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Bioprospecting of the probiotic potential of yeasts isolated from a wine environment

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ABSTRACT

Autochthonous yeasts of oenological origin are adapted to highly stressful and selective environments, which makes them potential candidates for probiotics. The objective of the present study was to explore the probiotic potential of 96 native yeasts of oenological origin, their biosafety, resistance to gastrointestinal tract conditions and adhesion properties. Regarding biosafety, 66 isolates shown negative hemolytic activity, negative urease activity and susceptibility to 3 or more antifungals. After the gastrointestinal resistance test, 15 isolates were selected that showed growth at different temperatures, tolerance to low pH and the presence of bile salts in in vitro tests. In general, survival after simulated conditions of the gastrointestinal tract was high and more restrictive was the duodenal. The results of the adhesion properties showed highly variable hydrophobicity and a high percentage of autoaggregation at 24 h. The maximum production of biofilm was detected in the *Pichia* strains. Of a total of 96 yeast strains, 15 non-*Saccharomyces* yeasts presented suitable properties as probiotic candidates. The native winemaking strains performed better than the reference probiotic strain, *Saccharomyces cerevisiae* var. *boulardii* CNCM I-745, which reaffirms that these strains are promising probiotic candidates and further studies are necessary to confirm their probiosis.

1. Introduction

Yeasts are a broad and heterogeneous group of eukaryotic microorganisms belonging to ascomycetes and basidiomycetes, which are widely distributed and ubiquitous in the environment. They are often isolated from fruit microbiota, plant exudates, soil and insects (Tikka et al., 2013). Unconventional yeasts, also called non-*Saccharomyces* strains, represent an interesting industrial alternative for the development of new products (Holt et al., 2017; Steensels et al., 2014). The yeasts are used in biotechnology for different purposes such as production of fermented foods and beverages, synthesis of recombinant proteins and vitamins, and biological control (García-Hernández et al., 2012; Hatoum et al., 2012). Particularly in the food industry, non-*Saccharomyces* strains can be used as starters in the production of various foods and beverages such as bread, wine, beer, kefir, koumiss and table olives (Arroyo-López et al., 2012; Moreira et al., 2011) and they are also involved in the ripening of some cheeses (Binetti et al., 2013).

A new trend in food microbiology is the use of multifunctional microorganisms such as starter cultures with probiotic activity (Perricone et al., 2014). Probiotics are viable, biologically active microorganisms that, when administered in adequate amounts, transmit a beneficial effect to host health (FAO / WHO, 2006). They should meet the following criteria: they must be safe, and thus not cause toxicity or disease to the host, they must be administered in large quantities and must be able to persist and multiply in the gastrointestinal tract (GIT). Hence, they must be resistant to gastric acid juice, basic pancreatic juice, lysozyme and bile salts. Besides, they should have the ability to self-aggregate and form a normal supporting microbiota and they must be technologically usable (Hill et al., 2014; Gut et al., 2018).

The most common probiotic species belong to the *Lactobacillus* and

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Bifidobacterium bacterial genera. Regarding yeasts, *Saccharomyces cerevisiae* var. *boulardii* (*Saccharomyces boulardii*) is only supported by a regulatory framework and commercial acceptance and it is available in several human probiotic formula (di Cagno et al., 2020; Czerucka et al., 2007). Quarella et al. (2016) reported *Kluyveromyces marxianus fragilis* B0399 as the first non-*Saccharomyces* yeast approved as a probiotic for animal feed and human consumption according to EFSA (2004) and Ministero della Salute (2016). Therefore, it is of increasing interest to evaluate additional functional properties in previously approved yeasts for probiotic traits (Gil-Rodríguez et al., 2015).

