture. Furthermore, since clonal heterogeneity may be present even within a batch (commercial yeast), two subclone isolates from both commercial probiotic yeasts available in Hungary were used. Detailed data on patients and isolates allowed us to compare mycosis-causing and non-mycosis-causing isolates. The conclusions made based on the results are explained in detail in the following subsections.

6.2.1 There is no considerable difference in *in vitro* virulence factors between groups of *S. 'boulardii'* isolates

The 14 isolates showed variability in some phenotypic properties, as well as in extracellular virulence factors already known in the case of the opportunistic pathogen *C. albicans*. In the case of α-hemolysis, the commercial isolates showed higher hemolytic activity after the first day of the experiment compared to the isolates of the other two groups. Mycosis-causing isolates showed higher activity only in β-hemolysis on the third day of the experiment. In addition, the commercial isolates produced significantly more phospholipase than the non-mycosis isolates. The latter in turn showed more active extracellular protease production. Based on the results, mycosis-causing isolates were not outstanding in the production of extracellular virulence factors, despite the fact that these properties are considered important in the development of yeast infections. This was also true for other phenotypes associated with virulence, such as pseudohyphae formation and high temperature tolerance. The latter was characteristic of two commercial isolates (PY0001, PY0002). It is worth noting that the non-mycosis isolate 2251/2018 differed from the other tested yeasts: it was characterized by low pseudohyphae formation and moderate thermotolerance.non-mycosis

6.2.2 *S. 'boulardii'* shows high resistance to fluconazole

Based on antimycotic resistance studies, most of the the isolates showed similar sensitivity to amphotericin B and caspofungin. For *Candida* species, the CLSI MIC breakpoint of amphotericin B is above 1 μg/mL, which is four times higher than the highest value measured for our isolates. The breakpoints of caspofungin in *Candida* species are highly variable, making it difficult to compare with the values we measured. In the case of fluconazole, resistance can be considered when measuring MIC values ≥ 8 μg/mL in most *Candida* species. In our case, seven *Saccharomyces* isolates reached this limit, including both blood isolates.

6.2.3 The interaction between yeast and intestinal cells is negligible

Previous studies using an *in vitro* Caco-2 epithelial model demonstrated that neither commercial nor clinical isolates of *S. cerevisiae* (including a *S. 'boulardii'* probiotic isolate) were able to penetrate the intestinal epithelium after 48 h. This is consistent with the results we obtained, since we did not see yeast cell transmigration through the applied epithelium model even after 6 days. Two isolates (DE3912, 551/2018) managed to pass throught, but the amount of such yeast cells was negligible. In another study, however, it was found that *S. cerevisiae*, albeit to a small extent, is able to pass through the epithelium, but *S. 'boulardii'* isolates were not used during this work. It is important to note that Klingberg *et al.* observed that TEER in epithelial cell monolayers of Caco-2 increased after inoculation of *S. cerevisiae* epithelium, which may indicate enhanced epithelial "barrier" function (considered an important probiotic property). However, under the same experimental conditions, we found that the increase in TEER is due solely to the accumulation of non-adherent yeast cell mass, which is easily detached from the epithelium upon replacement of a medium. Therefore, probiotic yeast is unlikely to have an effect on the barrier function of the epithelium *in vivo*. Our isolates did not damage the epithelium model we used. Thus, the pathogenic interactions of the tested isolates with the epithelium were negligible.

