Microbiological Research 227 (2019) 126298

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

A new, rapid multiplex PCR method identifies frequent probiotic origin among clinical *Saccharomyces* isolates

Alexandra Imre[a], Hanna Viktória Rácz[a], Zsuzsa Antunovics[b], Zoltán Rádai[c], Renátó Kovács[d,e], Ksenija Lopandic[f], István Pócsi[a], Walter P. Pfliegler[a,*]

[a] Department of Molecular Biotechnology and Microbiology, University of Debrecen, Egyetem tér 1, Debrecen, H4032, Hungary
[b] Department of Genetics and Applied Microbiology, University of Debrecen, Egyetem tér 1, Debrecen, H4032, Hungary
[c] Department of Evolutionary Zoology and Human Biology, University of Debrecen, Egyetem tér 1, Debrecen, H4032, Hungary
[d] Department of Medical Microbiology, Faculty of Medicine, University of Debrecen, Egyetem tér 1, Debrecen, H4032, Hungary
[e] Faculty of Pharmacy, University of Debrecen, Egyetem tér 1, Debrecen, H4032, Hungary
[f] Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 11/1/3, Vienna, A1190, Austria

ARTICLE INFO

Keywords:
Probiotics
Saccharomyces boulardii
Fungemia
Mycoses
Mycological typing techniques

ABSTRACT

An increasing number of infections originating from probiotic use are reported worldwide, with the majority of such cases caused by the yeast *Saccharomyces 'boulardii'*, a subtype of *S. cerevisiae*. Reliably linking infectious cases to probiotic products requires unequivocal genotyping data, however, these techniques are often time-consuming and difficult to implement in routine diagnostics. This leads to a widespread lack of genetic data regarding the origin of *Saccharomyces* infections. We propose a quick and reliable PCR-based protocol for the identification of *S. 'boulardii'* based on a combined analysis of interdelta fingerprinting and microsatellite typing. By applying various typing methods and our proposed method to the clinical yeast collection of a Hungarian hospital we show that probiotic origin is common among clinical *Saccharomyces*, and that the new multiplex method enables rapid and unequivocal identification of probiotic yeast infections. This method can be applied for the identification of yeast infection sources, helping decisions on probiotic use.

1. Introduction

Hospitalized, especially critically ill patients are often vulnerable to infections aquired during medical procedures and treatment. Besides nosocomial bacterial and fungal strains, an additional, emerging group of microbes began to draw attention: probiotic microorganism that are self-administered, prescribed to patients, or are merely inadvertently carried from one patient to another by healthcare personnel (Costa et al., 2018; Hennequin et al., 2000; Pérez-Torrado and Querol, 2015). According to a recent study, the majority of probiotic-derived infectious complications worldwide are caused by *Saccharomyces* yeasts (50.6% of the cases), surpassing probiotic bacterial genera (*Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Pediococcus* and *Escherischia*) (Costa et al., 2018). Occasional infectious side-effects of these probiotics mostly involve infants, immunocompromised, and elderly patients (Costa et al., 2018). Contamination of the air, environmental surfaces, and hands of health care personnel following the opening of probiotic yeast packets have been linked to catheter-related fungemias (Hennequin et al., 2000). Furthermore, *S. 'boulardii'* is the only known probiotic organism that caused a hospital infectious outbreak (Cassone et al., 2003). Because of their pathogenic potential, the yeast probiotics, their side-effects, and their epidemiology should be studied in more detail.

Several species of non-*albicans* yeasts have recently been identified as emerging or potentially emerging fungal pathogens and emphasis has been put on reliably identifying these species with methods that can be integrated into routine diagnostics (e.g. Arastehfar et al., 2019, 2018). However, intraspecific diversity of yeast pathogens is so far neglected. *Saccharomyces* yeast probiotics are marketed as *S. 'boulardii'*, although they do not constitute a species separate from *S. cerevisiae*, they only form a subclade of the Wine/European clade of the species that is notable for its lack of Ty1 retrotransposons and their asporogenous nature (Khatri et al., 2017; Peter et al., 2018).

S. cerevisiae is a potentially emerging fungal pathogen (Enache-Angoulvant and Hennequin, 2005; Muñoz et al., 2005) and clinical

Abbreviations: ITS, Internal Transcribed Spacer; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time Of Flight spectrometry; MLST, Multi-Locus Sequence Typing; MSP-PCR, Microsatellite-Primed PCR; TBE, Tris-Borate-EDTA

* Corresponding author.
E-mail address: pfliegler.valter@science.unideb.hu (W.P. Pfliegler).

https://doi.org/10.1016/j.micres.2019.126298
Received 13 May 2019; Received in revised form 20 June 2019; Accepted 12 July 2019
Available online 15 July 2019
0944-5013/

isolates have been identified in several of its phylogenetic clades (Peter et al., 2018). Yet, the diversity of the species is often not taken into account in case studies, even if probiotic origin of an infectious case is suspected. Our analysis of 40 publications on *S. cerevisiae* infections from the last 20 years shows that more than half of these did not use any genetic method to test the kinship of the infectious yeasts to commercial products (Supplementary file 1). Publications that used genotyping applied a wide variety of methods with various discriminatory power, and subtyping techniques based either on interdelta analysis (fingerprinting focussed on Ty transposone terminal repeat sequences) or on microsatellites, proposed earlier, have not gained use in recent years (de Llanos et al., 2004; Hennequin et al., 2001; Posteraro et al., 2005). This widespread lack of subtyping hinders the correct risk assesment of both the probiotics and the species as a whole.

