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ORIGINAL ARTICLE

Influence of culture conditions on co-aggregation of probiotic yeast *Saccharomyces boulardii* with *Candida* spp. and their auto-aggregation

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Abstract

Systemic infections caused by pathogenic *Candida* species pose a significant threat to public health in the past decades due to increasing resistance to existing antifungal drugs. Given this scenario, probiotics have been suggested as an alternative approach for managing *Candida* infections. Hence, the purpose of this study was to evaluate whether probiotic yeast *Saccharomyces boulardii* co-aggregate with *Candida* spp. as well as to determine their auto-aggregation ability in dependence on temperature (28 °C, 37 °C, 42 °C) and pH (4.5, 7.0, 8.5) after 5 h and 24 h. Our results revealed that the aggregation of tested yeasts was lower in the first 5 h but increased significantly after 24 h. All strains were able to auto-aggregate in different degrees ranging from 47.46 to 95.95% assessed at 24 h of incubation. Among them the highest auto-aggregation values had *C. albicans* and *C. krusei* strains followed by probiotic strain *S. boulardii*, while the less were observed in *C. glabrata* strains. In addition, co-aggregation between probiotic and *Candida* strains was strain-specific. It was evident that *S. boulardii* significantly inhibited the aggregation of *C. albicans* ATCC 10261, *C. krusei* ATCC 6258, and *C. glabrata* ZIM 2369. However, in *C. glabrata* ZIM 2382, the aggregation was even enhanced. Temperature and pH also affected the ability to aggregate in a different way only after 5 h of incubation, with the highest cell aggregation evidenced at temperature 37 °C in most cases and pH 4.5. These findings may be of importance when trying to establish probiotic use against pathogenic *Candida* species.

Introduction

Candida species are commensal yeast belonging to the normal microbiota of healthy individuals. Nevertheless, under certain circumstances, *Candida* spp. can switch from a harmless form into a pathogenic form that can cause infections in humans by colonizing the mucosal surfaces of the genital, urinary, respiratory, and gastrointestinal tracts and the oral cavity (Mundula et al. 2019; Jørgensen et al. 2017). Impairment of immune functions permits the pathogen to disseminate into the body, into the blood stream, resulting in life-threatening systemic infections (Miranda et al. 2009). In the last few decades, opportunistic invasive *Candida* infections present a significant problem in both immunocompetent and immunocompromised individuals (Kunyeit et al. 2019). Infections are primarily caused by *Candida albicans* and by other species like *Candida glabrata* and *Candida krusei* (Bassetti et al. 2006; Fidel et al. 1999). Although the antifungal agents used to treat *Candida* infections are very effective, they present some critical points, such as frequent side effects and, in particular, antifungal resistance (Sanglard and Odds 2002). This has stimulated efforts to discover new alternative therapeutic strategies. As a promising method in order to achieve these purposes are probiotics that are known to be beneficial for overall health (Jørgensen et al. 2017; Tomičić et al. 2016a).

Probiotic organisms are increasingly incorporated into food as dietary supplements or consumed as non-food preparations. Most of the commercialized probiotics contain bacteria from the genera *Lactobacillus* and *Bifidobacterium*; however, there is a growing interest in researching yeasts for their beneficial effects (Burgain et al. 2011; Ogunremi et al. 2015; Yildiran et al. 2019). Some yeast strains, viz.,

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Saccharomyces cerevisiae and *Saccharomyces boulardii*, are particularly attractive for probiotic applications and generally regarded as they are exerting some influence on the intestinal flora (Hossain et al. 2020; Lazo-Vélez et al. 2018). For yeasts intended to be used as probiotics, each strain must meet numerous criteria before its commercial usage (Moslehi-Jenabian et al. 2010; Ragavan and Das 2017). A criteria of utmost importance in selecting a potential candidate are acid and bile tolerances, the ability to aggregate and adhere to epithelial cells, immunostimulation, and antagonistic activity against pathogens (Food and Agriculture Organization of the United Nations 2002).

In therapeutic terms, probiotic yeasts present a safe and cost-effective method of keeping *Candida* under control, improving the health and wellness of the patient (Krasowska et al. 2009; Kunyeit et al. 2019; Tomičić et al. 2016a). Beneficial effects of *S. boulardii* involve different mechanisms, such as competition with pathogens for nutrients and receptors on the cell surfaces, thus preventing their adhesion and colonization on the mucosal surfaces (Kelesidis and Pothoulakis 2012; Tomičić et al. 2016b). Co-aggregation of probiotics with *Candida* spp. may act as a defense mechanism against pathogenic infection. Therefore, the phenomena of auto-aggregation and co-aggregation are desirable properties of probiotic yeast (Matsubara et al. 2016).