6.2.4 Different *S. 'boulardii'* isolates have a similar immunosuppressive effect

We also tested the effect of yeasts on the function of differentiated human DCs and T-cells. Our results show that the yeast isolates did not cause significantly different immune response as well as were not phagocytozed to a different degree by DCs. However, the differences were often donor dependent. The interactions of the yeast probiotic with the innate and adaptive immune system may be important not only in the case of infection, but also because the probiotic products are able to beneficially influence and modulate the host immune system, thereby exerting their beneficial effects on the body. For example, several studies have reported the anti-inflammatory effect of *S. 'boulardii'* in the intestinal tract, achieved through modulation of the functions of DCs. Different yeast strains may even have different effects on the inflammatory or proinflammatory responses of the intestinal tract. The immunogenicity of yeast strains is related to their cell wall composition, however, the way how immune modulation ability changes during commensalist-pathogen transition is not yet fully elucidated in the case of *Saccharomyces* species. Recognition of specific cell wall structures such as mannan plays a role in distinguishing and recognizing harmful (e.g., *C. albicans*) and generally harmless (e.g., *S. 'boulardii'*) yeasts. Based on these, an effective evolutionary strategy could be for a commensalist or probiotic strain to pursue a pathogenic lifestyle while retaining the properties that the immune system recognizes as "harmless". It is possible that for some of the probiotic isolates that cause fungemia used in our research, this allows survival in the bloodstream. However, in contrast to the other factors studied, the two mycosis isolates induced significant Th-17 polarization compared to commercial isolates, which may indicate higher inflammatory immune activation.

6.2.5 Variable virulence *in vivo* in a larva and mouse model

In most studies, the virulence of the probiotic yeast is assessed based on *in vitro* assays, however, this approach can be misleading. Therefore, to determine the level of virulence in the case of our strains, we used both insect and mammalian animal models.

Surprisingly, in larval infection experiments, isolates from the probiotic products showed the highest pathogenicity compared to the clinical specimens. Although the use of *Galleria* larvae is a common method for characterizing pathogenic fungi, the more relevant (from a human perspective) mammalian model was included in our studies, and our 14 *S. 'boulardii'* yeast isolates were used to infect immunosuppressed mice as well. Survival of mice was examined and colonization by the yeasts was determined by kidney burden analysis. Mice had a high survival rate when commercial isolates were injected, but high virulence was observed in some clinical isolates: 465/2018 (28% survival), DE45866, DE42533, and 2251/2018 (42% survival). Thus, in contrast to our expectations, not the blood isolates (DE6507, DE35762) showed high virulence in mice but some isolates belonging to the non-mycosis group. Based on all these, the results obtained in the larva and mouse model did not give similar results. It is important to note that in studies investigating *Saccharomyces* yeasts, it is not typical to use and discuss the larval and mouse models together. We are not aware of any publication that compared the two *in vivo* models in the case of *Saccharomyces* yeasts, so such a study would definitely be useful in the future. In the absence of this, we consider the conclusions drawn from the results of the mouse model to be much more relevant from a human perspective.

6.2.6 The health status of patients is likely to be an important factor in the development of *S. 'boulardii'* fungemia.

One of our most interesting findings was that the probiotic isolates that caused mycosis did not have a phenotype that would have helped them to actively penetrate the vascular system. Thus, the process of entry into the bloodstream and the development of systemic infection is still not fully understood. However, previous research suggests several mechanisms are likely to play a role. In addition to the normal oral route, probiotic yeast can enter the human body from the air or spread from person to person. Fungemia can occur because of catheter use or intentional injection, or through a damage of the gastrointestinal tract due to disease or surgery. Immunosuppressive therapies also predispose to fungal infections and, in combination with the conditions mentioned above, increase the risk of developing fungemia. Thus, the use of yeast probiotics is contraindicated in patients with severe medical conditions. Nevertheless, in many cases, those whose health is severely compromised receive *S. 'boulardii'* probiotic. In these patients, for example, probiotic yeast is used for the treatment of gastrointestinal dysbiosis, and this may be a source of infections, since the barrier function of the gut is likely to be impaired in such cases. The fact that probiotic products containing yeast can be dangerous in some cases has recently led a hospital to remove *S. 'boulardii'* products from its pharmacy.

Published stuides and our results suggest that the entry of yeast probiotics into the blood does not occur due to the virulence factors of the yeast, but depends on the current health status of the body. Thus, the survival and reproduction of probiotic strains in the bloodstream and in other sites of the human body is due to other properties of yeast not to virulence factors that promote penetration. Further research and preferably more isolates with different virulence would be necessary to explore the molecular processes and causes of probiotic yeasts becoming pathogenic.

