Article

Not peer-reviewed version

High-Resolution Melting Analysis Potential for *Saccharomyces cerevisiae* var. *boulardii* Authentication in Probiotic-Enriched Food Matrices

Monika Borkowska * , Michał Kułakowski , Kamila Myszka

Posted Date: 6 September 2024

doi: 10.20944/preprints202409.0492.v1

Keywords: *Saccharomyces cerevisiae* var. *boulardii*; identification; probiotic; dietary supplement; probiotic-enriched food; qPCR-HRM

 doi:10.20944/preprints202409.0492.v1

Article

High-Resolution Melting Analysis Potential for *Saccharomyces cerevisiae* var. *boulardii* Authentication in Probiotic-Enriched Food Matrices

Monika Borkowska *, Michał Kułakowski and Kamila Myszka

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-637 Poznań, Poland

* Correspondence: monika.borkowska@up.poznan.pl

Abstract: Since the mid-1990s probiotics have been proven to support the treatment of several gastrointestinal diseases. Additionally, probiotics have been taken as a common panacea for civilization diseases. To date, the only probiotic yeasts with evidence of health-promoting effects are *Saccharomyces cerevisiae* var. *boulardii*. The probiotic properties are strain-dependent, whereby it is crucial to develop methods to verify the probiotic-enriched food authentication including the growing number of dietary supplements with simplified commercialisation procedures. Due to the significant genetic similarity of *S. cerevisiae* var. *boulardii* to *S. cerevisiae*, qPCR-HRM analysis was tested, which has a very high sensitivity for polymorphism detection and enables simultaneous identification of the microorganism in the presence of the reference sample. The effectiveness of interspecies primer pairs and intragenus primer pairs designed to amplify heterogeneous regions was examined. HRM analysis using primers for *18SrRNA* and ITS allowed for identification of *S. cerevisiae* var. *boulardii* at the species level. *S. cerevisiae* var. *boulardii* was efficiently identified at the variety level with amplification of *HO* and *RPB2* in the single- and multi-component DNA extracts. The *RPB2* amplicon showed the highest intraspecies differentiation power. The predominance of *S. cerevisiae* var. *boulardii* in the mixtures tested was crucial for identification using qPCR-HRM analysis and quantitatively corresponded to the strain content in the probiotic-enriched food matrices.

Keywords: *Saccharomyces cerevisiae* var. *boulardii*; identification; probiotic; dietary supplement; probiotic-enriched food; qPCR-HRM

1. Introduction

As was established by the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) probiotics are live microorganisms that, when administered in adequate amounts, provide health benefits to the host (Hill et al. 2014). Health-promoting effects were predominantly demonstrated for specific probiotic strains of the bacteria genera *Lactobacillus* and *Bifidobacterium* (Fijan 2014), while the only yeast with a probiotic status evidenced clinically is *Saccharomyces cerevisiae* var. *boulardii* (*Sb*) (Łukaszewicz 2012). The probiotic yeast has unique physiological properties resulting in increased tolerance to stress conditions in the GI tract (Fietto et al. 2004). The mode of probiotic yeast action is not completely understood, whereby the beneficial effects have been confirmed in clinical studies (Czerucka et al. 2007). The clinical studies did not show any major side effects related to *Sb*. However, some cases of *Sb* fungemia are documented (Cesaro et al. 2000; Ellouze et al. 2016; Kara 2018; Santino et al. 2014; Thygesen et al. 2012). The most quoted cases concerned severely ill or immunocompromised patients.

Initially, *Sb* was categorized as a novel species of the genus *Saccharomyces*. The taxonomic position of *Sb* has been controversial over the years (Van Der Aa Kühle and Jespersen 2003; Cardinali and Martini 1994; Fietto et al. 2004; Kurtzman and Robnett 1998; McCullough et al. 1998; Molnar et al. 1995). McFarland indicated some crucial divergences at the physiological (i.e., lack of ability to use galactose as carbon source and lack of ability to produce ascospores) and molecular levels (i.e.,

 doi:10.20944/preprints202409.0492.v1

individual chromosome and gene copy numbers) between *Sb* and *Saccharomyces cerevisiae* (*Sc*) (McFarland 1996). Using comparative genomic hybridization for whole-genome analysis, *Sb*'s physiological and genotypic distinctive features were confirmed (Edwards-Ingram et al. 2007). The observations concerned the specific properties of Ty elements (yeast retrotransposons) and the copy number of genes in its subtelomeric regions. These were confirmed in the subsequent genomic comparative study, which additionally showed that *Sb* strains are closely related to *Sc* wine strains (Khatri et al. 2017). Pais and colleagues hypothesized that different phenotypes exhibited by *Sb* and *Sc* might result from variations in gene expression control. They demonstrated that *Sb* did not share conserved promoter regions and transcription factor binding sites with *Sc* (Pais et al. 2021).

So far, the search for methods of intraspecies differentiation of *Saccharomyces cerevisiae* has mainly involved clinical studies. By combining randomly amplified polymorphic DNA-PCR, restriction fragment length polymorphism analysis of rDNA spacer regions and pulsed-field gel electrophoresis, all examined *Sc* isolates were discriminated in clinical research. Moreover, probiotic Sb strains were demonstrated to form a separate cluster within the species (Mitterdorfer et al. 2002). Additionally, a powerful microsatellite-based technique was developed for intraspecies differentiation of probiotic and clinical *Sc* strains (Hennequin et al. 2001). Furthermore, a high level of discrimination was achieved by hybridization with retrotransposon Ty917 (Posteraro et al. 2005). Finally, a rapid multiplex PCR method unequivocally identified probiotic-derived *Sc* isolates (Imre et al. 2019).

A modern technique that allows differentiation of the amplicons at the single base resolution is the High-Resolution Melting (HRM) analysis of qPCR amplified DNA fragments. It is an alternative single-tube approach with no time-consuming post-PCR processing and the need for sequencing to detect DNA polymorphisms. HRM analysis was primarily used in clinical research and diagnostics, but its robust potential and technological progress allow it to enter other areas of life sciences. In food sciences, HRM analysis has already been used for the differentiation of food-derived yeast species including *Saccharomyces* spp (Nadai et al. 2018), sourdough yeast (Bazalová et al. 2022; Ripari et al. 2016) and spoilage yeast (Erdem et al. 2016; Kesmen et al. 2018a, b). All procedures presented a high potential for HRM analysis in yeast species differentiation based on the amplification of regions within rRNA genes or ITS non-coding sequences characterized by low intraspecific variability and high interspecific polymorphism (Baleiras-Couto et al. 1996; Valente et al. 1996). Most of the studies cited dealt with culture-dependent identification. Culture-independent identification of sourdough yeast by HRM analysis did not always give conclusive results (Bazalová et al. 2022; Ripari et al. 2016).

Probiotics have become increasingly popular over the past two decades as a result of proven health-promoting properties. Specialists have recommended probiotic supplements for disorders that frustrate conventional medicine, such as irritable bowel syndrome, or to delay the development of allergies in children. As a result, a wide range of probiotic-fortified dietary supplements have been available in European markets. According to EFSA food supplements are concentrated sources of nutrients or other substances with a nutritional or physiological effect that are marketed in " dose " form. Combining typical medicine dosage forms with misleading advertising can create a false impression of a supplement's therapeutic properties. Dietary supplements are currently regulated as foods and their legalization is facilitated by the law. The current state of the regulations and the expanded supplement market create an environment conducive to food adulterations, necessitating rapid testing to verify product status. The problem may soon affect a much wider range of foods. Concerning probiotic yeast, intense research activity arose in developing functional foods supplemented with *Sb* not only as a probiotic agent, but also as a key element for the generation of bioactives increasing the antioxidant capacity (Değirmencioğlu et al. 2016; Rekha and Vijayalakshmi 2010), improving nutritional value (Zamora-Vega et al. 2012), or stabilization of LAB strains throughout fermentation and storage (Chan et al. 2023; Karaolis et al. 2013).

It is crucial to verify the strain identity of *Sb* yeasts since their beneficial properties are considered to be strain-specific. Therefore, this study aimed to create a rapid genetic test to authenticate probiotic yeast strains. The *Sb* identification efficiency of interspecies and intragenus primer pairs was verified using qPCR-HRM analysis, conducted with reference *Sc* and *Sb* strains,

 doi:10.20944/preprints202409.0492.v1

along with *Kluyveromyces marxianus* and *Pichia fermentans* as negative controls. The utility of selected primer pairs previously optimised in culture-dependent studies was verified in dietary supplements and yeast cell mixtures corresponding in composition to kefir, which is a natural source of probiotic yeast origin (Goktas et al. 2021; Gut et al. 2019).

2. Materials and Methods

2.1. Biological Material

2.1.1. Strains

The following reference strains were used in the optimisation of qPCR-HRM analysis focused on identifying probiotic strains of *Saccharomyces cerevisiae*: (i) *Saccharomyces boulardii* CNCM I-745 (Enterol, Biocodex, Gentilly, France) (***Sb***$_{745}$) and *Saccharomyces boulardii* CNCM-I-3799 (Oslonik max extra, TZF Polfa, Warsaw, Poland) (***Sb***$_{3799}$) isolated from probiotic preparations under this project; (ii) and collection strains *Saccharomyces cerevisiae* ATCC 9763 (***Sc***$_{ATCC9763}$) and *Saccharomyces cerevisiae* Ethanol Red (Lesaffre; France) (***Sc***$_{EtRed}$). Additionally, (i) *Kluyveromyces marxianus* DSM 5422 (German Collection of Microorganisms and Cell Cultures GmbH; Germany) (***Km***), (ii) food-derived isolates *Saccharomyces cerevisiae* (***Sc***$_{D}$) and *Pichia fermentans* (***Pf***$_{D}$) deposited in the microbial collection of Department Biotechnology and Food Microbiology (DBFM), Poznan University of Life Sciences (PULS) (iii) *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072 (German Collection of Microorganisms and Cell Cultures GmbH; Germany) (***Ld*subl**), were used in the subsequent stages of testing the qPCR-HRM. All strains were maintained as glycerol stocks at -80°C until used. Yeast strains were recovered on YPD agar plates [(g L^{-1}): yeast extract, 10 (Biomaxima, Lublin, Poland), bactopeptone, 20 (BTL, Łódź, Poland), glucose, 20 (POCh, Gliwice, Poland) and agar, 15 (BTL)] and lactic acid bacteria (LAB) were retrieved with de man, Rogosa and Sharpe (M.R.S) agar (BTL).