The type of cell–cell interaction, termed auto-aggregation, is defined as the ability of the cells to self-adhere to cells of the same kind and colonize the environment, while co-aggregation is characterized by the highly specific recognition and adherence of different species of microorganisms to one another (Janković et al. 2012; Stevens et al. 2015). However, the effect of several culture conditions on the auto-aggregation and co-aggregation phenomenon was not fully evaluated until now. Considering effective but less exploited role of probiotic yeast as antifungal agent, the aim of this study was to investigate the ability of *S. boulardii* to co-aggregate with *Candida* spp. as well as their capability to auto-aggregate. The aggregation activities were conducted in response to different temperature, pH, and time. The experimental findings will give new insight for uses of *S. boulardii* in the battle against fungal infections.

Materials and methods

Strains and growth conditions

The probiotic *Saccharomyces boulardii* strain was used to examine its ability to co-aggregate with *Candida* strains. A total of four *Candida* strains were selected and tested because of their importance in clinical environment as the most frequently pathogens. Reference strains *C. albicans* ATCC 10261 and *C. krusei* ATCC 6258 were procured lyophilized from the American Type Culture Collection (Manassas, USA), while *C. glabrata* ZIM 2369 and *C. glabrata* ZIM 2382 strains were isolated from clinical samples (bronchoalveolar wash (BAL), urine from indwelling catheter, respectively). All yeast strains were obtained from the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, University of Ljubljana, Slovenia, and kept at − 80 °C in yeast peptone dextrose medium (Sigma-Aldrich, St. Louis, USA) (YPD) supplemented with 40% glycerol. Prior to performing auto-aggregation and co-aggregation assays, strains were revitalized from frozen stocks by cultivation on malt extract agar for microbiology (MEA) (Merck-KGaA, Darmstadt, Germany) for 24 h at 37 °C.

Auto-aggregation and co-aggregation assays

The auto-aggregation and co-aggregation abilities of tested yeasts were assessed according to the method of Kos et al. (2003) with certain modification. The yeast cells from malt extract agar (MEA) plates were harvested and washed twice with sterile phosphate-buffered saline (PBS; Oxoid, Hampshire, UK, pH 7.3) by centrifugation at 4000 *g* for 5 min. In order to analyze the effect of culture conditions such as temperature and pH on auto-aggregation and co-aggregation, cellular suspensions were made in PBS and adjusted with HCl (Merck KGaA, Darmstadt, Germany) to reach pH 4.0 and 7.0, or with NaOH (Merck KGaA, Darmstadt, Germany) to reach pH 8.5. The final cell concentration was measured at 600 nm and readjusted to an absorbance of 1 (A_{T0}) using a microplate reader (Varioskan Lux, Thermo Fisher Scientific).

For auto-aggregation assay, 4 mL of each yeast suspension was divided into sterile test tubes. The tubes were mixed by vortexing, and mono-cultures were incubated in PBS (pH 7.3) at three temperatures (28 °C, 37 °C, 42 °C) or three different pH (4.5, 7.0, 8.5) at 37 °C. The A_{600} of the carefully pipetted supernatants (upper layers) were measured immediately (0 h), and then after 5 h and 24 h of incubation. The auto-aggregation percentages were determined according to Eq. (1).

$$\%\ \text{auto} - \text{aggregation} = \left(1 - \frac{A_{Time}}{A_{T0}}\right) \times 100 \quad (1)$$

where A_{Time} represents the A_{600} at the specified times (5 h, 24 h), while A_{T0} represents the A_{600} at time zero ($t = 0$ h).

For co-aggregation assay, yeast suspensions were prepared in the same way as for the auto-aggregation assay. Equal volumes (2 mL) of probiotic *S. boulardii* suspension were mixed with the other *Candida* suspensions, as required (i.e., *S. boulardii* plus *C. albicans*; *S. boulardii* plus *C. krusei*; *S. boulardii* plus *C. glabrata*) in sterile test tubes. The tubes were mixed by vortexing, and co-cultures were

incubated in PBS (pH 7.3) at three temperatures (28 °C, 37 °C, 42 °C) or three different pH (4.5, 7.0, 8.5) at 37 °C. The A_{600} of the carefully pipetted supernatants (upper layers) were measured immediately (0 h), and then after 5 h and 24 h of incubation. The co-aggregation percentages were determined according to Eq. (2).

$$\%\ \text{co} - \text{aggregation} = \left[1 - \frac{A_{Mix}}{\frac{A_{Probiotic}+A_{Candida}}{2}}\right] \times 100 \qquad (2)$$

where A_{Mix} represents the A_{600} of the co-cultures (as indicated above), while $A_{Probiotic}$ and $A_{Candida}$ represent the A_{600} of the yeast mono-cultures (i.e., *S. boulardii*, *C. albicans*, *C. krusei*, *C. glabrata*), at the specified times (5 h, 24 h).