Our group recently applied complex genetic typing to unequivocally identify a probiotic-derived clinical isolate (Pfliegler et al., 2017). However, this complex typing approach is unsuitable for diagnostic purposes where probiotic origin is of interest due to its length and specialized equipment need. Thus, there is a need for the rapid and reliable identification of the *S. cerevisiae 'boulardii'* subtype that we aim to address in this work, helping diagnostics and the identification of possible nosocomial probiotic infections or equipment contamination (compare to Cassone et al., 2003; Hennequin et al., 2000). Furthermore, differentiating dietary, probiotic and other *Saccharomyces* may be useful in assessing colonization potential and overall health effects of the probiotic yeasts. Using multiple typing methods, we identified probiotic-derived clinical *Saccharomyces* isolates from a local university clinic and developed a rapid multiplex PCR method that can unequivocally identify which *S. cerevisiae* isolate is probiotic-derived in a matter of hours.

2. Materials and methods

2.1. Isolation, identification, isolate maintenance, DNA isolation, sporulation tests

We utilized the yeast isolate collection of the University Clinics of Debrecen to genotype clinical *Saccharomyces* isolates. Isolates originate from samples collected during routine diagnostics from August 2015 to March 2018 at the clinics of the University of Debrecen and identified by MALDI-TOF, mass spectra were generated with Microflex Biotyper (Bruker Daltonics, Billerica, MA, USA) using the manufacturer's standard settings. Mass fingerprints were acquired using the flexControl version 3.0 software (Brucker Daltonics), analyzed over a mass range from 2000 to 20000 Da, and compared with the Bruker Daltonics database. Any culture identified as *Saccharomyces* during routine diagnostics was later incorporated into our genetic study. Additionally, two batches from two locally available yeast probiotic products were obtained commercially. Single-colony isolates from clinical samples and from products were stored in GYP medium [1% yeast extract (Alfa Aesar, Ward Hill, MA, USA), 2% peptone (Oxoid, Basingstoke, UK), 2% glucose, pH 5.8] supplemented with 30% glycerol at −70 °C. Various *Saccharomyces* strains of different clades were also involved in testing the method (Supplementary file 3). A list of isolates is presented in Table 1.

Genomic DNA was isolated according to Hanna and Xiao (2006), concentration was standardized to 100 ng/µl. DNA samples were stored in 1 × TE buffer at −20 °C. Colony DNA for colony PCR tests was isolated according to Lõoke et al. (2011) and stored in 1 × TE.

2.2. MLST

MLST involved sequencing of four nuclear genes and the nuclear ITS region, all on different chromosomes, using the primers CCA1f (5′-GTGCTTTGGCACACC-3′), CCA1r (5′-ATTCTTGATCAGTCCCT GTA-3′) (Fay and Benavides, 2005); CYT1f (5′-CATTTTCATTACACTA TATCATCTACTA-3′), CYT1r (5′-CAATTCAGTATGCTCTACTAATA-3′) (Fay and Benavides, 2005); HMX1f (5′-GCTTAGTCTAAGGAGGAGC TAT-3′), HMX1r (5′-TGCTGTTTTTCCTTCCCTATTC-3′) (this study); NUP116f (5′-AAGCAACTGTCACCAACACG-3′), NUP116r (5′-CTTCCCC ATCGTTCTTTGAG-3′) (Ayoub et al., 2006); ITS1 (5′-TCCGTAGGTGA ACCTGCGG-3′) (White et al., 1990), ITS4 (5′-TCCTCCGCTTATTGATA TGC-3′) (Gardes and Bruns, 1993) for genes *CCA1*, *CYT1*, *HMX1*, *NUP116* and the ITS, respectively, using Pwo polymerase (Sigma Aldrich, St. Louis, MO, USA). PCR protocol was as follows: 94 °C for 2 min, 34 × (94 °C for 20 s, 55 °C for 50 s, 68 °C for 80 s), 68 °C for 5 min. 0.2 mM each dNTP and 10 pmol of primers were used in each reaction. For amplification, an Applied Biosystems (Foster City, CA, USA) 2720 thermal cycler and a final volume of 50 µl were used. PCR products were loaded onto 1.2% agarose gels for electrophoresis at 90 V for 45 min and UV transillumination was used to check the product size. PCR products were cleaned with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL, USA) and sequenced using the same primers (Microsynth AG, Austria). Amplified and sequenced products spanned the whole *CYT1* and *HMX1* genes, an 788 bp fragment of *CCA1* and a 427 fragment of *NUP116*. Complete ITS1-5.8S rDNA-ITS2 sequence was extracted from reads using ITSx (Bengtsson-Palme et al., 2013). Reads were checked and edited using Chromas 2.6.5. (Technelysium Pty. Ltd.). Sequences were deposited in GenBank (Supplementary file 2). For phylogenetic analysis, sequences were aligned (using MUSCLE) and concatenated using MEGA X (Kumar et al., 2018). Model selection and the generation of Maximum Likelihood phylograms with 1000 bootstrap replicates were conducted in the same software. Dendrograms were visualized using iTOL (Letunic and Bork, 2019). Haplotypes in one of the heterozygous isolates were phased out by cloning into pJET1.2 vectors using CloneJet (Thermo Fisher Scientific) according to the manufacturer's recommendations and by subsequent sequencing. Alignments were deposited in FigShare (Supplementary file 2).

2.3. PCR-fingerprinting

For MSP-PCR reactions 50 ng gDNA was used in 12.5 µl final volumes (Pfliegler et al., 2014). Reactions with primer $(GTG)_5$ were performed with the following programme: 95 °C for 5 m in. 30 × (94 °C 30 s, 45 °C 30 s, 72 °C 50 s), 72 °C 5 min. For amplification, 1.5 units of GoTaq DNA Polymerase was used with GoTaq Green Buffer and $MgCl_2$ at 2.5 mM end concentration, with 0.2 mM each dNTP. 10 pmol primer were used in each reaction. Reactions were carried out in a C1000 Touch (BioRad, Hercules, CA, USA) thermocycler.