2.1.2. Probiotic Supplements

Four probiotic preparations classified as dietary supplements and available in local pharmacies were analysed with the optimised qPCR-HRM method. The composition of chosen products is shown in Table 1.

Table 1 Composition of tested dietary supplements

LABEL	COMPOSITION
PS1	*Saccharomyces cerevisiae*
PS2	*Lactiplantibacillus plantarum*, *Bifidobacterium breve*, *Saccharomyces boulardii*
PS3	*Saccharomyces boulardii* and fructooligosaccharides
PS4	*Saccharomyces boulardii*, *L. rhamnosus* GG, fructooligosaccharides

2.2. Microbiological Methods

2.2.1. *S. boulardii* Reference Strains Isolation

The reference strains of *Sb* used in this study were isolated from two different commercial products, one with proven therapeutic effects and the other classified as a dietary supplement. To revive the yeast, the contents of each capsule (250 mg) were suspended in 20 mL of YPD in a sterile 50 mL Falcon tube. The mixture was then shaken at 150 rpm at 30°C overnight. Afterwards, the log

 doi:10.20944/preprints202409.0492.v1

serial dilutions were made from the overnight culture. 100 µL of each dilution was transferred in duplicate onto **Y**east extract **G**lucose **C**hloramphenicol agar (YGC) (BTL, Łódź, Poland). Incubation was carried out overnight at 30°C. Single colonies were then reinoculated onto fresh YGC agar. The yeast isolates were first verified based on morphological characteristics with microscopy and then subjected to MALDI TOF mass spectrometry identification.

2.2.2. Sporulation Test

2.2.2.1. Procedure of Sporulation Induction

5 mL of YPD medium in a 50 mL Falcon tube was inoculated with a fresh yeast colony for determination of the ascospore formation. Each culture was incubated at 30°C in a shaking incubator at 150 rpm for 18-20 hours. 200 µL of the overnight culture were transferred into 5mL of liquid YPD and incubated at 30°C in a shaking incubator until the cell suspension reached OD equal to 1. Then the culture was centrifuged at 1811 x g for 5 min and the supernatant was discarded. The pellet was resuspended in 5mL of pre-sporulation medium (g L^{-1}: yeast extract, 10; pepton, 20; potassium acetate, 10 (POCh, Gliwice, Poland)) and grew for 18-24 hours at 30°C in shacking incubator. The culture was centrifuged at 1811 x g for 5 min and the supernatant was discarded. Finally, the pellet was resuspended in 5mL of sporulation medium (g L^{-1}: potassium acetate, 10) and allowed to sporulate for 48 hours at 30°C in a shaking incubator. The tested strains were subjected to the sporulation procedure in two independent replicates.

2.2.2.2. Cells' Ziehl-Neelsen Staining

The basic dye, concentrated carbol fuchsin (g L^{-1}: fuchsin, 33,3 (Chempur, Piekary Śląskie, Poland), phenol, 66,7 (Chempur) and 167 mL of ethanol (POCh, Gliwice, Poland)) was applied to the fixed smear of yeast on a degreased basic slide for 15 min. During staining the slide was heated with a burner (to the so-called "three pairs"). Then the preparation was discoloured in a 3% solution of hydrochloric acid (Honeywell, Charlotte, USA) in ethanol (POCh, Gliwice, Poland) (acid alcohol). Afterwards, the contrast dye 0.1% (w/v) methylene blue (Chempur) was applied for 10 min. Finally, the preparation was observed in the light microscope Primo star (Zeiss, Oberkochen, Germany) under 1000x magnification using immersion.

2.2.3. The Mixtures of Lactic Acid Bacteria and Yeast Cells

5 mL of fresh M.R.S Broth or liquid YPD in a 50 mL Falcon tube was inoculated with a bacterial (*Ld*subl) or yeast colony (Sc_D, Sb_{745}, *Km*, Pf_D), respectively. The cultures were incubated at 30°C with 250 rpm shaking for 20 h. The average cells' concentration of each overnight culture was determined using CellDrop FL (DeNovix INC, Wilmington, USA). Subsequently, mixtures of microorganisms were prepared in saline water (0.85% w/v NaCl). Suspensions included Sc_D and Sb_{745} cells, where *Sb* accounted for 10% (Mx_0.1), 50% (Mx_0.5) and 90% (Mx_0.9) of the total amount of yeast cells. Another group of suspensions consisted of those that contained a fixed number of *Ld*subl cells and yeast cells with a 60% share of Sc_D (Mx_*Sc*) or *Sb* (Mx_*Sb*), 38% of *Km* and 2% of Pf_D. In this series of mixtures, the ratio of *Sc* cells to *Sb* cells was the same as in suspensions composed only of *Sc* cells (Figure S1).

2.3. MALDI-TOF Mass Spectrometry

Mass spectrometry analysis was conducted in the Microbiological Laboratory of the Jagiellonian Center of Innovation (Cracow, Poland). MALDI-TOF mass spectrometer Microflex LT (Bruker Daltonics, Bremen, Germany) was used for matrix-assisted laser desorption/ionization with time-of-flight analysis. Identification of the microorganisms was based on the unique ribosomal proteins' profile compared to a representative database of bacteria, yeast-like fungi, filamentous fungi and dermatophytes, using the MALDI Biotyper system (Bruker Daltonics). Identification confidence was expressed by identification indicator (*Ii*). An identification indicator higher or equal to 2,00 means

 doi:10.20944/preprints202409.0492.v1

high-confidence identification. *Ii* within the range 1,70 - 1,99 means low-confidence identification, and below 1,69, no species identification is possible.

2.4. Total Genomic DNA Extraction Procedures

2.4.1. Total Genomic DNA Isolation from Yeast Culture

Prior to genomic DNA extraction, all the yeast strains were freshly cultured on YPD agar at 30°C for 20 h. The biomass was collected with a 10 µl inoculation loop and resuspended in 0,5 ml saline solution, and the cells were pelleted by centrifugation. DNA extraction from cells' pellets was performed using a Genomic mini AX yeast spin kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol, including an optional step of yeast lysis with lyticase at 30°C, followed by vigorous vortexing. The concentration and purity of the isolated DNA were determined using a UV spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). The OD_{260}/OD_{280} ratio of the DNA samples, reflecting their average purity, ranged between 1,8 and 2,0. The quality and integrity of the DNA samples were verified through agarose gel electrophoresis according to a standard method (Sambrook and Russell, 2001). Only DNA samples exhibiting sharp and intensive bands were forwarded to qPCR. The extracted DNA was stored at –20°C.

2.4.2. Total Genomic DNA Isolation from Dietary Supplements and Microbial Mixtures

The capsule contents of each dietary supplement were crushed in liquid nitrogen in a mortar. The fresh yeast and bacterial cultures were used for preparing mixtures (**Section 2.2.3**). 2 mL of each mixture was pelleted by centrifugation. Total genomic DNA was extracted from 100 mg of each food supplement and each mixture pellet using Genomic mini AX Food kit (A&A Biotechnology, Gdynia, Poland). The procedure was conducted according to the manufacturer's protocol. The first step was lysis with proteinase K at 30°C, followed by vigorous vortexing. DNA was isolated and purified from the lysate with column work through gravity and then eluted, precipitated and dissolved in sterile water. The concentration and purity of the isolated DNA were determined using a UV spectrophotometer NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). The quality and integrity of the DNA samples were verified through agarose gel electrophoresis according to a standard method (Sambrook and Russell, 2001). The extracted DNA was stored at –20°C.

2.5. Quantitative Real-Time PCR – High-Resolution Melting Analysis (qPCR-HRM)

2.5.1. Primer Pairs

rDNA cluster sequences (*18S rRNA*, *26S rRNA* and ITS region) of the yeast strains under study were amplified with primer pairs designed previously (Borkowska and Celińska 2023). As part of this work, additional primer pairs were designed. *S. cerevisiae* S288C genes' sequences were retrieved from the NCBI database. Their accession numbers are listed in Table 2. The targeted sequences were aligned and compared to *Saccharomyces* spp using multiple sequence alignment tool from NCBI. The multiple sequence alignment was searched for conservative sequence segments allowing the attachment of intragroup primers, flanking DNA regions showing intragroup heterogeneity. Amplicon length was kept within the range of 100 to 250 bp. The primers were designed with the Primer3-BLAST tool, and synthesized by Merck KGaA (Darmstadt, Germany). Initially, annealing temperature optimisation of all primer pairs was performed using Perpetual OptiTaq PCR MasterMix according to the manufacturer's instructions (EURx, Gdansk, Poland) in a temperature gradient of 58 - 63°C. The amplification efficiency was verified through agarose gel electrophoresis according to a standard method (Sambrook and Russell 2001). *TDA8* primer pair was eliminated from the further study due to a lack of amplification on *Sc* samples in the optimisation step. Furthermore, amplification products of all studied primer pairs were positively verified for expected lengths (Figure S2).

 doi:10.20944/preprints202409.0492.v1

Table 2 Description of the primer pairs designed to amplify up to 250 bp regions within seven genes. Accession numbers correspond to the sequences of *Saccharomyces cerevisiae* S288C