To achieve the research goals, for aggregation after 24 h of incubation at 37 °C and pH 7, the pictures were taken using the microscope with camera (Leica DFC290) with × 40 magnification (images are shown for *C. glabrata* strains).

Statistical analysis

The statistical analysis was performed using StatSoft Statistica, ver. 10 (IBM, Armonk, NY, USA). The mean value and standard deviation of 4 readings were recorded ($n = 4$), and all results were presented as a percentage ± standard deviation (± SD) of auto-aggregation and co-aggregation. To evaluate the effects of different temperature, pH, and time on auto-aggregation and co-aggregation, statistical significances were determined using analysis of variance (ANOVA) and the post hoc Tukey's HSD test and were considered significant at $P < 0.05$.

Results

In the present study, we examined whether the presence of the probiotic yeast *Saccharomyces boulardii* affects the aggregation of the pathogenic yeast *Candida albicans*, *Candida krusei*, and *Candida glabrata* in dependence on growth temperature and pH.

The effect of temperature on co-aggregation of *Saccharomyces boulardii* with *Candida* strains and their auto-aggregation

The co-aggregation ability of *S. boulardii* with *Candida* strains and their auto-aggregation were evaluated after 5 h and 24 h of incubation at three different temperatures (28 °C, 37 °C, 42 °C), and the results were expressed as percentages. As can be seen in Fig. 1, the aggregation of all tested yeasts is lower in the first 5 h but increased significantly after 24 h. The highest degree of auto-aggregation was observed in *C. albicans* ATCC 10261 (up to 94.42%) and *C. krusei* ATCC 6258 (up to 95.95%) strains followed by probiotic strain *S. boulardii* (up to 84.56%), while the less auto-aggregative ability had *C. glabrata* ZIM 2369 (up to 69.95%) and *C. glabrata* ZIM 2382 (up to 47.46%) strains estimated after 24 h of incubation. At 5 h, the aggregation percentages showed a high level of variability, oscillating with temperature. Further, our results indicate that the presence of *S. boulardii* equally affected the aggregation of *C. albicans* and *C. krusei*, but this effect was highly dependent on *C. glabrata* strains. In the case of the strains *C. albicans* ATCC 10261, *C. krusei* ATCC 6258 ZIM, and *C. glabrata* ZIM 2369, the effect was antagonistic with values up to 78.25%, 75.11%, and 59.87%, respectively, which means that the aggregation of these *Candida* strains was significantly inhibited by *S. boulardii* in comparison to mono-cultures at 24 h ($P < 0.05$). However, in the strain *C. glabrata* ZIM 2382, the effect was even synergistic (with value up to 61.47%). Regarding the temperature, statistical analysis showed that growth temperature is a very important factor which considerably affects the co-aggregation of probiotic strain *S. boulardii* with *Candida* strains and their auto-aggregation at 5 h of incubation, while after 24 h, it had no effect. We observed two different patterns, the strains *C. krusei* and both *C. glabrata* aggregated in significantly higher degree at 37 °C than at 28 °C and 42 °C ($P < 0.05$), whereas in the case of *C. albicans*, aggregation was significantly stimulated at 42 °C and decreased at 28 °C and 37 °C ($P < 0.05$), as in mono and in co-culture with *S. boulardii*.