6.3 Potential role of *HMX1* in the pathogenicity of the probiotic yeast

Numerous publications describe that probiotic yeasts can cause mycosis, which can lead to life-threatening health conditions. However, much less is known about the contribution of genetic characteristics of yeast in the development of such infections. Thus one of our goals was to reveal these, primarily by examining changes due to loss of function of the *HMX1* gene. The gene has already been shown to be invariant among our probiotic yeasts based on the MLST data of our multiplex PCR development study, and we also examined the environment of the gene during whole genome assembly, allowing the design of a CRISPR/Cas9-based gene knockout. The gene plays a central role in the iron metabolism of *S. cerevisiae*, including *S. 'boulardii'*, when the concentration of available iron in the cell environment is low. Creating and maintaining an iron-deficient environment around pathogenic microorganisms is a common strategy in the human body. The *HMX1* gene might play an important role in yeast survival under such conditions due to its hem degrading activity, so we hypothesized that the *HMX1* gene may play a role in the development of pathogenicity and blood survival of the probiotic yeast. We tested the effect of gene deletion on *S. 'boulardii'* isolates with different genetic backgrounds. Following successful deletion of the gene, we tested the growth of strains and isolates under iron-deficient conditions (BPS iron chelator containing agar medium and 50% human serum) and assessed their α- and β-hemolytic activity. In addition, animal experiments were performed to determine yeast virulence and survival in an immunosuppressed mouse model.

6.3.1 Genetic differences of isolates selected for gene knockout

Using the newly assembled and annotated genome of one of the commercial isolates as reference, we found that the isolates are indeed close relatives but contain unique mutations and thus represent different genetic backgrounds. No large differences in genomic structure were found, however, hundreds of mutations affected protein functions in the isolates and a large loss of heterozygosity was also observed. Of the genes that interacted with *HMX1*, 11 had mutations with high (two genes) or moderate (ten genes) effect that affected protein function. In addition, similar mutations were found in several other genes (high-effect mutations in 43 genes and moderate-effect mutations in 73 genes), but these were not found in all isolates. The latter, larger group of genes involved in mutations sheds light on how different the genetic background may be even among closely related probiotic yeasts. These results are similar to the virulence data presented by McCullough et al. (1998), who showed that virulence can be different for two different batches of the same product. Such differences in the genetic background may explain the different outcome of iron starvation and mouse infection experiments, similar to how a recent study showed that differences in the genetic background of *S. cerevisiae* significantly affect the effects of loss-of-function mutations on yeast fitness. For example, deletion of the heme oxygenase gene had a different effect in some strains [generally reduced the fitness of a given isolate during iron starvation, but in one case (DE35762) it proved to be a beneficial mutation].

Other genomic characteristics were also tested in probiotic yeasts used for knockout experiments (SNP analysis, ploidy, CNV analysis, mitochondrial DNA, 2μ plasmid, rDNA, Ty2 and Ty5 transposon copy number, LOH and GOH events). However, no adaptations and differences were found that were specific for clinical strains or strains that showed high virulence in animal model. The results illustrated that although all isolates selected for gene knockout were closely related *S. 'boulardii'*, they had different genetic backgrounds.

6.3.2 Tolerance to iron starvation is a potential virulence factor for *S. 'boulardii'*

The iron starvation tolerance of isolates and mutant strains was variable in medium supplemented with BPS iron chelator and in liquid medium containing 50% human serum and 50% RPMI-1640. The CFU values obtained in the 50% human serum were lower for the AI0001, AI0003 and AI0009 deletion strains than for the wild-type isolates (although the difference was not significant for AI0009). Interestingly, AI0011 showed significantly higher growth compared to the isolate DE35762. These results were very similar to the results of the spot-plate experiment with BPS. Opposite results were obtained for strains AI0005 and AI0007, as they showed weaker growth in the spot-plate experiment but stronger growth in serum compared to isolates 465/2018 and 2251/2018. For the latter, the difference in growth was not significant.