For interdelta fingerprinting, primers delta12 (5′-TCAACAATGGA ATCCCAAC-3′) and delta2 (5′-GTGGATTTTTATTCCAACA-3′) (Legras and Karst, 2003) were used. For each 25 µl reaction 50 ng DNA and 0.75 unit of GoTaq G2 DNA polymerase (Promega, Madison, WI, USA) was used with GoTaq Green Buffer (Promega) supplemented with $MgCl_2$ (Thermo Fischer Scientific, Waltham, MA, USA) to 2.5 mM end concentration with 12.5 pmol of each primer and 0.2 mM each dNTP according to Pfliegler and Sipiczki (2016). Reactions were carried out in a TProfessional (Biometra, Göttingen, Germany) thermocycler. PCR protocol was as follows: 95 °C for 5 min, 30 × (94 °C for 50 s, 55 °C for 50 s, 72 °C for 50 s), 72 °C for 5 min.

PCR products (5 µl) were loaded onto 1.2% (MSP) or 2% (interdelta) agarose gels, electrophoresis was carried out at 110 V for 75 min in 1 × TAE buffer. 1 kb DNA marker (Thermo Fischer Scientific) was used for each gel run. Band patterns obtained after transillumination using Gel Red (Fremont, CA, USA) were analysed by the UPGMA algorithm available at http://genomes.urv.es/UPGMA/ with the Dice coefficient. Dendrograms were visualized using iTOL.

2.4. Microsatellite typing

Reactions were carried out with primers YLR177wf (5′-CTTAAAC

Table 1
Collection and patient data for the isolates used in this study. Isolates shown to be probiotic-derived in this study are marked. ICU = intensive care unit.

Identifier	Type	Formulation	Component	Place of aquisition	Date of aquisition	Country of manufacturing	Species
PY0001	probiotic supplement	active dry	single component	Debrecen, Hungary	Mar-2015	France	*S. cerevisiae*
PY0002	probiotic supplement	active dry	single component	Debrecen, Hungary	Nov-2017	France	*S. cerevisiae*
PY0003	probiotic supplement	active dry	multicomponent	Debrecen, Hungary	Sep-2017	Czechia	*S. cerevisiae*
PY0004	probiotic supplement	active dry	multicomponent	Debrecen, Hungary	Nov-2017	Czechia	*S. cerevisiae*

Identifier	Type	Age (yr), sex, and underlying disease of patient, mycosis symptoms	Anatomical origin/ sample type	Date of sampling	Geographic origin, unit	Species
DE6507	clinical isolate (probiotic-derived)	63 yr, ♂, pneumonia, mycosis	haemoculture	18-Feb-17	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE35762	clinical isolate (probiotic-derived)	66 yr, ♀, respiratory failure, mycosis	haemoculture	5-Nov-15	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE27290	clinical isolate	71 yr, ♂, aortic stenosis, non-mycosis	bronchus (sampling during intubation)	25-Aug-15	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE27020	clinical isolate (probiotic-derived)	40 yr, ♀, sepsis (bacterial), non-mycosis	bronchus (sampling during intubation)	23-Aug-15	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE29607	clinical isolate	61 yr, ♂, lung cancer, non-mycosis	sputum	16-Sep-15	Debrecen, University Clinic, inpatient care	*S. cerevisiae*
DE11595	clinical isolate	45 yr, ♂, subarachnoid hemorrhage, mycosis	haemoculture	22-Mar-18	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE42533	clinical isolate (probiotic-derived)	2 yr, ♂, fluid homeostasis disorder, non-mycosis	throat	15-Dec-17	Debrecen, University Clinic, inpatient care	*S. cerevisiae*
DE42651	clinical isolate	59 yr, ♂, 90 per cent burn, non-mycosis	nose	02-Dec-17	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE42807	clinical isolate (probiotic-derived)	1 yr, ♀, diarrhea, non-mycosis	vagina	04-Dec-17	Debrecen, University Clinic, inpatient care	*S. cerevisiae*
DE43763	clinical isolate	73 yr, ♀, sepsis (bacterial), non-mycosis	bronchus (sampling during intubation)	11-Dec-17	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE45866	clinical isolate (probiotic-derived)	64 yr, ♂, cerebral infarction, non-mycosis	bronchus (sampling during intubation)	29-Dec-17	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE342	clinical isolate	40 yr, ♀, vaginitis, mycosis	cervix	03-Jan-18	Debrecen, University Clinic, ambulatory care	*S. cerevisiae*
DE722	clinical isolate	29 yr, ♀, high risk pregnancy, non-mycosis	cervix	08-Jan-18	Debrecen, University Clinic, inpatient care	*S. cerevisiae*
DE3912	clinical isolate (probiotic-derived)	85 yr, ♂, pneumonia, non-mycosis	trachea (sampling from tracheal cannula)	31-Jan-18	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE10397	clinical isolate	67 yr, ♂, cardiovascular disease, non-mycosis	trachea (sampling from tracheal cannula)	14-Mar-18	Debrecen, University Clinic, ICU	*S. cerevisiae*
DE22293	clinical isolate	36 yr, ♂, acute pancreatitis, non-mycosis	bronchus (sampling during intubation)	11-Jul-15	Debrecen, University Clinic, ICU	*C. krusei*