GENE			PRIMERS				
NAME (SYMBOL)	ACCESSION NUMBER	RANGE [bp]	LABEL	START	STOP	SEQUENCE (5'->3')	PRODUCT LENGTH [bp]
Homothallic switching endonuclease (*HO*)	NC_001136.1	46810 - 47130	HO_Fw	46817	46836	TGAAGTTGTTCCCCCAGCAA	198
			HO_Rv	47014	46995	GGCGAAGGCCCTGAATCTTA	
DNA-directed RNA polymerase II core subunit (*RPB2*)	NC_001147.6	615169 - 615510	RPB2_Fw	615217	615239	ACGGTTCAAAACCTGAGAAACAC	229
			RPB2_Rv	615445	615424	AGGTCCATTATTGGCCCAACTT	
tRNA adenylyl transferase (*CCA1*)	NC_001137.3	522230 - 522503	CCA1_Fw	522282	522302	CCAGATGCTTGGATTTCTCGG	213
			CCA1_Rv	522494	522474	AGCCATTGACTCTTCGGATCA	
Hexose transporter (*HXT9*)	NC_001142.9	19800 - 20028	HXT9_Fw	19824	19846	AGAATGGGTTTGATCGTCTCAAT	197
			HXT9_Rv	20020	19996	AGGCCAGAAATAATTCTTCCAATGA	
Alpha-glucosidase permease (*MAL11*)	NC_001139.9	1074890 - 1075090	MAL11_Fw	1074891	1074910	TTTCTCACCAACCACCAGGG	196

2.5.2. qPCR Protocol

PCR mixtures contained 5 µL of commercial qPCR master mix with Eve Green dye (Bio-Rad Laboratories, Inc., CA, USA), 0,5 µL of 10 µM forward and reverse primer and 1- 4 µL of DNA sample (5 ng of single-yeast DNA or 20 ng of microbial-mix DNA per reaction) in a total volume of 10 µL. The amplification reactions were carried out in clear-walled PCR 96-well plates using the CFX96 cycler (Bio-Rad Laboratories, CA, USA). The cycling conditions were as follows: 95°C for 3 min was followed by 30 - 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C (primer pairs for sequence segment within *18SrRNA*, *26SrRNA*, ITS, *TEF1alpha*, *HO*, *RPB2*, *MAL11* and *HXT9*) or 58°C (primer pairs for *CCA1*) for 30 s and extension at 72°C for 30 s. Melting curves were obtained by cycling within a range of 65 to 95°C. Data acquisition was performed in 0,2°C increments, with a 10 s step. All DNA preparations were analysed in technical duplicates in at least two independent runs. To check the purity of reagents, No Template Control (NTC) for each primer pair was run in parallel.

2.5.3. HRM Analysis

The emerging qPCR amplicons were analyzed using Precision Melt Analysis Software (PMAS) (Bio-Rad Laboratories, Inc.). The pre-and post-melt regions, as well as the intersection point of the melt curves on temperature-shifted view, were carefully evaluated (and adjusted where necessary) for each amplicon. Clustering settings were adjusted to increase the sensitivity of clustering based on the shape of the melt curves. A T_m difference threshold of 0,2°C was set for all the melt curve analyses. Clustering confidence scores were assigned automatically by PMAS.

2.6. Statistical Analysis

The statistical significance of the observed differences based on clustering in HRM analysis of yeast strains for the four examined regions (*18SrRNA*, *26SrRNA*, ITS, *TEF1alpha*) was evaluated using the software DarWin version 6.0.21. Afterwards, the strains were grouped using the unweighted pair-group method with the arithmetic averages (UPGMA) clustering algorithm.

 doi:10.20944/preprints202409.0492.v1

3. Results

3.1. Isolation and Identification of Reference Strains Saccharomyces Cerevisiae var. boulardii

Two reference strains were identified with high confidence by MALDI-TOF mass spectrometry as *Saccharomyces cerevisiae. Ii* was 2,16 and 2,09 for *Sb*$_{745}$ and *Sb*$_{3799}$, respectively. The reference strains did not show the ability to sporulate (Figure 1a, b), as well as no amplification of regions *MAL11* and *HXT9* was detected for both (Figure 1e, f).

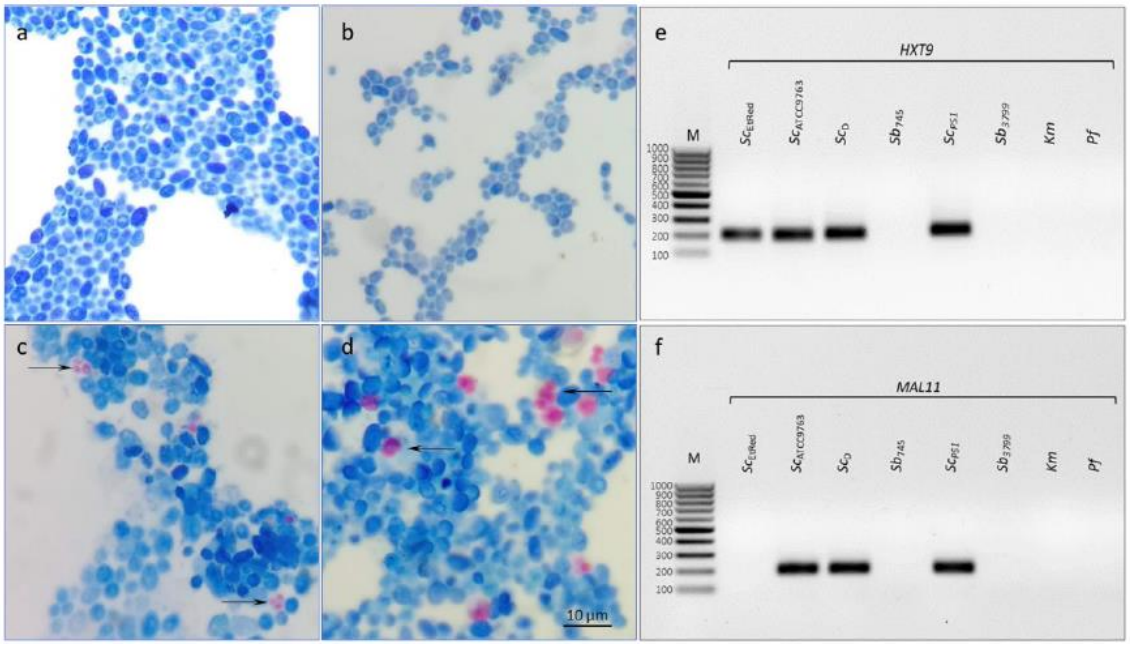

Figure 1. Verification of sporulation and *HXT9* or *MAL11* presence in *Sc* strains. Microscopic images of yeast cells of *Sb*$_{745}$ (**a**), *Sb*$_{3799}$ (**b**), *Sc*$_{PS1}$ (**c**), *Sc*$_{D}$ (**d**) strains. The cells' suspensions were induced to sporulate under starvation conditions and then stained with the Ziehl-Neelsen method. Electrophoretic separation of *HXT9* (**e**) and *MALL1* (**f**) amplicons obtained in PCR for *S. cerevisiae* var. *boulardii* reference strains (*Sb*$_{745}$, *Sb*$_{3799}$), *S. cerevisiae* strains (*Sc*$_{ATCC9763}$, *Sc*$_{EtRed}$, *Sc*$_{D}$ and *Sc*$_{PS1}$), *K. marxianus* (*Km*) and *P. fermentans* (*Pf*). M – DNA Marker 100bp LOAD (Syngen Biotech, Wroclaw, Poland).

3.2. Differentiation of Saccharomyces Cerevisiae Strains Using Interspecies Primer Pairs in qPCR-HRM Analysis

For all sequences tested, a homogeneous product was obtained for each sample as evidenced by single peaks (Figure 2a). HRM analysis of the *18SrRNA* region amplicon showed individual clustering of *Sc* and probiotic *Sb* strains at the differentiation thresholds used. The other species used in the study, the *Km* collection strain and the *Pf*$_{D}$ isolate were grouped separately (Figure 2b, ***18SrRNA***). The maximum RFU difference between the *Sb* and *Sc* clusters was 0,03, a slightly larger difference of 0,1 was between the *Sb* and *Sc* clusters combined and the *Pf*$_{D}$ cluster, and the largest difference of 0,3 was between the *Sb* and *Sc* clusters combined and the *Km* cluster. For the non-coding ITS sequence, all strains belonging to *Sc* species, including the probiotic ones, were in one cluster. *Km* and *Pf*$_{D}$ control strains formed separate clusters (Figure 2b, ITS). The maximum RFU difference was 0,6 between the *Sb*/*Sc* cluster and *Km*. Slightly less, 0,4, was shown by comparing the *Sb*/*Sc* and *Pf*$_{D}$ clusters. (Figure 2b, ITS). According to HRM analysis, the qPCR reaction with the primer pair for the *26SrRNA* region yielded a uniform product for all *Sc* strains as well as the *Km* strain. In this case, only *Pf*$_{D}$ was grouped separately (Figure 2b, *26SrRNA*). Amplification of a region selected within *TEF1alpha* resulted in a product whose denaturation profile was the same for all *Sc* and *Sb* tested except *Sc*$_{EtRed}$. The *TEF1alpha* region products obtained for the negative control strains were clustered individually (Figure 2b, *TEF1alpha*). The melting temperatures of the amplicons of each of the genomic DNA regions tested did not differ within the *Sc* species significantly (Table S1). HRM-based

 doi:10.20944/preprints202409.0492.v1

clustering of the selected sequences analyzed with DarWin software showed that the reference probiotic strains tested are genetically very similar and phylogenetically a variant of the species *Sc* (Figure S3).