The effect of pH on co-aggregation of *Saccharomyces boulardii* with *Candida* strains and their auto-aggregation

In order to examine the effect of pH on co-aggregation of *S. boulardii* with *Candida* strains and their auto-aggregation, the assays were performed over a pH range of 4.5–8.5 at 37 °C, what is pH range in relevant biological systems, and the results were measured after 5 h and 24 h of incubation and expressed as percentages. As in experiment with temperature, all obtained aggregation percentages were lower after 5 h and showed a high level of variability depending on pH. Namely, the highest degree of auto-aggregation had *C. albicans* ATCC 10261 (up to 94.14%) and *C. krusei* ATCC 6258 (up to 92.13%) followed by *S. boulardii* (up to 80.65%), *C. glabrata* ZIM 2369 (up to 71.61%), and *C. glabrata* ZIM 2382 (up to 45.37%) strains after 24 h of incubation as presented in Fig. 2. In addition, the presence of probiotic strain *S. boulardii* significantly inhibited the aggregation of *C. albicans* ATCC 10261, *C. krusei* ATCC 6258, and *C. glabrata* ZIM 2369 (with values up to 77.51%, 77.87%, and 53.02%, respectively) in comparison to their mono-cultures at 24 h ($P < 0.05$), which is confirmed in the photographs of sample microscope preparations for *C.*