Yeasts have certain advantages over bacteria such as -non-susceptibility to antibacterial treatments, -their larger size (at least 10 times larger, which could represent an important stearic hindrance for bacteria), -growth at low pH and, mainly, - transferring antibiotic resistance genes is extremely scarce (Czerucka et al., 2007; Daniali et al., 2020). In addition, yeasts can potentially support host health through several mechanisms, including stimulation of the immune system, induction of growth of other probiotics, degradation of bacterial toxins, and competition with pathogens for adhesion to gastrointestinal epithelial cells (Arevalo-Villena et al., 2017). It should be mentioned that several yeast species such as *Kluyveromyces lactis*, *K. marxianus*, *Issatchenkia orientalis*, *S. cerevisiae* and *Debaryomyces hansenii* have shown antifungal, antibacterial, anti-inflammatory and antitumor activity (Oh et al., 2002; Diniz et al., 2003; Lopitz-Otsoa et al., 2006). Furthermore, several studies have proposed the native yeast species *Wickerhamomyces anomalus*, *Candida famata*, *C. tropicalis*, *Kluyveromyces marxianus*, *K. lactis*, *Meyerozyma caribbica* and *Pichia kudriavzevii* as probiotic candidates (Amorim et al., 2018; (Arevalo-Villena et al., 2017; Greppi et al., 2017; Ogunremi et al., 2015). Other authors have reported the genera *Rhodotorula*, *Yarrowia* and *Torulaspora* as possible probiotics (Pereira et al., 2018).

Several species of different yeast genera are involved in winemaking, by transforming the grape must into wine. They are present both in the vineyard (mainly on the grape surface) and in the winery facilities, and they are especially characterized by their adaptation to hostile conditions given the highly selective nature of the winemaking process (Chambers et al., 2015; Marsit and Dequin, 2015). In contrast to beer and bread fermentation matrices, which are species-poor environments and which are more biotechnologically controlled, grape must is a dynamic and especially challenging habitat for yeasts. It has a high selection pressure which is both the result of physical conditions (osmolarity, low pH) and chemical parameters (limited nitrogen availability, high ethanol concentrations), and also attributable to the many different competing microbial species (Bauer and Pretorius, 2000; Kurtzman et al., 2011). The wine ecosystem provides an ecological niche in which biotic pressures, in the form of ecological interactions, significantly alter the evolutionary trajectory of participating yeasts (Conacher et al., 2019). Added to this, several species exhibit properties of hydrophobicity, exopolysaccharide production and/or biofilm formation, special characteristics that allow them to better adhere to the grape surface, in addition to creating a protective barrier against other microorganisms and/or adverse physicochemical factors (Renouf et al., 2005).Therefore, tolerance to hostile winemaking conditions (with some similarities to those of the gastrointestinal tract) along with structural and functional similarities to other conventional probiotics, make enological yeasts potential probiotic candidates to exert beneficial effects on human health.

The Cuyo region is traditionally recognized as the main wine producing area in Argentina. Several studies have been carried out to contribute to the growth and development of the industry in the region. Our research group has isolated several autochthonous yeast species throughout the winemaking process and during different vintages. These yeasts have been thoroughly characterized from an enological point of view and evaluated for various purposes (Kuchen et al., 2018, 2019; Maturano et al., 2015a,b; Mestre Furlani et al., 2017; Maturano et al., 2012). Importantly, microorganisms from these niches with extreme environmental conditions are able to exert selection pressure on other microbial populations in the same niche, so it is a challenge to evaluate them in other new scenarios. Consequently, these yeasts could be used for different purposes, which would make them multifunctional microorganisms.

The main objective of the present study was to explore the probiotic potential of autochthonous yeasts adapted to the wine environment by subjecting the yeasts to a series of in vitro analyses to evaluate: a) their biosafety; b) their resistance to conditions in the gastrointestinal tract; and c) their adhesion-related properties. These evaluations are part of a first step in the selection of yeasts, isolated from viticultural environments, to be used as probiotics.

2. Materials and methods

2.1. Microorganisms

The present study examined 96 yeasts isolated from environments associated with winemaking such as soil, grape clusters, and grape musts throughout different stages of spontaneous fermentations during 1992, 1994, 2004 and 2011 vintages (Cuyo region, Argentina).

The ascomycetes and basidyomycetes yeasts belonged to 25 different species (Table 1), included in the genera *Candida* (7 strains), *Cryptococcus* (3), *Debaryomyces* (3), *Hanseniaspora* (13), *Issatchenkia* (1), *Kluyveromyces* (5), *Metschnikowia* (12), *Pichia* (13), *Rodhotorula* (3), *Saccharomyces* (29), *Torulaspora* (2) and *Wickerhamomyces* (5). *Saccharomyces cerevisiae* var. *boulardii* CNCM I-745 was used as control. All strains were identified using conventional morphological, physiological and biochemical assays according to Kurtzman et al., (2011), and a molecular assay as described by Esteve-Zarzoso et al. (1999). All isolates belong to the Culture Collection of Autochthonous Microorganisms of the Institute of Biotechnology, School of Engineering – UNSJ, San Juan, Argentina.