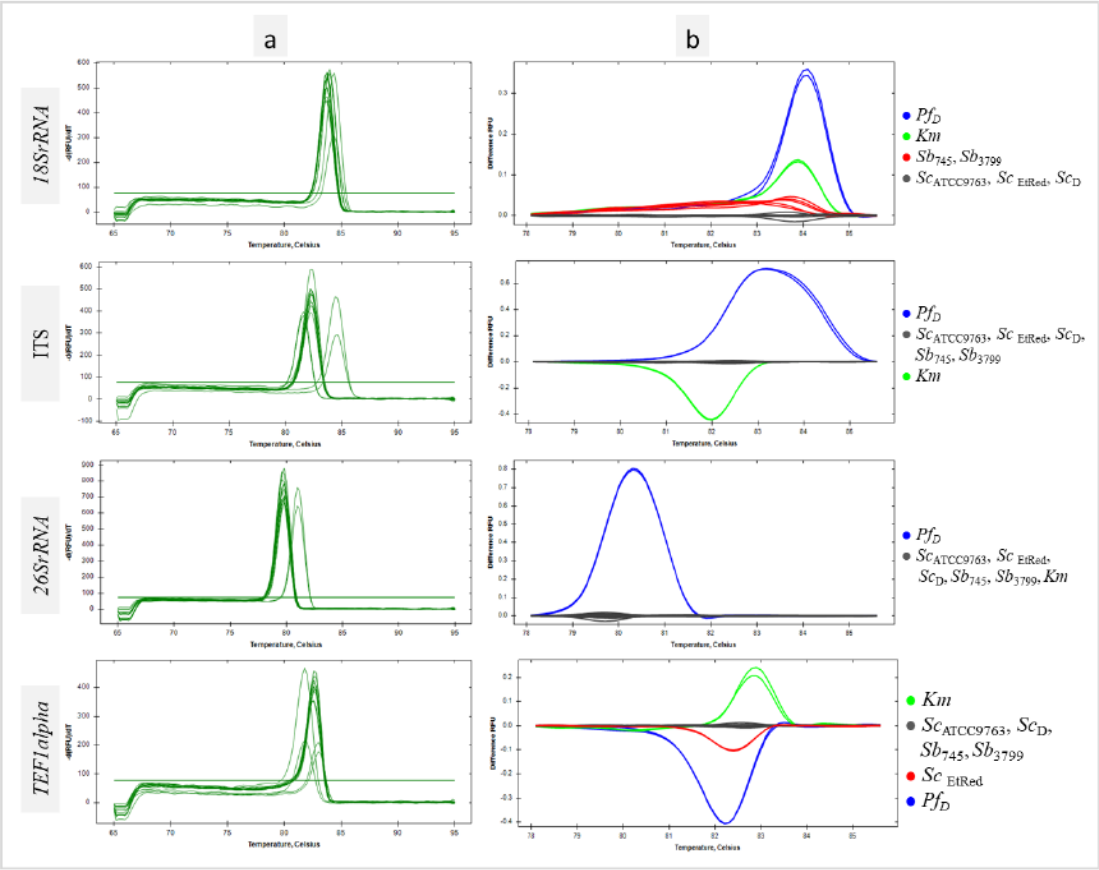

Figure 2. Differentiation of yeast with interspecies primer pairs in qPCR-HRM analysis. Melt peaks (**a**) and difference curves grouped as colour-marked clusters (**b**) were obtained by qPCR-HRM analysis of *18SrRNA*, ITS, *26SrRNA*, and *TEF1alpha* regions. DNA templates of *S. cerevisiae* var. *boulardii* reference strains (*Sb*$_{745}$, *Sb*$_{3799}$), *S. cerevisiae* strains (*Sc*$_{ATCC9763}$, *Sc*$_{EtRed}$, *Sc*$_{D}$), *K. marxianus* (*Km*) and *P. fermentans* (*Pf*$_{D}$) strains were amplified in technical duplicate.

3.3. Differentiation of Saccharomyces Cerevisiae Strains Using Intragenus Primer Pairs in qPCR-HRM Analysis

No products were demonstrated for *Km* and *Pf*$_{D}$ control templates using primer pairs designed for the genus *Saccharomyces* (Figures 3 and S2). Furthermore, no amplification of the *MAL11* and *HXT9* regions was observed for the reference strains *Sb* (Figure 3, *MAL11*, *HXT9*, Figure 1e, f). In addition, no sequence amplification in the *MAL11* region was detected for the *Sc*$_{EtRed}$ template (Figure 3 *MAL11*, Figure S2). For all sequences tested, a homogeneous product was obtained for the remaining samples as evidenced by single peaks (Figure 3a). The peak melting curves of *RPB2* amplicons for *Sc*$_{D}$ had shoulders, indicative of polymorphisms in amplified sequence. HRM software grouped *Sb* probiotic strains and *Sc*$_{D}$ isolate in one cluster at *CCA1* region analysis (Figure 3 *CCA1*). A similar result was the joint clustering of probiotic strains and *Sc*$_{EtRed}$ regarding a melting temperature and profile of *HO* sequence (Figure 3 *HO*). The maximum RFU difference in the *HO* melting profile was 0,1 between the *Sb*$_{745}$ cluster and the *Sc*$_{ATCC9763}$ cluster (Figure 3b *HO*). The amplicon obtained with the primer pair designed for *RPB2* was essential in the differentiation of the probiotic strains against the other *Sc* tested in this study. The RFU difference between the clusters was 0,2 (Figure 3b *RPB2*). Significant differences were found in the melting temperatures of *CCA1* PCR products for all *Sc* strains tested, including the two probiotic strains. Significantly the melting

 doi:10.20944/preprints202409.0492.v1

temperature of *RPB2* amplicon was the same for both *Sb* strains and differed from the others by 0,2°C (Table S1).

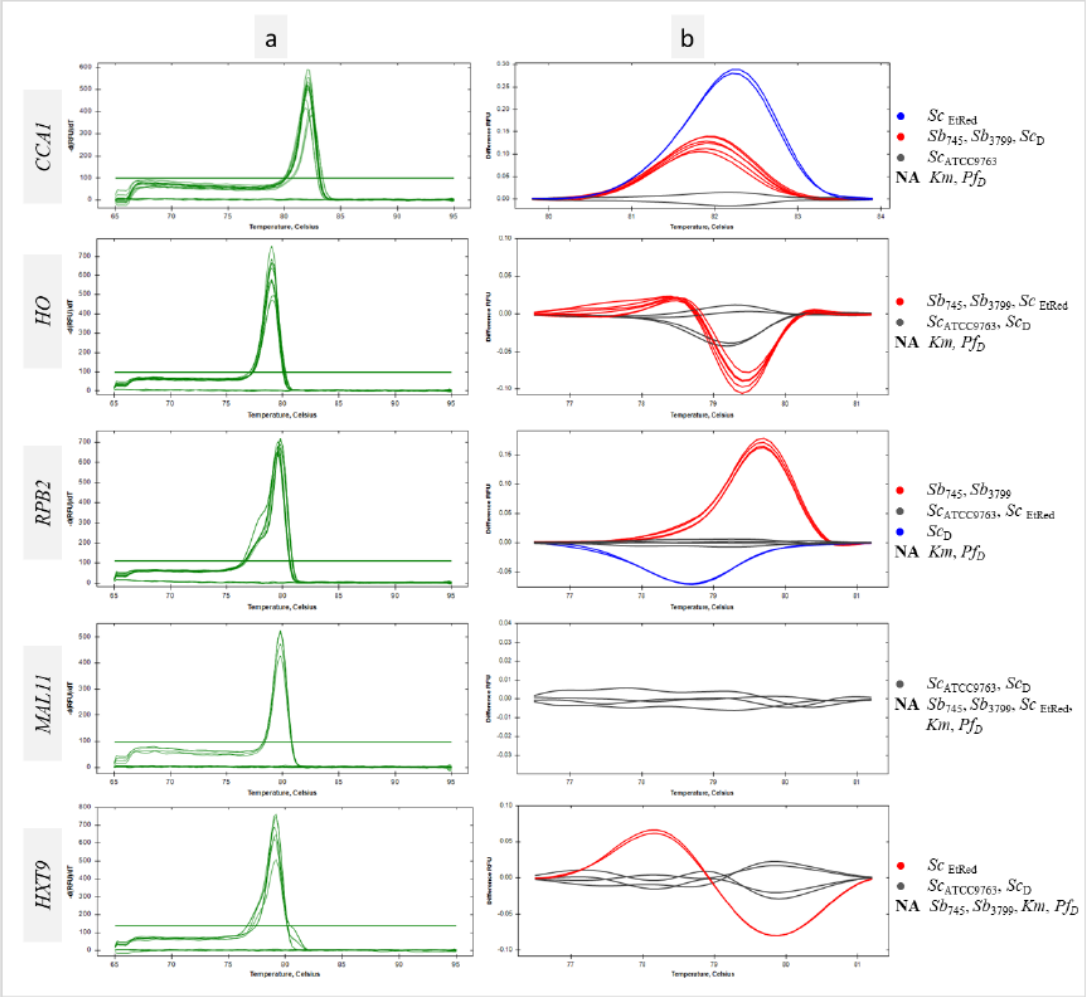

Figure 3. Differentiation of *Sc* strains using intragenus primer pairs in qPCR-HRM analysis. Melt peaks (**a**) and difference curves grouped as colour-marked clusters (**b**) were obtained by qPCR-HRM analysis of *CCA1*, *HO*, *RPB2*, *MAL11* and *HXT9* regions. DNA templates of *S. cerevisiae* var. *boulardii* reference strains (*Sb745*, *Sb3799*), *S. cerevisiae* strains (*ScATCC9763*, *ScEtRed*, *ScD*), *K. marxianus* (*Km*) and *P. fermentans* (*PfD*) strains were amplified in technical duplicate. **NA** – not amplified.

3.4. Identification of Probiotic Yeast in Dietary Supplements with qPCR-HRM Based on a Verified Set of Primer Pairs

In the present study, a genetic analysis of four probiotic supplements available in pharmacies was performed. Their composition is presented in Table 1. DNA extracts obtained from the preparations along with reference templates of *Sb745* and *ScATCC9763* were subjected to the qPCR reactions with the selected primer pairs. According to HRM analysis, the *18SrRNA* sequences for each of the test samples were grouped with the references. The same effect was observed for the amplicons of the ITS region (Figure 4.1a, b). If *Saccharomyces*-specific primer pairs were used, only PR2, PR3 and PR4 samples were found to cluster with the *Sb745* reference for *HO* sequences, as well as *RPB2*. The PR1 sample remained in separate clusters for both studied sequences with *ScATCC9763* amplicons (Figure 4.2a, b). PS1 strain was identified by MALDI-TOF mass spectrometry as *Saccharomyces cerevisiae* with high confidence (*Ii* was 2,15). The amplification of *MAL11* and *HXT9* regions on the DNA template of the PR1 supplement was found (Figure 1e, f). In addition, the isolated strain of the PR1 supplement was able to sporulate (Figure 1c).