AACAGCTCCCAAA-3′) and YLR177wr (5′-ATGAATCAGCGCATCAGA AAT-3′) (Malgoire et al., 2005) for the microsatellite region in *YLR177w*, and with YOR267cf (5′-ATGACTGCAGCAATGAATCG-3) and YOR267cr (5′-TCCTCTGTGCTGTTGACTCG-3′) (Jubany et al., 2008) for microsatellite region in *HRK1* (*YOR267c*) using the following programme: 98 °C for 30 s, 18× (*YOR267c*) or 20× (*YLR177w*) (98 °C for 10 s, 55 °C for 20 s, 72 °C for 15 s), 72 °C for 5 min. For each reaction, 0.25 unit Phusion polymerase (Thermo Fischer Scientific), 20 ng of genomic DNA, 10 pmols of each primer, and 0.2 mM each dNTP in 12.5 µl end volume were used. This optimized short programme and the high fidelity polymerase were chosen to avoid polymerase stutterning and other potential amplification problems common for microsatellite regions. Reactions were carried out in a C1000 Touch thermocycler. Gel electrophoresis was conducted in 3% low electroendosmosis TBE agarose gel stained with GelRed, at 110 V for 5 h. 5 µl product was used for each run. Primer annealing sites and expected product sizes were determined beforehand by BLASTing into probiotic yeast genomes (Khatri et al., 2017) using NCBI's Nucleotide BLAST service. Microsatellite alleles were determined using 20 bp size marker (Sigma Aldrich) and by using the software GelAnalyzer (Lazar & Lazar, http://www.gelanalyzer.com/) for product size and hence repeat number determination. Due to the presence of mono-, di- and triallelic isolates, repeat numbers were compared using the POLYSAT 1.7 package in R (Clark and Jasieniuk, 2011) with no a priori assumption of ploidy. Bruvo's distances were calculated for pairwise comparisons. Minimum spanning networks were visualized using POPPR (Kamvar et al., 2014).

2.5. Sporulation test

Sporulation capability was tested by inoculating overnight GYP cultures onto potassium acetate sporulation medium (0.05% glucose, 1% potassium acetate, 0.1% yeast extract, 2% agar). Sporulation was evaluated after 5 days of incubation at room temperature, using phase contrast microscopy and 400× magnification.

3. Results

3.1. Species identification and MLST

Altogether 16 yeast isolates, all collected during routine diagnostic work at the clinics of the University of Debrecen, were identified as *Saccharomyces cerevisiae* by the MALDI-TOF method. Of these, a single isolate proved to be misidentified based on sequencing the ITS barcode region. This *Pichia kudriavzevii* (=*Candida krusei*) isolate, DE22293 was omitted from further analysis. Besides the ITS region, gene sequencing was conducted for four nuclear genes located on different chromosomes of the species, and commercial probiotic isolates were also included. For three isolates, the gene *HMX1* was unreadable or partially readable and for six isolates, the ITS region was unreadable using Sanger sequencing due to the presence of alleles differing in insertion/deletion mutations (Supplementary file 2). Heterozygous, yet readable sequences with various single nucleotide polymorphisms (SNPs) were found in all sequenced genes and the ITS. Using the concatenated dataset of 3854 nucleotides, phylogenetic analysis identifed eight clades. Among these, eight clinical isolates formed a uniform clade with commercial probiotic yeasts (Fig. 1A). However, one of these, DE10397 displayed heterozygous SNPs for genes *CCA1*, *CYT1*, *HMX1*, and *NUP116* (while its ITS was unreadable), which were subsequently phased out by cloning into vectors. Sequenced clones revealed that this isolate possessed haplotypes identical to those of the probioitics as well as diverged haplotypes with 1–7 SNP differences. The remaining seven isolates in the clade (DE6507, DE27020, DE35762, DE3912, DE42533, DE42807, DE45866) displayed no nucleotide differences from the probiotics along the five sequenced regions and even shared a single heterozygous position in the ITS1 region (A/G, position 121). All other sequenced regions were homozygous in these seven isolates.

3.2. Fingerprinting and microsatellite typing, sporulation

MSP-PCR had a low resolution, discriminating merely two groups. The probiotics and the seven probiotic-like isolates described above were uniform but indistinguishable from additional isolates (Fig. 1B, Supplementary file 3). Interdelta fingerprinting identified nine groups among the isolates. The probiotic yeasts and the seven potentially probiotic-derived clinical isolates were indistinguishable with this method, too (Fig. 1C, Supplementary file 3). Microsatellite typing identified homozygous alleles for all probiotics and clinical yeasts in the case of *YLR177* (chromosome XII), with 4–12 CAG repeats. Homo-, di- and trizygousity was found for *YOR267c*, with 11–41 CAA repeats (Supplementary file 3). All probiotics and the derived seven clinical isolates were clearly distinguished from other isolates (Fig. 1D) and all of them were homozygous for both microsatellites. These seven probiotic-like isolates showed no sporulation, similarly to the four sequenced commercial probiotic yeasts, while all other isolates sporulated (Fig. 1E) on potassium acetate medium.

3.3. New multiplex PCR identification method

We aimed to combine PCR-based methods with high diagnostic value (applicable for strain-level differentiation) that do not require specialized equipment and extensive preparation. Based on MLST, MSP-PCR, interdelta and microsatellite typing results as well as and other works concerning interdeltas and microsatellite typing (Hennequin et al., 2001; Legras and Karst, 2003; Posteraro et al., 2005), we combined primers for the following: interdeltas, *YLR177w*, *YOR267c*, and the ITS region into a single multiplex reaction. To avoid large intensity differences of product bands, primer concentrations were optimized (Table 2).

For probiotics and probiotic-derived clinical isolates, our multiplex PCR resulted in a conspicuous band pattern of seven bands in three groups (600–850 bp, 210–270 bp, and 100–140 bp ranges), plus a single band of < 75 bp with the enzyme GoTaq (Fig. 2A). The band patterns were invariable among the probiotics and the seven probiotic-related clinical isolates. Sufficient resolution was achieved already after 1 h of gel electrophoresis at 100 V in 2% TBE agarose gel.

The other, non-probiotic related clinical isolates displayed different band patterns and formed seven separate groups, clearly distinguishing them from the probiotic group (Fig. 2B). Furthermore, as expected, the *C. krusei* isolate displayed no bands from the *S. cerevisiae*-specific sequences, but displayed a clear control ITS band (Fig. 2A).

The method was subsequently tested both with purified genomic and colony DNA, and also with DreamTaq enzyme (Thermo Fischer Scientific, Waltham, MA, USA) (Supplementary file 3). DreamTaq polymerase also produced identical patterns for the probiotic and derived clinical yeasts. Two bands of the patterns were changed in size and relative intensity compared to the pattern with GoTaq, but this did not affect evaluation of the band patterns.