The pathogens microorganisms *Pseudomona aeruginosa* ATCC® 27,853 and *Shigella sonei* ATCC® 9290 were used as positive controls for the evaluation of hemolytic and urease activity, respectively. These strains were provided by American Type Culture Collection ATCC (Rockville, MD, USA).

All microorganisms were cryogenically preserved at −80 °C. Yeasts were propagated in YEPD broth (g/L: Yeast extract 10, Peptone 20, Glucose 20) and bacteria in CN broth (g/L: Pluripeptone 5, meat extract, 3) for 24 h prior to experimental use.

2.2. First Screening: Biosafety assessment

2.2.1. Hemolytic activity

Yeast strains were spot-inoculated onto blood-agar medium, supplemented with 5 % defibrinated sheep blood, and incubated for 48 h at 37 °C (Lara-Hidalgo et al., 2019). Hemolytic activity was evidenced by the development of clear zones around the colonies. *Pseudomona aeruginosa* ATCC® 27,853 was used as positive control. Plates were examined for signs of α-hemolysis (green-hued zones around colonies) and β-hemolysis (clear zones around colonies) (Bonatsou et al., 2018).

2.2.2. Urease activity

Urease activity was measured using Christensen medium according to the method by Seeliger (1956) with modifications. The medium (urea base agar) consisted of the following basal components (g/L): peptone 10, glucose 10, sodium chloride 5, potassium dihydrogen phosphate 2, phenol red 0.012, agar 15; final pH was 6.8 ± 0.2. The medium (2.85 mL) was dispensed into test tubes and autoclaved for 15 min at 121 °C. Each tube of the autoclaved medium was supplemented with 0.15 mL of a 40 % filtered urea solution. The contents were allowed to solidify with the tubes held in tilted position.

An inoculum from previously activated yeasts on YEPD-agar (generally within 24 to 48 h and cultured at optimal growth

Table 1
Autochthonous yeast isolates and assigned nomenclature for the study.

Phylum	Species	Isolates	Nomenclature
Ascomycota	*Candida famata*	2	PB1, PB2
	Candida intermedia	1	PB3
	Candida membranaefaciens	2	PB4, PB5
	Candida sake	2	PB6, PB7
	Cryptococcus albidus	1	PB8
	Cryptococcus magnus	2	PB9, PB10
	Debaryomyces hansenii	1	PB11
	Debaryomyces vanrijiae	2	PB12, PB13
	Hanseniaspora sp	1	PB14
	Hanseniaspora guilliermondii	3	PB15, PB16, PB17
	Hanseniaspora uvarum	6	PB18, PB19, PB 20, PB21, PB22, PB23
	Hanseniaspora vineae	3	PB24, PB25, PB26
	Issatchenkia orientalis	1	PB27
	Kluyveromyces sp	2	PB28, PB29
	Kluyveromyces lactis	2	PB30, PB31
	Kluyveromyces maxianus	1	PB32
	Metschnikowia pulcherrima	12	PB33, PB34, PB35, PB36, PB37, PB38, PB39, PB40, PB41, PB42, PB43, PB44
	Pichia fabiani	1	PB45
	Pichia kluyveri	2	PB46, PB47
	Pichia kudriavzevii	6	PB48, PB49, PB50, PB51, PB52, PB53
	Pichia manshurika	1	PB54
	Pichia occidentalis	3	PB56, PB57, PB58
	Saccharomyces cerevisiae	29	PB62, PB63, PB64, PB65, PB66, PB67, PB68, PB69, PB70, PB71, PB72, PB73, PB74, PB75, PB76, PB77, PB78 PB79, PB80, PB81, PB82, PB83, PB84, PB85, PB86, PB87, PB88, PB89, PB90
	Torulaspora delbrueckii	2	PB91, PB92
	Wickerhamomyces anomalus	5	PB93, PB94, PB95, PB96, PB97
Basidiomycota	*Cryptococcus albidus*	1	PB8
	Cryptococcus magnus	2	PB9, PB10
	Rhodotorula glutinis	2	PB59, PB60
	Rhodotorula mucilaginosa	1	PB61
	Total	96	

temperature) was striated with a loop on the surface of the medium in slant culture. Tubes were then incubated at 37 °C for 18–24 h. Hydrolysis of urea was shown by a distinctive color change of the indicator from yellow orange to deep pinkish red that began in the sloping middle part and rapidly progressed to the deep part.