In addition, we found a significant difference between the three groups studied (commercial, mycosis, non-mycosis) in the spot-plate experiment. The non-mycosis group showed the highest tolerance compared to the other two groups regardless of whether *HMX1* was deleted or not. It is worth noting that members of the non-mycosis group caused high mouse mortality, whereas it was not possible to differentiate strains based on hemolytic activity, despite the latter method is often used to assess the virulence of pathogenic yeasts and the safety of probiotic yeasts. Based on our results, we concluded that testing for tolerance to iron starvation might be a better method to assess virulence than agar plate testing for hemolytic activity, since the latter was not suitable for identifying virulent isolates that caused high mortality in mice.

6.3.3 *S. 'boulardii'* heme oxygenase-1 (*HMX1*) gene as a potential antivirulence factor

Of the wild-type strains, 465/2018 and 2251/2018 significantly (p < 0.0001) reduced the survival of mice, making them the most virulent of the isolates tested. PY0001, PY0002, and DE35762 were shown to be avirulent, while DE6507 showed moderate virulence. Our hypothesis was that deleting the *HMX1* gene would significantly increase the survival of mice due to the inability of yeast cells to utilize the iron-containing heme within the cell. In contrast, when *HMX1* deletion strains were inoculated into mice, the survival of the mice was yeast strain dependent, despite the strains were genetically very close (probiotic-derived strains that presumably underwent only in-host microevolution). Virulence decreased in case of AI0005 and AI0007 strains, but this change was not significant compared to the original wild-type strains (465/2251 and 2251/2018). The virulence of AI0001, AI0009, and AI0011 was higher compared to wild-type strains, but it was not significant for AI0009. The survival of mice in case of the strain AI0003 was unchanged compared to the wild-type strain PY0002. However, loss of *HMX1* resulted in a significant increase of CFU values for all strains except for strain AI0005. Interestingly, the wild-type isolate of this strain caused the highest mouse mortality. It was also observed that the deviation of CFU values was high for every strain, suggesting that the susceptibility of mice to yeast infection was individual-dependent. Despite of this comparing CFU values and survival data showed that deletion of *HMX1* significantly increased the growth of probiotic strains in the kidneys of the applied mouse model.

Virulence factors are properties of fungal pathogens that damage the host (directly or through the host response) or promote fungal growth and survival during infection. The latter are also called virulence determinants. Our results show that, in contrast to the homologue of *C. albicans, HMX1* in *Saccharomyces* is not considered a virulence factor gene. The increased fitness of the deletion strains in the infected mice suggests that yeast heme oxygenase may be considered as potential antivirulence factor. This phenomenon has recently been defined for human pathogenic fungi. Loss or deletion of such genes leads to increased virulence, either by influencing the host response, or by the compensatory stress responses of the fungal cell, which overcompensates for the detrimental effects of gene loss and leads to fitness increase in the stressful environment of the host organism.

The results presented here are the first evidence for the presence of an antivirulence-like gene in *S. 'boulardii'*, suggesting that not only the acquisition of a virulence factor but also a loss-of-function mutation may contribute to increased probiotic yeast viability in a mammalian host. Similar results were only observed in the context of cell wall composition and higher virulence (gene *SSD1*) and cell wall composition and higher *in vitro* immunoactivation (gene *MCD4*) in *S. cerevisiae*. This draws attention to the fact that deletion of a gene may have unexpected adaptive benefits that potentially promote pathogenicity. In this study, the mammalian pathogenicity model showed this surprising effect in the form of a significantly higher kideny burden. *In vitro* experiments, even serum growth tests, did not correlate with these, highlighting the fact that mammal models are definitely recommended when testing the potential pathogenic nature of *Saccharomyces* strains. For that reason, genetic engineering and strain development of the *S. 'boulardii'* probiotic yeast should be performed with caution and bearing in mind that it may be a source of health-related problems in the future. *In vivo* virulence tests of novel probiotic strains, including engineered ones, are therefore highly recommended.