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 September 2024 doi:10.20944/preprints202409.0492.v1

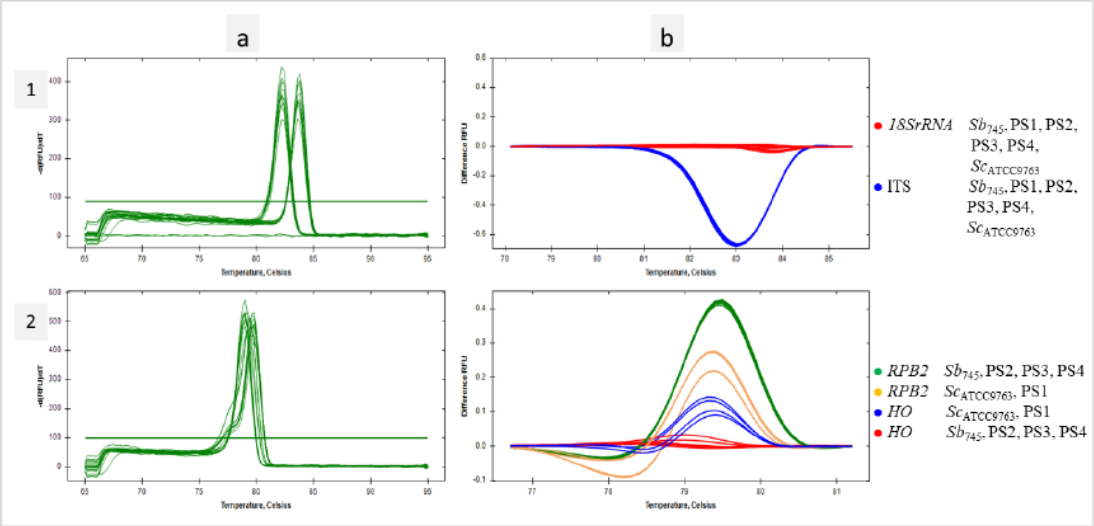

Figure 4. Identification of *Sb* in dietary supplements with qPCR-HRM. Melt peaks (**a**) and difference curves grouped as colour-marked clusters (**b**) were obtained by qPCR-HRM analysis of *18SrRNA* and ITS regions (**1**), *HO* and *RPB2* regions (**2**). DNA template of *S. cerevisiae* var. *boulardii* reference strain (Sb_{745}) as positive control and the dietary supplements' DNA templates (PS1, PS2, PS3, PS4) were amplified in technical duplicate.

3.5. Identification of Probiotic Yeasts in Microbial Mixtures with qPCR-Based HRM Analysis

The mixed suspensions (Mx) for DNA isolation containing a fixed amount of *Ld*subl (log 8 CFU mL^{-1}), *Km* (3,8 log 6 CFU mL^{-1}) and Pf_D (2 log 5 CFU mL^{-1}) cells were prepared. Successively, the suspensions Mx_Sc, Mx_0.1, Mx_0.5, Mx_0.9 and Mx_Sb contained increasing numbers of Sb_{745} cells. In addition, suspensions S_0.1, S_0.5, and S_0.9 were prepared, containing only Sc_D and Sb_{745} cells, which were combined in analogous ratios to suspensions Mx (Figure S1). DNA was extracted from the obtained cells' pellets with a kit designed for foods. The resulting templates and Sb_{745} positive control DNA were amplified using the primer pairs for *18SrRNA*, ITS, *HO* and *RPB2* regions. Considering the *18SrRNA* sequence, the melting temperatures (T_m) of the amplicons obtained for the following samples were in the range of 83,80 – 84,0°C (Figure 5a). In the same range remained the T_m of the *18SrRNA* amplification products of the yeast species studied, namely Sc_D, *Km* and Pf_D (Table S1). The software grouped all tested samples into one cluster (Figure 5b). In HRM analysis of ITS sequences, more clusters were obtained (Figure 5d). The first grouped the reference strain Sb_{745} and mixtures with Sb_{745} and Sc_D (S_0.1, S_0.5 i S_0.9). In this cluster were amplicons with a T_m of 82,4°C (Figure 5c), which corresponded to the melting point of the ITS product for Sc_D (Table S1). The remaining clusters included samples containing *Km*, Pf_D, Sc_D and Sb_{745} in different proportions (Figure 5d). These amplification products showed double peaks with T_m of 81,6°C and 84,2°C. (Figure 5c). The T_m indicated in the figure corresponded to the melting temperatures of ITS sequences of *Km* and Pf_D species, respectively (Table S1). In each case, the product with a melting point corresponding to Pf_D was quantitatively predominant. Exploring the *HO* sequence, the Mx_Sb, Mx_0.9 and S_0.9 samples were clustered with the positive control in the HRM analysis. The remaining Mx_Sc, Mx_0.1, Mx_0.5, S_0.1, and S_0.5 were grouped in a separate cluster (Figure 5e). The same result was obtained for HRM analysis of *RPB2* amplicons (Figure 5f). For both marker sequences, a gradational distribution of differential melting curves was observed depending on the initial Sb_{745} cell content of the sample. The smaller the proportion of Sb_{745} cells in the suspension mix, the further the differential melting curve was from the reference curve. Regarding the adopted clustering parameters, the denaturation curve profiles of Mx_0.5 and S_0.5 products were significantly different from the Sb_{745} reference, as shown in the detailed graphs (Figure 5g, h). The melting point of the *HO* product of all the mixtures tested was 79,0°C, which is consistent with Table S1 data on *HO* T_m for Sc_D and Sb_{745} samples. T_m of *RBP2* products amplified with Sb_{745} and Mx_Sb DNA templates was 79,8°C and

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 September 2024 doi:10.20944/preprints202409.0492.v1

corresponded to *Sb*$_{745}$ amplicons. *RPB2* amplification products of the other mixed samples achieved T_m of 79,4 – 79,6°C, in which range the melting point of the *Sc*$_D$ sequence falls (Table S1).

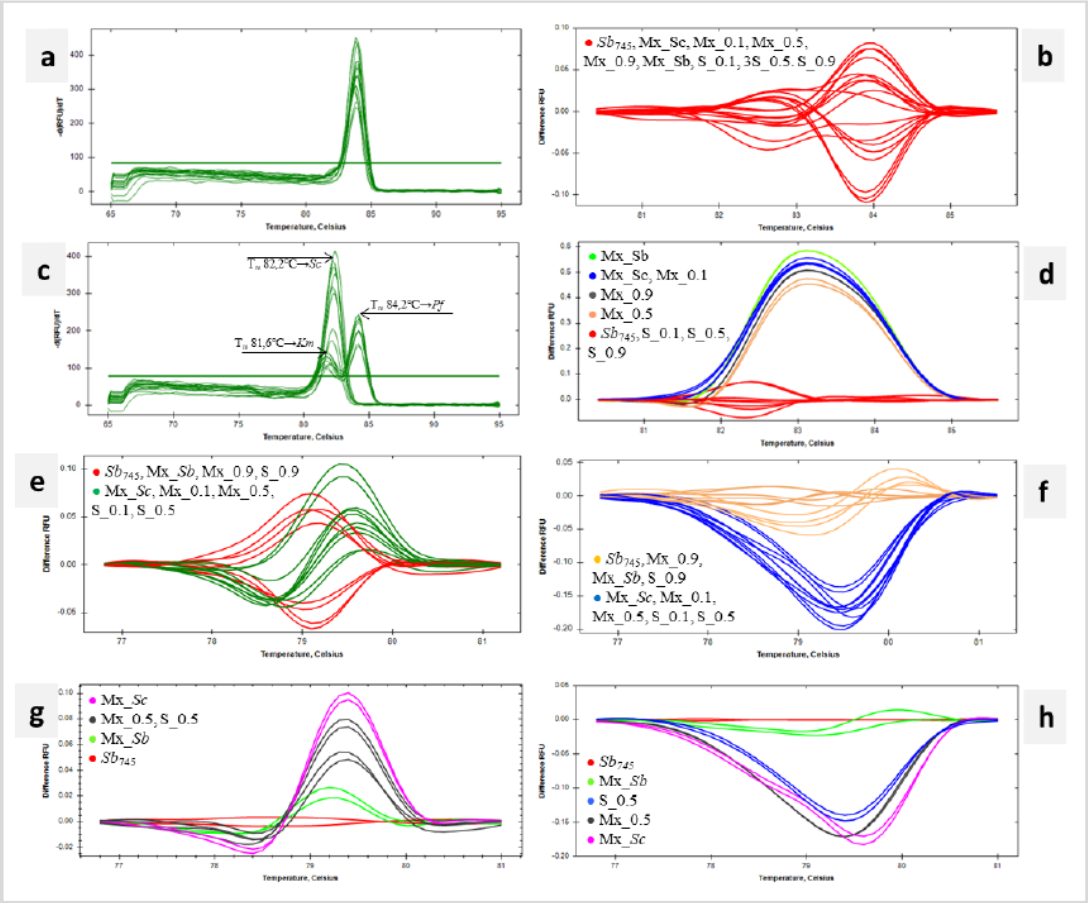

Figure 5. Identification of *Sb*$_{745}$ in microbial mixtures with qPCR-based HRM analysis. Melt peaks (**a**) and difference curves grouped as colour-marked clusters (**b**) were obtained by qPCR-HRM analysis of *18SrRNA*. Melt peaks (**c**) – the arrows indicate species-specific peaks at T_m 81,6°C, 82,2°C and 84,2°C for *Km*, *Sc*$_D$ and *Pf*$_D$ respectively and difference curves (**d**) grouped as colour-marked clusters detected for ITS amplicon. Difference curves grouped as colour-marked clusters obtained by qPCR-HRM analysis of *HO* sequence presented for all samples (**e**) and selected samples (**f**), and *RPB2* sequence presented for all samples (**g**) and selected samples (**h**). DNA templates of *S. cerevisiae* var. *boulardii* reference strain (*Sb*$_{745}$) as positive control and the microbial mixtures' DNA extracts (Mx_Sb, Mx_0.9, Mx_0.5, Mx_0.1, Mx_Sc, S_0.9, S_0.5, S_0.1) were amplified in technical duplicate.