Finally, we also tested further wine, food, bioethanol and laboratory *S. cerevisiae* isolates for their band patterns with the multiplex method. The resulting band patterns were markedly different from that of the probiotics, allowing for a reliable subtyping of *'boulardii'* yeasts (Supplementary file 3).

4. Discussion

PCR-based identification methods for species determination and PCR-fingerprinting methods for subtyping (i.e. differentiating among different lineages of a single species) are both applicable in food and clinical microbiology to circumvent or complement specialized and time-consuming (Pfliegler et al., 2017) diagnostic techniques. The intraspecific diversity of food and clinical yeasts has received considerable attention lately, with *S. cerevisiae*, a widely utilized yet occasionally pathogenic yeast as a prime example for both groups (Legras et al.,

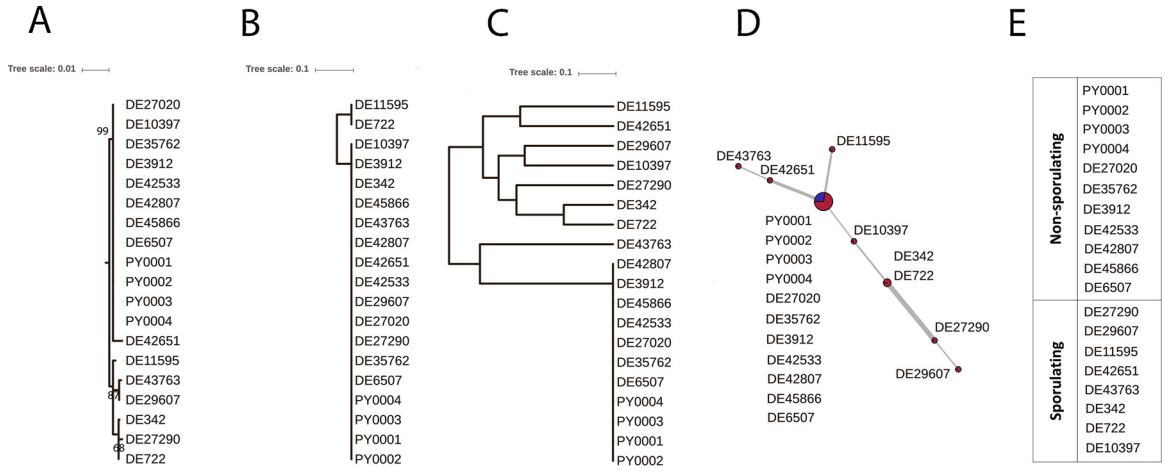

Fig. 1. Results of the application of various typing techniques applied for the isolate collection of the University of Debrecen. A: maximum likelihood phylogram for the concatenated dataset of five sequences with bootstrap support values. B: UPGMA dendrogram obained from MSP-PCR analysis. C: UPGMA dendrogram obtained for interdelta analysis. D: Minimum spanning network of microsatellite allele similarities. E: sporulation data for the isolates.

2018; Peter et al., 2018; Pfliegler et al., 2017). Routine clinical mycological diagnostics rarely focuses on subtyping methods, although these would be a prequisite to understand how and why infections may arise from products containing live yeasts, such as probiotics.

Our newly described approach to combine rapid typing methods with high diagnostic value to identify probiotic-derived infections aims to address this diagnostic challenge. Recent works have highlighted that the application of different genotyping methods can lead to different results (Csoma et al., 2018; Pfliegler et al., 2014; Pfliegler and Sipiczki, 2016), thus the combination of methods into a single, multiplex reaction is highly desirable. Our method was developed on the isolate collection of the University of Debrecen clinics by sequencing of five regions, interdeltas, and MSP fingerprinting, and by typing of two microsatellites. Of these, interdeltas, two microsatellites, and the ITS region as a control were combined into a multiplex method. Interdelta primers are specific to Ty1- and Ty2-transposon long terminal repeats (the delta sequences), the former of which lack from the probiotic genome (Khatri et al., 2017) resulting in uniqe fingerprinting band patterns, while alleles of the used microsatellites have been shown to be specific to *S. 'boulardii'* in our study and in earlier papers (Hennequin et al., 2001; Posteraro et al., 2005). It is noteworthy that the relatively time-consuming MLST method showed inferior resolution and methodological constraints in the case of isolates with heterozygous in/del mutations. Probiotic yeasts are known to be asporogenous, and among our isolates, all probiotic derived ones retained this unusual trait.

By using our multiplex method, the equally spaced groups of bands in the case of the probiotics allows a quick visual evaluation even without a computerized gel analysis pipeline. As MALDI-TOF, the most widespread species identification method in clinical microbial diagnostics, involves testing live colonies, our method may be applied directly to the remaining cell mass after such a species identification has been conducted. This can be achieved by running a colony PCR and a subsequent agarose gel. We estimate that the complete workflow if yeast colonies are available can be finished within 3 h after MALDI-TOF identifies a *S. cerevisiae* yeast. The fungal ITS-region specific primers in the multiplex PCR account for a positive control product in case of samples incorrectly identified as *Saccharomyces* (as in Fig. 2A).

Using this multiplex PCR, we were able to show that probiotic-derived clinical yeasts are common among *S. cerevisiae* isolates and are recovered from various anatomical sites of a wide range of patients: 16 isolates from 16 different patients were identified as *S. cerevisiae* using the MALDI-TOF platform, of which a single isolate proved to be misidentified. The 15 *S. cerevisiae* isolates that were confirmed by sequence analysis originated from various anatomical sites and specimen types. The seven probiotic-derived isolates included samples from all of these body sites and were collected from three female and four male patients, ranging from infants to elderly patients, suggesting that probiotic colonization (without mycosis symptoms) and infection (mycosis/fungaemia) are relatively common. Merely three fungaemia cases were recorded during the study period, of these, two originated from probiotics, while the additional probiotic-drived isolates likely represented colonizers.