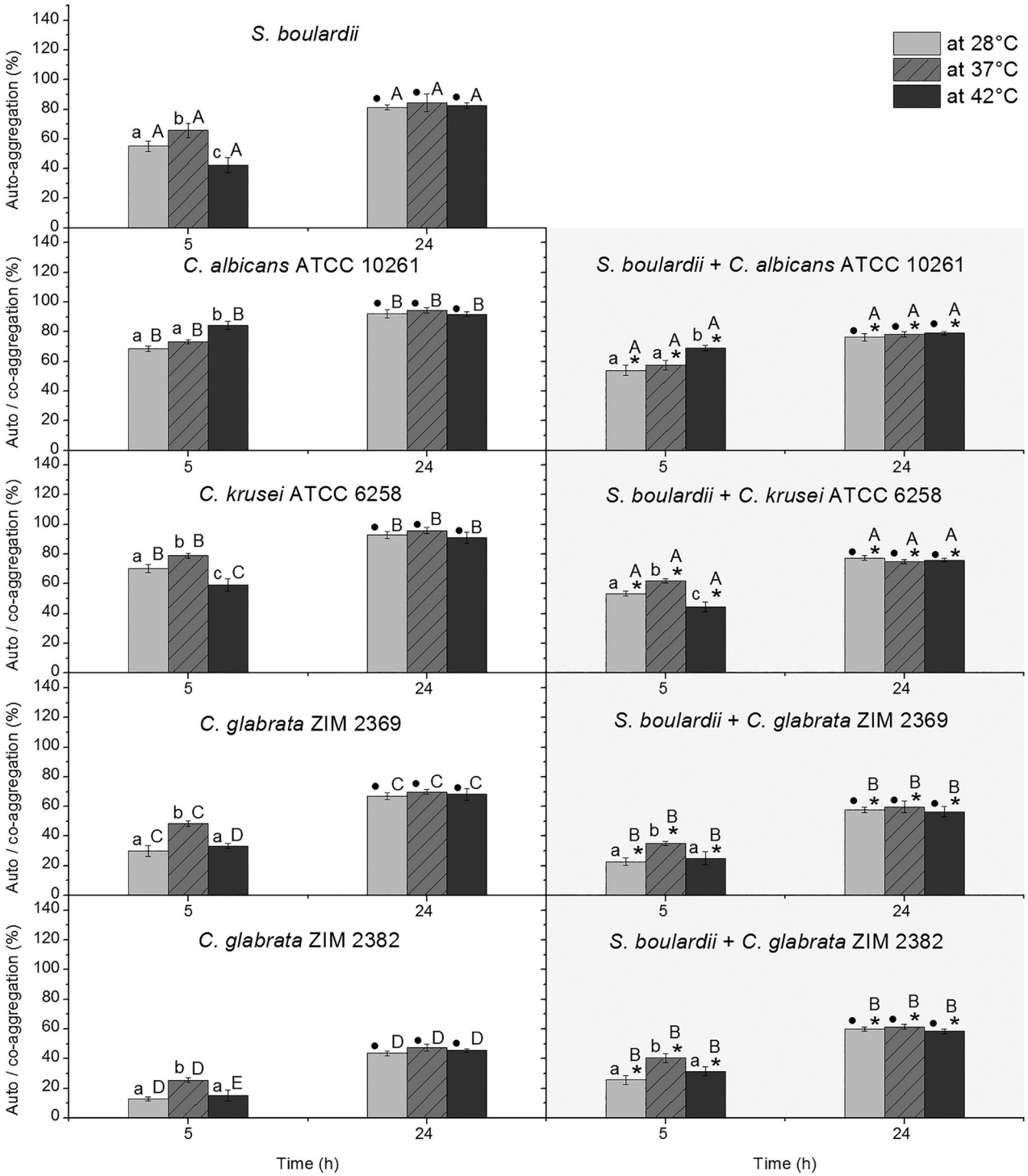

Fig. 1 The effect of temperature on aggregation of *Saccharomyces boulardii* and *Candida* strains resuspended in PBS (pH 7.3) as mono-cultures or mixed co-cultures after 5 h and 24 h. Data are means ± standard deviations (SD) of four replicates. Different letters (a, b, c) mark significant differences among temperatures at the same time, while asterisks (•) mark significant differences between time at the same temperature for each mono-culture or mixed co-culture ($P < 0.05$). The asterisks (*) indicate that there is significant difference between auto-aggregation of *Candida* strains and their co-aggregation with *S. boulardii*, while different letters (A, B, C) indicate significant differences among mono-cultures or mixed co-cultures, at the same temperature and time ($P < 0.05$). Unmarked terms are not significant