4. Discussion

In the early 2000s molecular data obtained by DNA analyses, including rDNA sequences, strongly indicated a close relatedness of *Sb* to *Sc* and thereby supported the recognition of *Sb* as a subtype of *Sc*, not as a separate species regardless of some genotypic differences (Van Der Aa Kühle and Jespersen 2003; Edwards-Ingram et al. 2007; Fietto et al. 2004; Posteraro et al. 2005). The comparative genomic research demonstrated that *Sb* and *Sc* share more than 99% genomic relatedness as determined by Average Nucleotide identity (ANI). The results revealed that the *Sb* probiotic strains are closer to wine strains of *Sc* than industrial or baking strains (Khatri et al. 2017). In the present work, a dendrogram was generated based on the clustering obtained in the HRM analysis. The clustering was conducted based on the melt curves progression obtained for the four analyzed regions: *18SrRNA*, *26SrRNA*, ITS and *TEF1alpha*. All *Sc* strains were grouped and reference probiotic Sb strains created a separate sub-cluster within as indicated.

 doi:10.20944/preprints202409.0492.v1

Since the health-promoting effects of *Sb* are strain-dependent, the preparations referred to as probiotics must include strains with appropriate phenotypic and genetic characteristics. The purpose of this study was to develop a qPCR-HRM-based analysis to detect and identify *Sb* in probiotic-fortified foods. Initially, two probiotic strains described in detail by the manufacturers were isolated to serve as references. The specific features such as the non-sporulation phenotype and absence of selected genes, including *MAL11* and *HXT9*, confirmed the strains to be probiotic subtypes. After prolonged incubation of *Sb* on a sporulation medium some changes in cell morphology were observed. Cells appeared enlarged and highly granular. An analogous effect was observed earlier, presumed to be the result of the initiation of the sporulation process combined with the inability to complete meiosis (Edwards-Ingram et al. 2007).

Reviewing the melting temperatures of each rDNA amplicon, there were no significant differences between the *Sc* and *Sb* strains tested. A pair of primers designed for the *18SrRNA* region tested on pure cultures enabled the clustering of subtype *Sb* outside the *Sc* cluster. HRM analysis results concerning *26SrRNA* and ITS sequences resulted in joint clustering of *Sb* and Sc strains. In comparative genomic studies, the sequence ITS1-5.8S rDNA-ITS2 of *Sb* displayed some subtle differences and 99% resemblance. As regards the sequence of the D1/D2 domain of the *26SrRNA* had a similarity value of 100% compared to the *Sc* genomes. The probability of constructing an effective differentiation sequence within rDNA is very low, with the scarcity of polymorphisms and the strict requirements for HRM analysis (Van Der Aa Kühle and Jespersen 2003; Fietto et al. 2004; Posteraro et al. 2005). The problematic rDNA regions were used in attempts to differentiate species of the genus *Saccharomyces* using HRM analysis. Four primer combinations were designed spanning the *26SrRNA* polymorphic region of 10 *Saccharomyces* species aligned. The highest discrimination level was achieved with five clusters for 10 type strains examined (Nadai et al. 2018). The combination of *26SrRNA* and ITS regions in another HRM analysis of *Saccharomyces* species enabled discrimination of the most studied yeasts at the species level. However, differentiation of *Sc*, *Sb* and *Saccharomyces uvarum* in the pure cultures or the mixed samples failed (Bazalová et al. 2022).

Genes encoding translation elongation factor (*TEF1alpha*), actin (*ACT1*), RNA polymerase subunit (*RPB1*) and *COX2*, were stated to be more adequate in correct differentiation of the problematic/closely related species (Cappello et al. 2010; Eizaguirre et al. 2018; Hulin et al. 2014). Moreover, *TEF1alpha* sequencing and MALDI-TOF mass-spectrometry were found more relevant for differentiation within the *Pichia cactophila* clade, than sequencing of standard barcoding regions ITS and D1/D2 (Guitard et al. 2015). Nowadays, *Sc* promoter regions were found not to be fully conserved, in terms of nucleotide sequence nor predicted transcription factor (TF) binding sites, in homolog *Sb* genes. Some of the differentially expressed genes in *Sb* strains were found to have gained or lost TF binding sites in their promoter regions (Pais et al. 2021). Therefore, a pair of primers for the yeast polymorphic region within *TEF1alpha* was included in this work. It was effective in interspecies discrimination while showing a very low degree of intraspecies differentiation. Only the *Sc*$_{\text{EtRed}}$ strain remained in a distinct cluster from the other *Sc* and *Sb* strains tested.

As intended, the primer pairs designed for this work for regions within *CCA1*, *HO*, *RPB2*, *HXT9* and *MAL11* were not amplified in species other than *Sc*. Amplification of *HXT9* and *MAL11* sequences was found only in *Sc* strains. The exception was *Sc*$_{\text{EtRed}}$, which showed no *MAL11* amplification. *Sc*$_{\text{EtRed}}$ closely relates to wine strains therefore it might show high similarities to *Sb* (Gronchi et al. 2022). MLST (Multilocus Sequence Typing) involved the sequencing of four nuclear genes *CCA1*, *CYT1* (ubiquinol-cytochrome-c reductase catalytic subunit gene), *HMX1* (heme oxygenase gene), *NUP116* (FG-nucleoporin gene) and ITS region resulted in the uniform clade of clinical isolates and commercial probiotic yeasts (Imre et al. 2019). In this study, a primers pair designed for the *CCA1* sequence was not sufficient to provide differentiation of probiotic strains. In contrast, the expected effect was found for the *RPB2* sequence. Moreover, HRM analysis with *RPB2* amplicon effectively clustered separately newly isolated *Saccharomyces paradoxus* strain (data not shown). *HO* sequence clustered *Sb* strains with *Sc*$_{\text{EtRed}}$ indicating the close affinity of *Sb* and *Sc* wine strains.

 doi:10.20944/preprints202409.0492.v1

In further research, *18S rRNA* and ITS sequences were selected for qPCR-HRM analysis of collected dietary supplements. Comparative analysis with rDNA regions confirmed the presence of *Sc* strains in the dietary supplements tested. Moreover, the selected intraspecies sequences, *HO* and *RPB2*, confirmed the presence of *Sb* in PS2, PS3, PS4, and *Sc* in PS1, according to their composition as declared by the manufacturers. The preparations did not include detailed specifications of the yeast strains declared. As established, *MAL11*, *MAL13* (transcription factor gene), and *ARN2* (siderophore transporter gene) were present in more than 70% of the strains of different subgroups of *Sc* strains but were absent in all the probiotic strains. The large hexose transporter family comprises *HXT11* and *HXT9* which were absent from all strains of *Sb* (Khatri et al. 2017). Therefore, *HXT9* and *MAL11* sequences were amplified on PS2 - PS4 templates. As a result, no amplification products of the *MAL11* region were confirmed, while only for PS2 the *HXT9* amplicon was not detected. Thus, the strains included in the examined supplements except PS2, do not completely correspond to those with health-promoting properties.

HRM analysis of DNA extracted from seed mixtures showed reduced sensitivity detection of the selected template compared to the analysis of mixed DNA samples (Emenyeonu et al. 2018). The yield of bacterial DNA from sourdough fermented with different strains of the same species may differ up to 100,000-fold even if the organisms are present at the same cell counts (Zheng et al. 2015). The observations support the assumption that the structure of the food matrix could lower the recovery of the nuclear or organellar DNA. It was declared that qPCR-HRM analysis detects but does not identify organisms if they account for 0.1–1% of the bacterial and yeast population, respectively (Lin and Gänzle 2014). Therefore, the effectiveness of selected sequences in identifying probiotic strains was tested in mixtures containing three yeast species. Mixtures were prepared that microbiologically corresponded in composition to kefirs (Goktas et al. 2021; Kalamaki and Angelidis 2017; Nejati et al. 2020; Özer and Kirmaci 2014; Wang et al. 2008). The application of commercial kefirs in the study failed. The reason was the very low yeast content, no more than log 2 CFU mL^{-1} of cells was determined in commercial products (data not shown). Due to very small differences in melting temperature (T_m) and melting profile of *18SrRNA* amplicons for Sb_{745}, Sc_D, *Km* and Pf_D all mix-yeast samples were clustered together. If the ITS sequence was considered, common clustering of samples containing only strains of *Sc* species was observed (Sb_{745} and Sc_D). In contrast, the other samples with additional *Km* and Pf_D presence formed several separate clusters. ITS region was the most variable in DNA primary structure amongst analyzed rDNA sequences. Consequently, it has the highest strength of interspecies differentiation in the HRM analysis. Amplification with a pair of ITS primers on DNA extracted from mixtures yielded heterogeneous products as a result of the increased affinity of the primers for the *Pf* sequence, as a consequence of nucleotide changes in the annealing regions of the *Sc* and *Km* sequences (Borkowska and Celińska 2023).