5. Conclusion

The research of the effect of probiotics, including their safety still lags behind their widespread application (Costa et al., 2018; Suez et al., 2019). With our multiplex method, PCR followed by gel electrophoresis and visual inspection is sufficient to establish the connection between yeast probiotics and yeast infections. This can aid a better assessment of

Table 2
Specifications of the proposed Multiplex PCR method.

List of primers	δ12 (5'-TCAACAATGGAATCCCAAC-3'), δ2 (5'-GTGGATTTTTATTCCAACA-3'), YLR177wf (5'-CTTAAACAACAGCTCCCAAA-3'), YLR177wr (5'-ATGAATCAGCGCATCAGAAAT-3'), YOR267cf (5'-ATGACTGCAGCAATGAATCG-3'), YOR267cr (5'-TCCTCTGTGCTGTTGACTCG-3'), ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS4 (5'-TCCTCCGCTTATTGATATGC-3')
PCR mix and programme (GoTaq)	PCR mix for a 50 µl reaction: 1× GoTaq Flexi Buffer; 4.0 mM $MgCl_2$; 0.2 mM each dNTP; 20 pmol of primers δ12, δ2, YLR177wf, YLR177wr each; 10 pmol of primers YOR267cf, YOR267cr each; 3 pmol of primers ITS1, ITS4 each; 2.5 u GoTaq G2 Hot Start Polymerase; 1 ng genomic DNA (or 1 µl colony DNA) Programme: 95 °C 3 min, 25× (95 °C 30 sec, 55 °C 30 sec, 72 °C 1 min), 72 °C 5 min
PCR mix and programme (DreamTaq)	PCR mix for a 50 µl reaction: 1× DreamTaq Buffer incl. $MgCl_2$; 0.2 mM each dNTP; 20 pmol of primers δ12, δ2, YLR177Wf, YLR177Wr each; 10 pmol of primers YOR267Cf, YOR267Cr each; 3 pmol of primers ITS1, ITS4 each; 1.25 u DreamTaq Polymerase; 1 ng genomic DNA (or 1 µl colony DNA) Programme: 95 °C for 3 min, 25x (95 °C 30 sec, 50 °C 30 sec, 72 °C 1 min), 72 °C 5 min