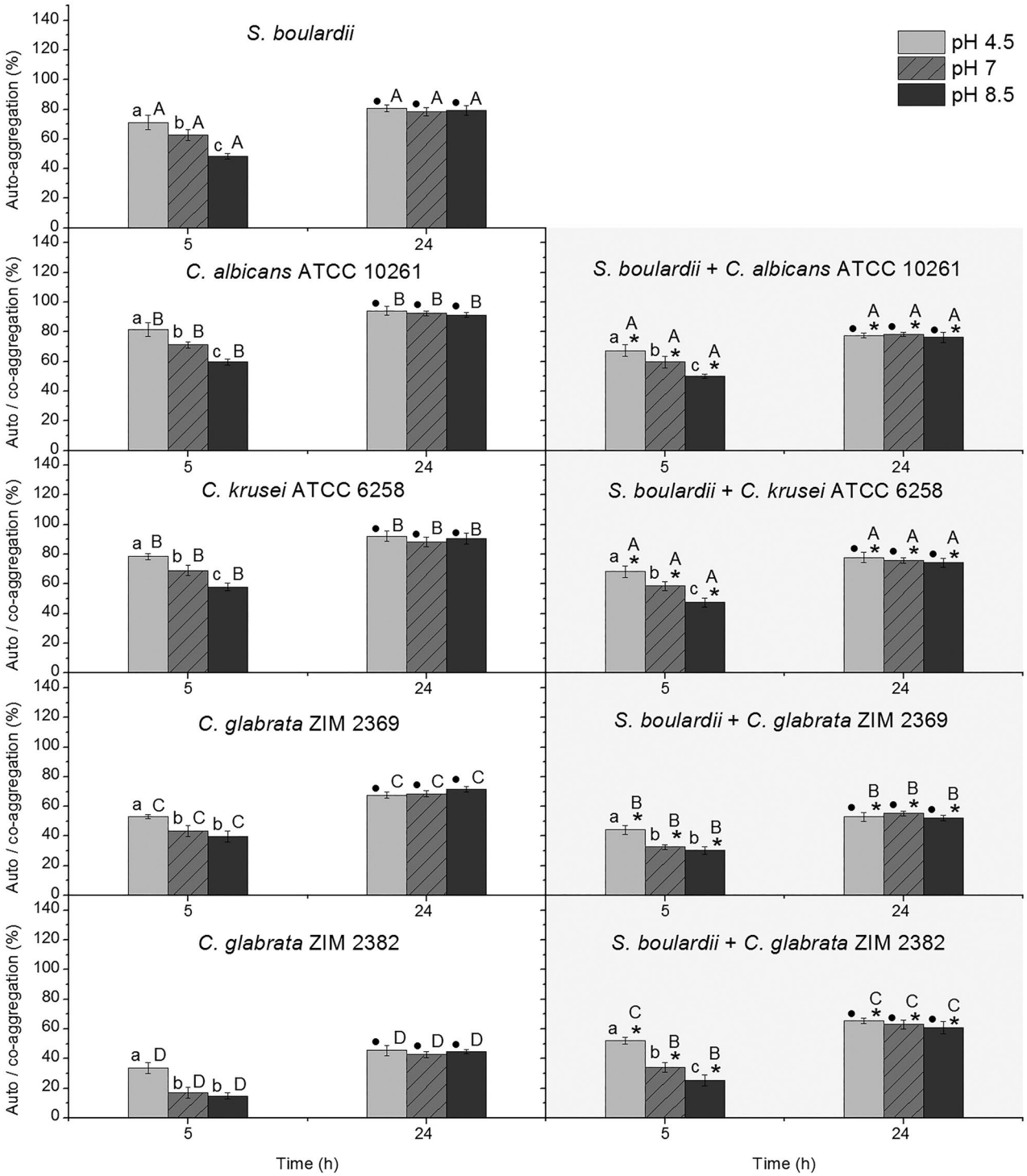

Fig. 2 The effect of pH on aggregation of *Saccharomyces boulardii* with *Candida* strains resuspended in PBS (pH 7.3) and adjusted to pH 4.0, 7.0 and 8.5 as mono-cultures or mixed co-cultures after 5 h and 24 h at 37 °C. Data are means ± standard deviations (SD) of four replicates. Different letters (a, b, c) mark significant differences among pH at the same time, while asterisks (•) mark significant differences between time at the same pH for each mono-culture or mixed co-culture ($P < 0.05$). The asterisks (*) indicate that there is significant difference between auto-aggregation of *Candida* strains and their co-aggregation with *S. boulardii*, while different letters (A, B, C) indicate significant differences among mono-cultures or mixed co-cultures, at the same pH and time ($P < 0.05$). Unmarked terms are not significant

glabrata ZIM 2369 strain as in mono and in co-culture with *S. boulardii* (Fig. 3A, B). However, probiotic strain acted differently on *C. glabrata* ZIM 2382, enhancing its aggregation up to 65.51% (Fig. 3C, D). Our results also indicate that the co-aggregation between *S. boulardii* and *Candida* strains and their auto-aggregation were highly dependent on pH after 5 h, with the highest aggregation at pH 4.5 and lowest at pH 8.5 ($P < 0.05$). While after 24 h of incubation, pH had no effect.

Discussion

Infections caused by *Candida* species have been increased dramatically worldwide in recent years (Mundula et al. 2019), although several strategies for the prevention and treatment of candidiasis have been adopted at the clinical level such as the use of various antifungal drugs (Kabir and Ahmad 2012; Pfaller and Diekema 2007). Resistance to antifungals represent current threats for public health requiring the use of alternative antifungal therapy (Sanglard and Odds 2002; Sardi et al. 2013). Consequently, there are a growing number of potential health benefits attributed to probiotics. They include certain bacteria (Bustamante et al. 2020), while among yeasts, only *Saccharomyces boulardii* is widely used and represents an effective biotherapeutic agents against *Candida* infections (Krasowska et al. 2009; Matsubara et al. 2016; Mundula et al. 2019). These probiotic products are usually well accepted by patients and often marketed as a dietary supplement (Burgain et al. 2011; Lazo-Vélez et al. 2018). However, the use of antagonistic bacteria to inhibit pathogenic microorganisms is extensively documented (Aarti et al. 2018; Ekmekçi et al. 2009; Fonseca et al. 2021; Jørgensen et al. 2017; Pino et al. 2019), but very little attention has been paid to yeasts in a similar role. Aggregation properties have been suggested to be important