The designed intragenus primer pairs for *HO* or *RPB2* regions were the best in the identification of *Sb* in the mixtures. The *RPB2* sequence had the highest differentiation power. The reference probiotic strain was detectable and identifiable by qPCR-HRM when the mixture contained at least log 7 CFU mL^{-1} of *Sb* cells, and Sb_{745} significantly exceeded the Sc_D strain quantitatively. Figure 5h shows that the presence of other yeast species does not significantly change the product melting profile for M_Sb. In contrast, a reduction in the number of Sb_{745} cells to Sc_D resulted in a significant increase in difference RFU of 0,2 to the reference. The limits for accurate quantification of *Sc* in wine artificially contaminated, with real-time PCR using specific primers, were established for log 5 CFU mL^{-1} in sweet wine and log 6 CFU mL^{-1} in red wine (Martorell et al. 2005). Such high detection thresholds by qPCR indicate that the stated threshold for identification of *Sb* cells using qPCR-HRM analysis is plausible. A required minimum dose of *health-boosting microorganisms* is log 6 CFU mL^{-1} or CFU g^{-1} for the food product to be labelled as a probiotic (White and Hekmat 2018). Since the viability of microorganisms is the key to achieving health benefits (log 6 – log 7 CFU per g during the expected shelf-life of the probiotic food or beverage, according to WHO/FAO, 2006), some researchers even suggest increasing the dose up to log 7 CFU mL^{-1} or CFU g^{-1} (Fiocco et al. 2020). This is sufficient enough to detect and identify the probiotic strain of *Sb* yeast in probiotic-enriched foods with qPCR-HRM analysis using the *RPB2* marker.

5. Conclusions

qPCR-HRM analysis using interspecies *18SrRNA* and ITS sequences optimized in culture-dependent analysis identified *Sb* at the species level, while intraspecies *HO* and *RPB2* sequences at the variety level in single-yeast dietary supplements. Enhancement of *S. cerevisiae* var. *boulardii* differentiation was achieved by amplification of *HXT9* and *MAL11* regions with presence-absence variation. The low variability in sequences amplified by interspecies primer pairs in qPCR-HRM analysis prevented the differentiation of *Sb* in mixtures of three yeast species. In contrast, the identification of *Sb* using designed intragenus primer pairs was successful in *Sc* mixtures and mixtures with kefir microbial composition. The *RPB2* sequence showed the highest intraspecies differentiation power. However, qPCR-HRM analysis identified *Sb* only with the variety predominance in the microbial mix. Therefore, the presented qPCR-HRM analysis can be considered an appropriate tool for identifying *Sb* in probiotic-enriched food matrices.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Monika Borkowska, Michał Kułakowski and Kamila Myszka. The first draft of the manuscript was written by Monika Borkowska and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding: This work was supported by a subvention from the PULS fund (number 506.771.03.00) received from the Ministry of Science and Higher Education.

Data Availability statement: The datasets generated during the current study are available from the corresponding author upon request.

Competing interests: The authors have no relevant financial or non-financial interests to disclose.

References

1. Baleiras-Couto MM, Eijsma B, Hofstra H, Huis In'T Veld JH, Van Der Vossen JM (1996) Evaluation of Molecular Typing Techniques to Assign Genetic Diversity among Saccharomyces Cerevisiae Strains. *Applied and Environmental Microbiology* 62(1), 41 – 46. DOI: 10.1128/aem.62.1.41-46.1996
2. Bazalová O, Cihlář JZ, Dlouhá Z, Bár L, Dráb V, Kavková M (2022) Rapid Sourdough Yeast Identification Using Panfungal PCR Combined with High Resolution Melting Analysis. *Journal of Microbiological Methods* 199. DOI: 10.1016/j.mimet.2022.106522
3. Borkowska M, Celińska E (2023) Multiple Region High Resolution Melting-Based Method for Accurate Differentiation of Food-Derived Yeasts at Species Level Resolution. *Food Microbiology* 109. DOI: 10.1016/j.fm.2022.104120
4. Cappello MS, Poltronieri P, Blaiotta G, Zacheo G (2010) Molecular and Physiological Characteristics of a Grape Yeast Strain Containing Atypical Genetic Material. *International Journal of Food Microbiology* 144(1), 72 – 80. DOI: 10.1016/j.ijfoodmicro.2010.08.013
5. Cardinali G, Martini A (1994) Electrophoretic Karyotypes of Authentic Strains of the Sensu Strict0 Group of the Genus Saccharomyces? International Journal of Systematic Bacteriology 44(4), 791 – 797. DOI: 10.1099/00207713-44-4-791
6. Cesaro S, Chinello P, Rossi L, Zanesco L (2000) Saccharomyces Cerevisiae Fungemia in a Neutropenic Patient Treated with Saccharomyces Boulardii. *Supportive Care in Cancer* 8(6), 504 – 5. DOI: 10.1007/s005200000123
7. Chan MZA, Tan LT, Heng SWQ, Liu SQ (2023) Effect of Co-Fermentation of Saccharomyces Boulardii CNCM-I745 with Four Different Probiotic Lactobacilli in Coffee Brews on Cell Viabilities and Metabolic Activities. *Fermentation* 9(3). DOI: 10.3390/fermentation9030219
8. Czerucka D, Piche T, Rampal P (2007) Review article: Yeast as Probiotics - Saccharomyces Boulardii. *Alimentary Pharmacology and Therapeutics* 26(6), 767 – 78. DOI: 10.1111/j.1365-2036.2007.03442.x
9. Değirmencioğlu N, Gurbuz O, Şahan Y (2016) The Monitoring, Via an In Vitro Digestion System, of the Bioactive Content of Vegetable Juice Fermented with Saccharomyces Cerevisiae and Saccharomyces Boulardii. *Journal of Food Processing and Preservation* 40(4), 798 – 811. DOI: 10.1111/jfpp.12704

10. Edwards-Ingram L, Gitsham P, Burton N, Warhurst G, Clarke I, Hoyle D, Oliver SG, Stateva L (2007) Genotypic and Physiological Characterization of Saccharomyces Boulardii, the Probiotic Strain of Saccharomyces Cerevisiae. *Applied and Environmental Microbiology* 73(8), 2458 – 67. DOI: 10.1128/AEM.02201-06
11. Eizaguirre JI, Peris D, Rodríguez ME, Lopes CA, De Los Ríos P, Hittinger CT, Libkind D (2018) Phylogeography of the Wild Lager-Brewing Ancestor (Saccharomyces Eubayanus) in Patagonia. *Environmental Microbiology* 20(10), 3732 – 43. DOI: 10.1111/1462-2920.14375
12. Ellouze O, Berthoud V, Mervant M, Parthiot JP, Girard C (2016) Septic Shock Due to Saccharomyces Boulardii. *Medecine et Maladies Infectieuses* 46(2), 104 – 5. DOI: 10.1016/j.medmal.2015.12.003
13. Emenyeonu LC, Croxford AE, Wilkinson MJ (2018) The Potential of Aerosol EDNA Sampling for the Characterisation of Commercial Seed Lots. *PLoS ONE* 13(8). DOI: 10.1371/journal.pone.0201617
14. Erdem M, Kesmen Z, Özbekar E, Çetin B, Yetim H (2016) Application of High-Resolution Melting Analysis for Differentiation of Spoilage Yeasts. *Journal of Microbiology* 54(9), 618 – 25. DOI: 10.1007/s12275-016-6017-8
15. Fietto JLR, Araújo RS, Valadão FN, Fietto LG, Brandão RL, Neves MJ, Gomes FCO, Nicoli JR, Castro IM, (2004) Molecular and Physiological Comparisons between Saccharomyces Cerevisiae and Boulardii. *Canadian Journal of Microbiology* 50(8), 615 – 21. DOI: 10.1139/w04-050
16. Fijan S (2014) Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. *International Journal of Environmental Research and Public Health* 11(5), 4745 – 67. DOI: 10.3390/ijerph110504745
17. Fiocco D, Longo A, Arena MP, Russo P, Spano G, Capozzi V (2020) How Probiotics Face Food Stress: They Get by with a Little Help. *Critical Reviews in Food Science and Nutrition* 60(9), 1552 – 80. DOI: 10.1080/10408398.2019.1580673
18. Goktas H, Dikmen H, Demirbas F, Sagdic O, Dertli E (2021) Characterisation of Probiotic Properties of Yeast Strains Isolated from Kefir Samples. *International Journal of Dairy Technology* 74(4), 715 – 22. DOI: 10.1111/1471-0307.12802
19. Gronchi N, De Bernardini N, Cripwell RA, Treu L, Campanaro S, Basaglia M, Foulquié-Moreno MR, Thevelein JM, Van Zyl WH, Favaro L, Casella S (2022) Natural Saccharomyces Cerevisiae Strain Reveals Peculiar Genomic Traits for Starch-to-Bioethanol Production: The Design of an Amylolytic Consolidated Bioprocessing Yeast. *Frontiers in Microbiology* 12. DOI: 10.3389/fmicb.2021.768562
20. Guitard J, Atanasova R, Brossas JY, Meyer I, Gits M, Marinach C, Vellaissamy S, Angoulvant A, Mazier D, Hennequin C (2015) Candida Inconspicua and Candida Norvegensis: New Insights into Identification in Relation to Sexual Reproduction and Genome Organization. *Journal of Clinical Microbiology* 53(5), 1655 – 61. DOI: 10.1128/JCM.02913-14
21. Gut AM, Vasiljevic T, Yeager T, Donkor ON (2019) Characterization of Yeasts Isolated from Traditional Kefir Grains for Potential Probiotic Properties. *Journal of Functional Foods* 58, 56 – 66. DOI: 10.1016/j.jff.2019.04.046
22. Hennequin C, Thierry A, Richard GF, Lecointre G, Nguyen HV, Gaillardin C, Dujon B (2001) Microsatellite Typing as a New Tool for Identification of Saccharomyces Cerevisiae Strains. *Journal of Clinical Microbiology* 39(2), 551 – 59. DOI: 10.1128/JCM.39.2.551-559.2001
23. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014) Expert Consensus Document: The International Scientific Association for Probiotics and Prebiotics Consensus Statement on the Scope and Appropriate Use of the Term Probiotic. *Nature Reviews Gastroenterology and Hepatology* 11(8), 506 – 14. DOI: 10.1038/nrgastro.2014.66.
24. Hulin M, Harrison E, Stratford M, Wheals AE (2014) Rapid Identification of the Genus Dekkera/Brettanomyces, the Dekkera Subgroup and All Individual Species. *International Journal of Food Microbiology* 187, 7 – 14. DOI: 10.1016/j.ijfoodmicro.2014.06.028
25. Imre A, Rácz HV, Antunovics Z, Rádai Z, Kovács R, Lopandic K, Pócsi I, Pfliegler WP (2019) A New, Rapid Multiplex PCR Method Identifies Frequent Probiotic Origin among Clinical Saccharomyces Isolates. *Microbiological Research* 227. DOI: 10.1016/j.micres.2019.126298
26. Kalamaki MS, Angelidis AS (2017) Isolation and Molecular Identification of Yeasts in Greek Kefir. *International Journal of Dairy Technology* 70(2), 261 – 68. DOI: 10.1111/1471-0307.12329