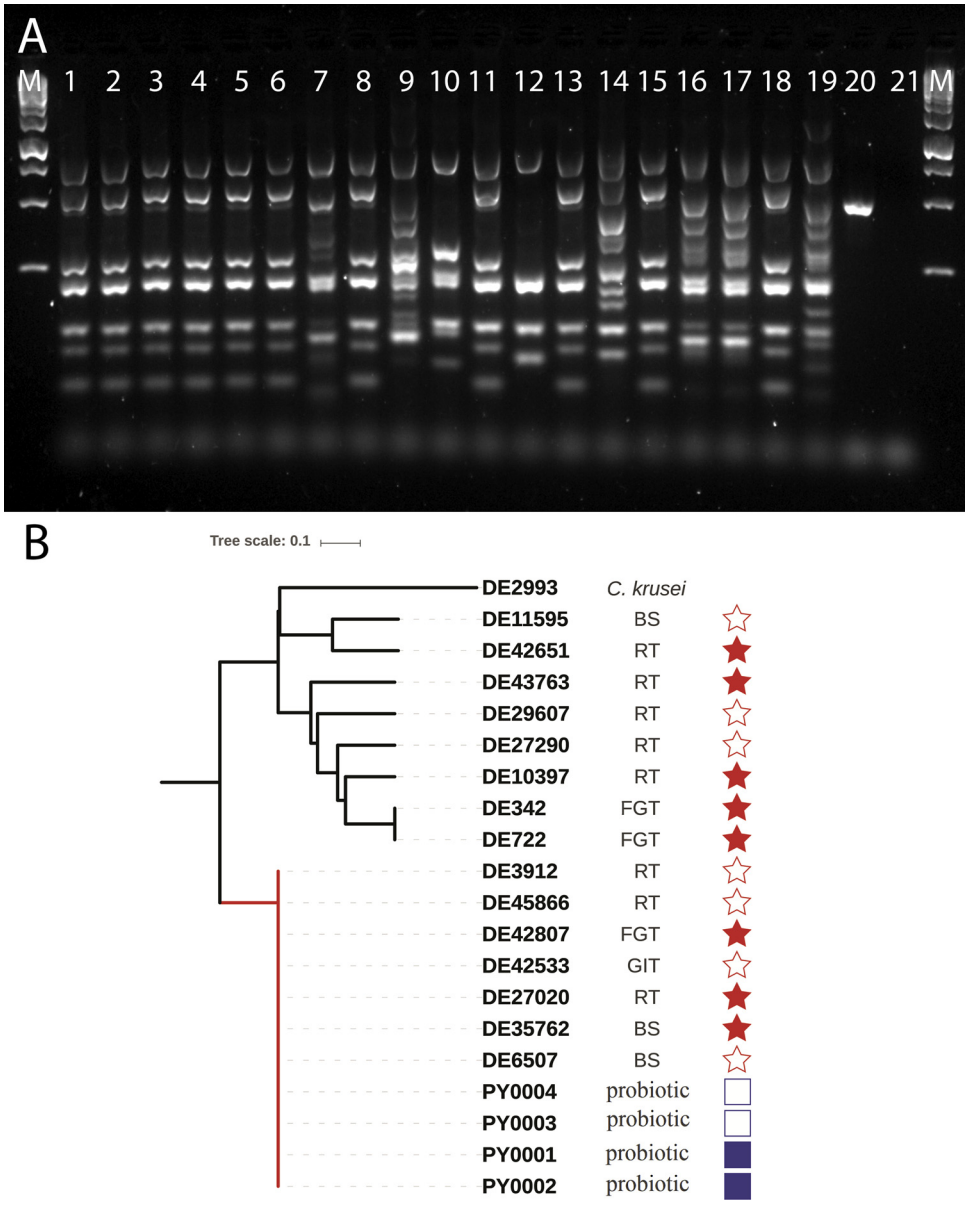

Fig. 2. Results obtained with the newly designed multiplex method. A: gel electrophoresis image (1 h, 2% TBE, 100 V, 5 µl product); M: Gene Ruler 1 kb marker (Thermo Fischer Scientific); 1: PY0001; 2: PY0002; 3: PY0003; 4: PY0004; 5: DE6507; 6: DE35762; 7: DE27290; 8: DE27020; 9: DE29607; 10: DE11595; 11: DE42533; 12: DE42651; 13: DE42807; 14: DE43763; 15: DE45866; 16: DE342; 17: 722; 18: DE3912; 19: DE10397; 20. DE22293; 21: negative control. B: UPGMA dendrogram obtained using the multiplex method; probiotic clade of isolates marked with red; filled squares: single component probiotics; empty squares: multicomponent probiotic products; filled stars: female patients; empty stars: male patients; BS: bloodstream; FGT: female genital tract; GIT: gastrointestinal tract; RT: respiratory tract.

probiotic safety in general. Using the method, we showed that probiotic-derived yeasts make up a large proportion of mycosis and non-mycosis human *Saccharomyces* isolates. This observation shall be taken into account not only by clinical mycologists, but also by researchers studying the mycobiome of hospitalized patients and the phenomenon of microbial dysbiosis.

Ethical approval

Patient data was handled in accordance with EU, state, and local regulations with a clinical study ethics approval from the Regional and Institutional Research Ethics Council of Debrecen (DE RKEB/IKEB 5194-2019).

Acknowledgements

This work was supported by the Austria-Hungary Action (98öu7; Walter P. Pfliegler and Ksenija Lopandic) and the ÚNKP-18-3-I-DE-4 New National Excellence Program of the Ministry of Human Capacities of Hungary (Alexandra Imre). We thank Reviewers for valuable comments.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126298.

References

Arastehfar, A., Fang, W., Badali, H., Vaezi, A., Jiang, W., Liao, W., Pan, W., Hagen, F., Boekhout, T., 2018. Low-cost tetraplex PCR for the global spreading multi-drug resistant fungus, *Candida auris* and its phylogenetic relatives. Front. Microbiol. 9, 1119. https://doi.org/10.3389/fmicb.2018.01119.

Arastehfar, A., Fang, W., Pan, W., Lackner, M., Liao, W., Badiee, P., Zomorodian, K., Badali, H., Hagen, F., Lass-Flörl, C., Boekhout, T., 2019. YEAST PANEL multiplex PCR for identification of clinically important yeast species: stepwise diagnostic strategy, useful for developing countries. Diagn. Microbiol. Infect. Dis. 93, 112–119. https://doi.org/10.1016/j.diagmicrobio.2018.09.007.

Ayoub, M.J., Legras, J.L., Saliba, R., Gaillardin, C., 2006. Application of Multi Locus Sequence Typing to the analysis of the biodiversity of indigenous *Saccharomyces cerevisiae* wine yeasts from Lebanon. J. Appl. Microbiol. 100, 699–711. https://doi.org/10.1111/j.1365-2672.2006.02817.x.

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., De Wit, P., Sánchez-García, M., Ebersberger, I., de Sousa, F., Amend, A., Jumpponen, A., Unterseher, M., Kristiansson, E., Abarenkov, K., Bertrand, Y.J.K., Sanli, K., Eriksson, K.M., Vik, U., Veldre, V., Nilsson, R.H., 2013. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes

for analysis of environmental sequencing data. Methods Ecol. Evol. 4, 914–919. https://doi.org/10.1111/2041-210X.12073.
Cassone, M., Serra, P., Mondello, F., Girolamo, A., Scafetti, S., Pistella, E., Venditti, M., 2003. Outbreak of *Saccharomyces cerevisiae* subtype *boulardii* fungemia in patients neighboring those treated with a probiotic preparation of the organism. J. Clin. Microbiol. 41, 5340–5343. https://doi.org/10.1128/JCM.41.11.5340-5343.2003.
Clark, L.V., Jasieniuk, M., 2011. polysat: An R package for polyploid microsatellite analysis. Mol. Ecol. Resour. 11, 562–566. https://doi.org/10.1111/j.1755-0998.2011.02985.x.
Costa, R.L., Moreira, J., Lorenzo, A., Lamas, C.C., 2018. Infectious complications following probiotic ingestion: a potentially underestimated problem? A systematic review of reports and case series. BMC Complement. Altern. Med. 18, 329. https://doi.org/10.1186/s12906-018-2394-3.
Csoma, H., Ács-Szabó, L., Papp, L.A., Sipiczki, M., 2018. Application of different markers and data-analysis tools to the examination of biodiversity can lead to different results: a case study with *Starmerella bacillaris* (synonym *Candida zemplinina*) strains. FEMS Yeast Res. 18. https://doi.org/10.1093/femsyr/foy021.
de Llanos, R., Querol, A., Planes, A.M., Fernández-Espinar, M.T., 2004. Molecular characterization of clinical *Saccharomyces cerevisiae* isolates and their association with non-clinical strains. Syst. Appl. Microbiol. 27, 427–435. https://doi.org/10.1078/0723202041438473.
Enache-Angoulvant, A., Hennequin, C., 2005. Invasive *Saccharomyces* infection: a comprehensive review. Clin. Infect. Dis. 41, 1559–1568. https://doi.org/10.1086/497832.
Fay, J.C., Benavides, J.A., 2005. Evidence for domesticated and wild populations of *Saccharomyces cerevisiae*. PLoS Genet. 1, 0066–0071. https://doi.org/10.1371/journal.pgen.0010005.
Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x.
Hanna, M., Xiao, W., 2006. Isolation of nucleic acids. Methods Mol. Biol. 313, 15–20. https://doi.org/10.1385/1-59259-958-3:015.
Hennequin, C., Kauffmann-Lacroix, C., Jobert, a, Viard, J.P., Ricour, C., Jacquemin, J.L., Berche, P., 2000. Possible role of catheters in *Saccharomyces boulardii* fungemia. Eur. J. Clin. Microbiol. Infect. Dis. 19, 16–20.
Hennequin, C., Thierry, A., Richard, G.F., Lecointre, G., Nguyen, H.V., Gaillardin, C., Dujon, B., 2001. Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains. J. Clin. Microbiol. 39, 551–559. https://doi.org/10.1128/JCM.39.2.551-559.2001.
Jubany, S., Tomasco, I., Ponce De León, I., Medina, K., Carrau, F., Arrambide, N., Naya, H., Gaggero, C., 2008. Toward a global database for the molecular typing of *Saccharomyces cerevisiae* strains. FEMS Yeast Res. 8, 472–484. https://doi.org/10.1111/j.1567-1364.2008.00361.x.
Kamvar, Z.N., Tabima, J.F., Grünwald, N.J., 2014. Poppr : an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2, e281. https://doi.org/10.7717/peerj.281.
Khatri, I., Tomar, R., Ganesan, K., Prasad, G.S., Subramanian, S., 2017. Complete genome sequence and comparative genomics of the probiotic yeast *Saccharomyces boulardii*. Sci. Rep. 7, 1–13. https://doi.org/10.1038/s41598-017-00414-2.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.
Legras, J.-L., Galeote, V., Bigey, F., Camarasa, C., Marsit, S., Nidelet, T., Sanchez, I., Couloux, A., Guy, J., Franco-Duarte, R., Marcet-Houben, M., Gabaldon, T., Schuller, D., Sampaio, J.P., Dequin, S., 2018. Adaptation of *S. cerevisiae* to fermented food environments reveals remarkable genome plasticity and the footprints of domestication. Mol. Biol. Evol. 35, 1712–1727. https://doi.org/10.1093/molbev/msy066.
Legras, J., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. FEMS Microbiol. Lett. 221, 249–255. https://doi.org/10.1016/S0378-1097(03)00205-2.
Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47, W256–W259. https://doi.org/10.1093/nar/gkz239.
Lõoke, M., Kristjuhan, K., Kristjuhan, A., 2011. Extraction of genomic DNA from yeasts for PCR-based applications. Biotechniques 50, 325–328. https://doi.org/10.2144/000113672.
Malgoire, J.Y., Bertout, S., Renaud, F., Bastide, J.M., Mallié, M., 2005. Typing of *Saccharomyces cerevisiae* clinical strains by using microsatellite sequence polymorphism. J. Clin. Microbiol. 43, 1133–1137. https://doi.org/10.1128/JCM.43.3.1133-1137.2005.
Muñoz, P., Bouza, E., Cuenca-Estrella, M., Marı, J., Jesu, M., Sa, M., Rinco, C., Hortal, J., Pela, T., Maran, G., 2005. *Saccharomyces cerevisiae* fungemia: an emerging infectious disease. Clin. Infect. Dis. 40, 1625–1634. https://doi.org/10.1086/429916.
Pérez-Torrado, R., Querol, A., 2015. Opportunistic strains of *Saccharomyces cerevisiae*: a potential risk sold in food products. Front. Microbiol. 6, 1522. https://doi.org/10.3389/fmicb.2015.01522.
Peter, J., De Chiara, M., Friedrich, A., Yue, J.-X., Pflieger, D., Bergstrom, A., Sigwalt, A., Barré, B., Freel, K., Llored, A., Cruaud, C., Labadie, K., Aury, J.M., Istace, B., Lebrigand, K., Barbry, P., Engelen, S., Lemainque, A., Liti, G., Schacherer, J., 2018. Genome evolution across 1,011 *Saccharomyces cerevisiae* isolates. Nature 556, 339–344. https://doi.org/10.1038/s41586-018-0030-5.
Pfliegler, W.P., Boros, E., Pázmándi, K., Jakab, Á., Zsuga, I., Kovács, R., Urbán, E., Antunovics, Z., Bácsi, A., Sipiczki, M., Majoros, L., Pócsi, I., 2017. Commercial strain-derived clinical *Saccharomyces cerevisiae* can evolve new phenotypes without higher pathogenicity. Mol. Nutr. Food Res. 61. https://doi.org/10.1002/mnfr.201601099.
Pfliegler, W.P., Horváth, E., Kállai, Z., Sipiczki, M., 2014. Diversity of *Candida zemplinina* isolates inferred from RAPD, micro/minisatellite and physiological analysis. Microbiol. Res. 169, 402–410. https://doi.org/10.1016/j.micres.2013.09.006.
Pfliegler, W.P., Sipiczki, M., 2016. Does fingerprinting truly represent the diversity of wine yeasts? A case study with interdelta genotyping of *Saccharomyces cerevisiae* strains. Lett. Appl. Microbiol. 63, 406–411. https://doi.org/10.1111/lam.12679.
Posteraro, B., Sanguinetti, M., Romano, L., Torelli, R., Novarese, L., Fadda, G., 2005. Molecular tools for differentiating probiotic and clinical strains of *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 103, 295–304. https://doi.org/10.1016/j.ijfoodmicro.2004.12.031.
Suez, J., Zmora, N., Segal, E., Elinav, E., 2019. The pros, cons, and many unknowns of probiotics. Nat. Med. 25 (5), 716–729. https://doi.org/10.1038/s41591-019-0439-x.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols. Academic Press, New York, pp. 315–322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1.