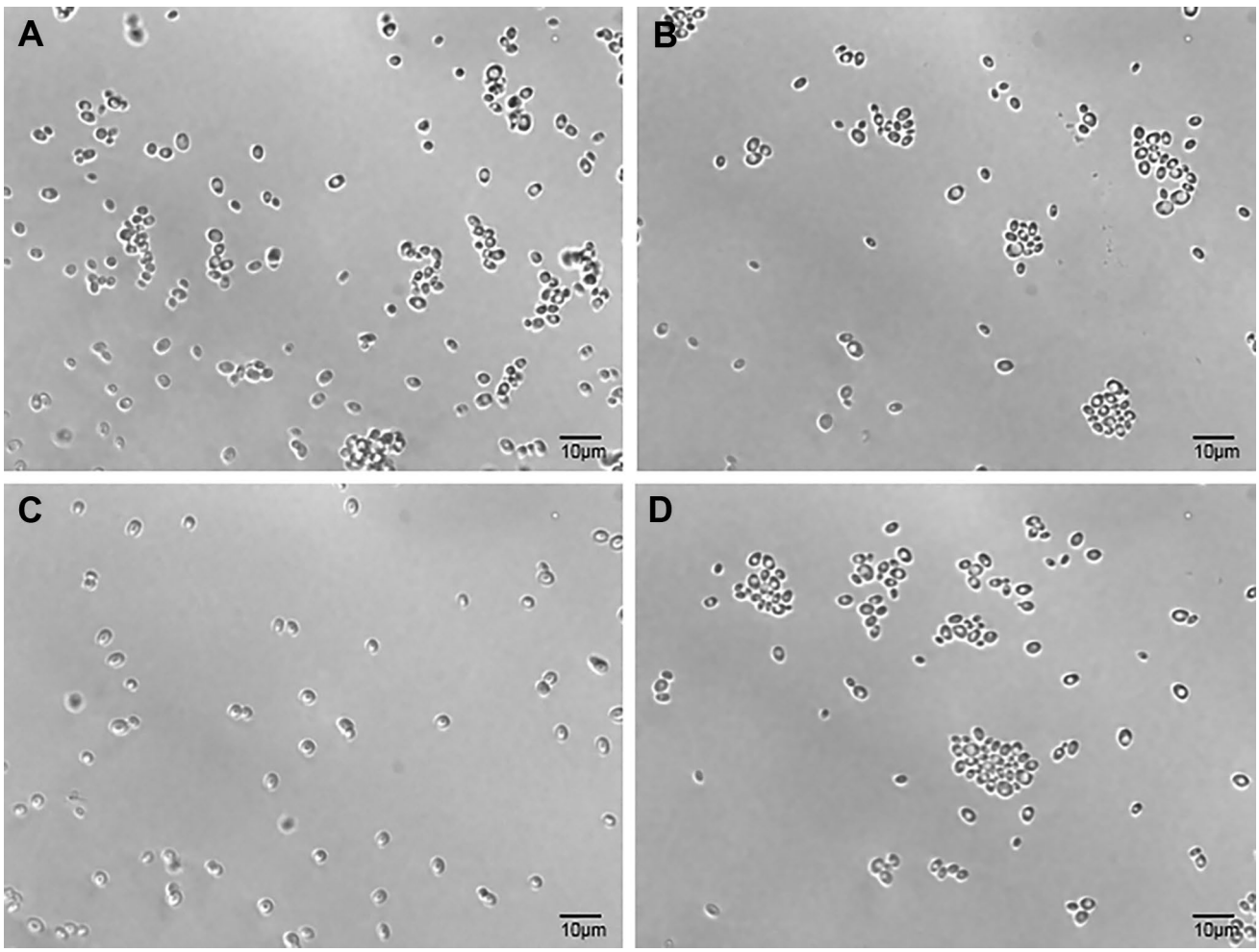

Fig. 3 Aggregation of *Candida glabrata* strains with probiotic yeast *Saccharomyces boulardii* after 24 h incubation at 37 °C and pH 7. Sample of microscope images (**A**) *C. glabrata* ZIM 2369; (**B**) *C. glabrata* ZIM 2369 + *S. boulardii*; (**C**) *C. glabrata* ZIM 2382; (**D**) *C. glabrata* ZIM 2382 + *S. boulardii*

characteristics of yeast strains used as probiotics (Suvarna et al. 2018; Ragavan and Das 2017). As a relationship between aggregation and adhesion ability has been reported in most cases (Balakrishna 2013; Collado et al. 2007; Kos et al. 2003), the current study was based on our previous finding that probiotic *S. boulardii* decreased *C. glabrata* adhesion (Tomičić et al. 2016a). With this in mind, we investigated co-aggregation of *S. boulardii* with *Candida* strains as well as their auto-aggregation ability under different culture conditions such as temperature and pH after 5 h and 24 h.