SUMMARY

S. ‘boulardii’ (*S. ‘boulardii’* CNCM I-745) is one of the most sought-after probiotic, the properties of which have been demonstrated in more than 80 randomized clinical trials. Despite its undeniable health benefits, there is a risk of fungemia in immunocompromised or severely ill patients, infants and the elderly. The number of such infections has increased in recent years.

Studying the literature, we revealed that in the case of *Saccharomyces* fungemia, typing methods were performed rarely (in only half of the cases) to determine if the infection was of probiotic origin, and even in these cases, mostly time-consuming methods were used. Combining existing typing methods (ITS, microsatellite, and interdelta analyzes), we developed a multiplex PCR method that can reliably determine in a short time (within 3 hours) whether or not a yeast infection was caused by the probiotic *S. ‘boulardii’*. This may facilitate the assessment of the safety of yeast probiotics and, due to its simple feasibility, may be used in clinical diagnostics. In addition, using our strain collection and the multiplex PCR method, we found that a significant proportion of *Saccharomyces* clinical isolates are of probiotic origin.

The phenotype of the isolates, their interactions with the intestinal epithelial model, and the changes in immune activation were analyzed for 14 *S. ‘boulardii’* isolates. Each yeast isolate had a different phenotype, but it was not possible to unequivocally distinguish between commercial and clinical or clinical non-mycosis and clinical mycosis-causing samples. This shows that *S. ‘boulardii’* is able to survive in the human host for a longer period of time without acquiring traits that previous studies have linked to pathogenicity. It is also notable that probiotic products have high resistance to fluconazole.

We examined the role of the *HMX1* gene in the development of pathogenicity and blood survival of the probiotic yeast by CRISPR/Cas9 gene deletion. Based on the data, the *HMX1* gene may be the first potential antivirulence gene described in *S. ‘boulardii’*. Thus, not only the development of a virulence factor but also a loss-of-function mutation may contribute to pathogenicity. This draws attention to the fact that deletion of a gene and subsequent metabolic changes can cause unexpected adaptation benefits that potentially promote pathogenicity. For this reason, the genetic engineering and strain development of the probiotic yeast *S. ‘boulardii’* should be done with caution, keeping in mind that it may be a source of health problems in the future. Therefore, monitoring the safety and virulence of probiotics that have undergone strain development is strongly recommended using *in vivo* animal models.

NEW SCIENTIFIC RESULTS IN THE DISSERTATION

The data in the dissertation support that:

- The use of interdelta, microsatellite (*YLR177w*, *YOR267c*) and ITS primers in a multiplex PCR reaction allows the diagnostic identification of *S. 'boulardii'* isolates on case of *S. cerevisiae* fungemia or in case of fungal infection of other origins.

- *S. 'boulardii'* isolates show signs of intra-host microevolution, with differences in phenotype, but based on *in vitro* studies the origin of the isolates (commercial, mycosis-causing clinical isolates) can not be determined.
- *In vivo* studies have shown that the virulence and kidney burden of clinical probiotic yeast isolates is higher than that of commercial isolates, although they show variability.

- Genomic analysis of strains selected for *HMX1* deletion experiments showed that the isolates were indeed close relatives but unique mutations and loss-of-heterozygosity were present, thus provided different genetic backgrounds during gene deletion. Individual mutations are considered signs of in-host microevolution.

- The *S. 'boulardii' HMX1* gene acts as a potential antivirulence factor for the yeast based on kidney burden analysis of immunosuppressed mouse model.

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List of publications related to the dissertation

1. **Imre, A.**, Kovács, R. L., Tóth, Z., Majoros, L., Benkő, Z., Pfliegler, V. P., Pócsi, I.: Heme Oxygenase-1 (HMX1) Loss of Function Increases the In-Host Fitness of the Saccharomyces 'boulardii' Probiotic Yeast in a Mouse Fungemia Model.
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Conference publications, presentations, posters:

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