27. Kara I, Yıldırım F, Özgen Ö, Erganiş S, Aydoğdu M, Dizbay M, Gürsel G, Kalkanci A (2018) Saccharomyces Cerevisiae Fungemia after Probiotic Treatment in an Intensive Care Unit Patient. *J Mycol Med* 28(1), 218 – 21. DOI: 10.1016/j.mycmed.2017.09.003
28. Karaolis C, Botsaris G, Pantelides I, Tsaltas D (2013) Potential Application of Saccharomyces Boulardii as a Probiotic in Goat's Yoghurt: Survival and Organoleptic Effects. *International Journal of Food Science and Technology* 48(7), 1445 – 52. DOI: 10.1111/ijfs.12111
29. Kesmen Z, Özbekar E, Büyükkiraz ME (2018a) Multifragment Melting Analysis of Yeast Species Isolated from Spoiled Fruits. *Journal of Applied Microbiology* 124(2), 522–34. DOI: 10.1111/jam.13645
30. Kesmen Z, Büyükkiraz ME, Özbekar E, Çelik M, Özkök FÖ, Kılıç Ö, Çetin B, Yetim H (2018b) Assessment of Multi Fragment Melting Analysis System (MFMAS) for the Identification of Food-Borne Yeasts. *Current Microbiology* 75(6), 716 – 25. DOI: 10.1007/s00284-018-1437-9
31. Khatri I, Tomar R, Ganesan K, Prasad GS, Subramanian S (2017) Complete Genome Sequence and Comparative Genomics of the Probiotic Yeast Saccharomyces Boulardii. *Scientific Reports* 7(1). DOI: 10.1038/s41598-017-00414-2
32. Kurtzman CP, Robnett CJ (1998) Identification and Phylogeny of Ascomycetous Yeasts from Analysis of Nuclear Large Subunit (26S) Ribosomal DNA Partial Sequences. Antonie Van Leeuwenhoek 73(4), 331 – 71. DOI: 10.1023/a:1001761008817
33. Lin XB, Gänzle MG (2014) Quantitative High-Resolution Melting PCR Analysis for Monitoring of Fermentation Microbiota in Sourdough. *International Journal of Food Microbiology* 186, 42 – 48. DOI: 10.1016/j.ijfoodmicro.2014.06.010
34. Łukaszewicz M (2012) Saccharomyces Cerevisiae Var. Boulardii – Probiotic Yeast. *Probiotics*. InTech Press, US. DOI: 10.5772/50105
35. Martorell P, Querol A, Fernández-Espinar MT (2005) Rapid Identification and Enumeration of Saccharomyces Cerevisiae Cells in Wine by Real-Time PCR. *Applied and Environmental Microbiology* 71(11), 6823 – 30. DOI: 10.1128/AEM.71.11.6823-6830.2005
36. McCullough MJ, Clemons KV, McCusker JH, Stevens DA (1998) Species Identification and Virulence Attributes of Saccharomyces Boulardii (Nom. Inval.). Journal of Clinical Microbiology 36(9), 2613 – 2617. DOI: 10.1128/JCM.36.9.2613-2617.1998
37. McFarland LV (1996) Saccharomyces boulardii is not Saccharomyces cerevisiae. *Clin. Infect. Dis.* 22, 200 – 201. DOI: 10.1093/clinids/22.1.200
38. Mitterdorfer G, Mayer HK, Kneifel W, Viernstein H (2002) Clustering of Saccharomyces Boulardii Strains within the Species S. Cerevisiae Using Molecular Typing Techniques. *J Appl Microbiol.* 93(4), 521 – 30. DOI: 10.1046/j.1365-2672.2002.01710.x
39. Molnar O, Messner R, Prillinger H, Stahl U, Slavikova E (1995) Genotypic Identification of Saccharomyces Species Using Random Amplified Polymorphic DNA Analysis. *Systematic and Applied Microbiology* 18(1), 136 – 45. DOI: 10.1016/S0723-2020(11)80461-3
40. Nadai C, Bovo B, Giacomini A, Corich V (2018) New Rapid PCR Protocol Based on High-Resolution Melting Analysis to Identify Saccharomyces Cerevisiae and Other Species within Its Genus. *Journal of Applied Microbiology* 124(5), 1232 – 42. DOI: 10.1111/jam.13709
41. Nejati F, Junne S, Kurreck J, Neubauer P (2020) Quantification of Major Bacteria and Yeast Species in Kefir Consortia by Multiplex TaqMan QPCR. *Frontiers in Microbiology* 11. DOI: 10.3389/fmicb.2020.01291.
42. Özer B, Kirmaci HA (2014) Fermented Milks: Products of Eastern Europe and Asia. *Encyclopedia of Food Microbiology: Second Edition*, 900 – 907. Elsevier Inc. DOI: 10.1016/B978-0-12-384730-0.00123-3
43. Pais P, Oliveira J, Almeida V, Yilmaz M, Monteiro PT, Teixeira MC (2021) Transcriptome-Wide Differences between Saccharomyces Cerevisiae and Saccharomyces Cerevisiae Var. Boulardii: Clues on Host Survival and Probiotic Activity Based on Promoter Sequence Variability. *Genomics* 113(2), 530 – 39. DOI: 10.1016/j.ygeno.2020.11.034
44. Posteraro B, Sanguinetti M, Romano L, Torelli R, Novarese L, Fadda G (2005) Molecular Tools for Differentiating Probiotic and Clinical Strains of Saccharomyces Cerevisiae. *International Journal of Food Microbiology* 103(3), 295 – 304. DOI: 10.1016/j.ijfoodmicro.2004.12.031
45. Rekha CR, Vijayalakshmi G (2010) Bioconversion of Isoflavone Glycosides to Aglycones, Mineral Bioavailability and Vitamin B Complex in Fermented Soymilk by Probiotic Bacteria and Yeast. *Journal of Applied Microbiology* 109(4), 1198 – 1208. DOI: 10.1111/j.1365-2672.2010.04745.x

46. Ripari V, Gänzle MG, Berardi E (2016) Evolution of Sourdough Microbiota in Spontaneous Sourdoughs Started with Different Plant Materials. *International Journal of Food Microbiology* 232, 35 – 42. DOI: 10.1016/j.ijfoodmicro.2016.05.025
47. Sambrook J, Russell D (2001) *Molecular Cloning: A Laboratory Manual*. 3rd ed. Cold Spring Harbor Laboratory Press, New York.
48. Santino L, Alari' A, Bono S, Tetp E, Bernardini A, Magrini L, Di Somma S, Teggi A (2014) Saccharomyces cerevisiae fungemia, a possible consequence of the treatment of Clostridium difficile colitis with a probioticum. International Journal of Immunopathology and Pharmacology 27 (1), 143 – 146. DOI: 10.1177/039463201402700120
49. Thygesen JB, Glerup H, Tarp B (2012) Saccharomyces Boulardii Fungemia Caused by Treatment with a Probioticum. *BMJ Case Reports*. DOI: 10.1136/bcr.06.2011.4412
50. Valente P, Gouveia C, De Lemos GA, Pimentel ' D, Van Elsas JD, Mendonqa-Hagler LC, Hagler AN (1996) PCR Amplification of the RDNA Internal Transcribed Spacer Region for Differentiation of Saccharomyces Cultures. FEMS Microbiol Lett. 137(2-3), 253 – 6. DOI: 10.1111/j.1574-6968.1996.tb08114.x
51. Van Der Aa Kühle A, Jespersen L (2003) The Taxonomic Position of Saccharomyces Boulardii as Evaluated by Sequence Analysis of the D1/D2 Domain of 26S RDNA, the ITS1-5.8S RDNA-ITS2 Region and the Mitochondrial Cytochrome-c Oxidase II Gene. *Systematic and Applied Microbiology* 26(4), 564 – 71. DOI: 10.1078/072320203770865873
52. Wang SY, Chen HC, Liu JR, Lin YC, Chen MJ (2008) Identification of Yeasts and Evaluation of Their Distribution in Taiwanese Kefir and Viili Starters. *Journal of Dairy Science* 91(10), 3798 – 3805. DOI: 10.3168/jds.2007-0468
53. White J, Hekmat S (2018) Development of Probiotic Fruit Juices Using Lactobacillus Rhamnosus GR-1 Fortified with Short Chain and Long Chain Inulin Fiber. *Fermentation* 4(2). DOI: 10.3390/fermentation4020027
54. Zamora-Vega R, Montañez-Soto JL, Martínez-Flores HE, Flores-Magallón R, Muñoz-Ruiz CV, Venegas-González J, De Jesús Ariza Ortega T (2012) Effect of Incorporating Prebiotics in Coating Materials for the Microencapsulation of Sacharomyces Boulardii. *International Journal of Food Sciences and Nutrition* 63(8), 930 – 35. DOI: 10.3109/09637486.2012.687364
55. Zheng J, Ruan L, Sun M, Gänzle M (2015) A Genomic View of Lactobacilli and Pediococci Demonstrates That Phylogeny Matches Ecology and Physiology. *Applied and Environmental Microbiology* 81(20), 7233 – 43. DOI: 10.1128/AEM.02116-15