The evaluation of aggregation in this study revealed that all tested yeasts had the ability to aggregate, which increased significantly over a period of 24 h for all combinations of probiotic and *Canida* strains. It was evident that *C. albicans* and *C. krusei* followed by probiotic strain *S. boulardii* showed the highest degree of auto-aggregation, whereas the lowest was noted in *C. glabrata* strains. This observation is supported by reports about favorable aggregation abilities in yeast strains (Ogunremi et al. 2015; Sourabh et al. 2011). When probiotic yeast was co-incubated with *Candida* strains, the aggregation of *C. albicans* ATCC 10261, *C. krusei* ATCC 6258, and *C. glabrata* ZIM 2369 was reduced (Figs. 1 and 2). The photographs of sample microscope preparations also confirm this effect of *S. boulardii* analyzed for *C. glabrata* ZIM 2369 strain using the microscope with camera (Leica DFC290) with ×40 magnification as shown in the Fig. 3. We could speculate that cells of probiotic strain aggregate with pathogens entrap them and in this manner interrupt aggregation. Many studies have shown that the ability of *S. boulardii* to bind bacterial pathogens has been associated with the presence of mannan and polysaccharides on the outer layer of yeast cell wall (Badia et al. 2012; Gedek 1999; Tiago et al. 2012). One of the possible roles for *S. boulardii* in managing pathogenic activity may also be related to the production of compounds with antimicrobial activity (Offei et al. 2019; Pais et al. 2020). *S. boulardii* appears to secrete an active compound such as capric acid which inhibits filamentation and partially adhesion and biofilm formation of *C. albicans* (Murzyn et al. 2010). On the other hand, our results indicate that the interaction between *C. glabrata* ZIM 2382 and *S. boulardii* happened differently (Fig. 3). The effect was even synergistic suggesting that co-aggregation is strongly strain dependent what is in agreement with our previous finding (Tomičić et al. 2016a) where the adhesion of the same strain was significantly stimulated. It is well known that co-aggregation is a recognized feature of the early biofilm formation which is tightly controlled by specific cell surface-associated receptor–ligand interactions, and these often result in increased levels of multispecies biofilm formation (Jørgensen et al. 2017; Trunk et al. 2018). Despite the contradiction with the discussion above, in this case, we could conclude that *S. boulardii* is able to support the growth of some organisms. Nevertheless, considering both observations, further studies are needed to examine which mechanisms are responsible for the different actions of probiotic.

The effect of different culture conditions such as temperature and pH on co-aggregation of *S. boulardii* with *Candida* strains as well as their auto-aggregation was also tested. These factors affected the aggregation ability in a different way only after 5 h of incubation, but had no effect after 24 h. We noticed two types of cell response to different temperatures; the strains *C. krusei* and both *C. glabrata* were aggregated to the highest extent at 37 °C, while *C. albicans* showed different behavior with a much greater propensity for aggregation at 42 °C, as in mono- and in co-culture with *S. boulardii* (Fig. 1). The result is not surprising, taking into account that these yeasts can adhere to abiotic surfaces over a wide range of temperatures as we reported (Tomičić and Raspor 2017; Tomičić et al. 2016a). This signifies that the ability to grow and aggregate in extreme conditions such as unfavorable high temperature (42 °C) characteristic for the fever of the host is also an important virulence trait of pathogenic *Candida* strains. Additionally, in the process of digestive tract infection, adaptation to a variable pH is a requirement for the survival of many pathogens, including basic pH in the mouth, acid pH in the stomach, and neutral pH in the large intestine (Madigan et al. 1997; Zupan and Raspor 2010). For *C. albicans*, it is known that the pH of the infection site regulates the expression of genes necessary for survival in that niche (De Bernardis et al. 1998; Peñalva and Arst 2002). To our best knowledge, nothing is known regarding the effect of *S. boulardii* cells on the aggregate properties of *Candida* strains at acidic or basic pH. If the mono- and co-cultures from our experiments are considered, uniform conclusions could be reached that in all cases tested yeasts were able to aggregate at all pH values, but with significant preference in a more acidic environment (Fig. 2). However, noteworthy is the fact that probiotic *S. boulardii* has ability to reduce aggregation of *Candida* spp. and thus can compensate the balance of the microenvironment of the intestinal tract and boost the host's immune system.

Conclusion

We clearly showed that probiotic *S. boulardii* exhibited the potential to inhibit the aggregation of the most common *Candida* species such as *C. albicans*, *C. krusei*, and *C. glabrata* what is of crucial importance for theoretical understanding of this phenomena, but can also have very influential impact on development of yeast probiotics. However, in partnership with one *C. glabrata* strain, *S. boulardii* had a significant stimulative effect. These findings highlight that the impact of probiotic is strain-specific and opens up new

research interest in examining cell wall surfaces of tested strains, which may explain these differences and open possibilities for target-specific probiotics strains as food supplements or even as specific treatment when antimicrobials are not effective any longer. So, our results suggest that there is a potential benefit associated with the use of *S. boulardii* to treat *Candida* infections. Besides to the specificity of the strain, when culture conditions are considered, aggregation of yeasts was also affected by temperature and pH. However, for a comprehensive understanding of the interactions between probiotic *S. boulardii* and *Candida* strains in respect to a special therapeutic application of this probiotic, further studies are of crucial importance.

Authors contribution Ružica Tomičić performed experimental work, wrote the manuscript, and contributed in conception, acquisition, analysis and interpretation of the data. Zorica Tomičić contributed in experimental work, acquisition, and analysis of the data. Peter Raspor contributed in analysis and interpretation of the data and reviewed the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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