2.2.3. Antibiotic susceptibility

Sensitivity to the following antibacterial agents (μg/mL): ampicillin 25, gentamicin 10, levofloxacin 10, rifampicin 20, tetracycline 80, chloramphenicol 60; and antifungals (μg/mL): ketoconazole 50, clotrimazole 10, fluconazole 10, itraconazole 10, miconazole 50, nystatin 100, was assessed. Each yeast, previously activated, was inoculated ($1x10^7$ CFU/mL) on YEPD-agar to form a lawn. Wells (≈ 5.5 mm) were punched in the agar and 50 μl of each antibiotic were added to the wells. Subsequently, plates were incubated for 48 h at 37 °C, according to Amorim et al. (2018) with changes. Sensitivity was detected by the formation of inhibition halos around the well and quantified with a digital caliper. Isolates that were biosafe were selected for subsequent assays.

2.3. Second Screening: Resistance to the gastrointestinal tract (GIT)

2.3.1. Tolerance to temperature

Yeasts, previously activated in YEPD broth, were inoculated in a physiological solution (NaCl 0.9 g/L) and shaken at 100 rpm for 48 h at 28 °C to deplete nutritional reserves. For this assay, the methodology proposed by Gut et al., (2018), with modifications, was used. Yeasts were spot inoculated onto YEPD agar and incubated at 37 °C (internal body temperature), 39 and 42 °C (fever states) for 48 h. Yeast growth was determined by comparison with a control at 25 °C (Ragavan and Das, 2017).

2.3.2. Tolerance to low pH

Previously activated yeasts ($1x10^6$ CFU/mL) were inoculated in YEPD broth at pH 2, 3 and 4.6 (control), and incubated at 100 rpm for 4 h at 37 °C. Yeasts were then inoculated on YEPD-agar after 0 and 4 h. Viable cell counts (CFU/mL) were performed after 48 h at 30 °C. Survival was determined as follows:

$$\%\text{survival} = \left(\frac{log\ n^o\ final\ CFU/mL}{log\ n^o\ initial\ CFU/mL}\right)*100$$

Where:

log n° initial CFU/mL is the natural logarithm of initial number colonies forming units
log n° final CFU/mL is the natural logarithm of final number colonies forming units

2.3.3. Bile tolerance

Bile tolerance was determined according to the modified ecometric method proposed by Kociubinski et al. (1999). The assay was performed by making 5 consecutive streaks on YGC-agar (g/L: Yeast extract 5, D (+) glucose 20, chloramphenicol 0.1, agar 20) (positive control) and YGC-agar + 1 % bile, using a calibrated 5 μl platinum wire loop without reloading or flame sterilization. Plates were incubated for 48 h at 37 ± 1 °C under anaerobic conditions. Growth was recorded for each streak. The degree of resistance was quantified by assigning a numeric value to positive growth in each streak on YGC agar-bile (1 for microbial growth in the first streak, 2 in the second streak, 3 in the third streak, 4 in the fourth streak, and 5 in the fifth streak). Absence of growth on YGC-bile was recorded as zero. The bile resistance index, RI_{bile}, was calculated as the sum of the values assigned to the five streaks. RI_{bile} ranged from 0 to 15, where the maximum value indicates a high degree of bile resistance.

Isolates showing biosafety and growth under conditions present in the GI tract were selected for subsequent assays.

2.4. Third Screening: Adhesion-related properties

2.4.1. Hydrophobicity

The hydrophobic capacity of yeasts was indirectly evaluated as the ability of cells to bind xylene. The assays were carried according to Gong et al. (2012), and was modified as follows: previously activated yeasts were harvested, centrifuged at 4000 rpm for 10 min and washed 3 times with PBS. The supernatant was removed and the pellet was resuspended in PBS. Tubes were prepared in triplicate with a final volume of 9 mL of each yeast at a concentration of $5x10^7$ CFU/mL and OD was measured at 600 nm. The hydrocarbon was subsequently added to the suspension at a ratio of 1 (xylene) to 3 (yeast suspension). Samples were shaken for 2 min and left under static conditions for 1 h at 37 °C; the ability of xylene to trap cells was measured. The hydrophobicity index (HI) was calculated as described by Collado et al. (2008):

$$\text{HI}\ (\%) = \left(\frac{A_0 - A}{A_0}\right)*100\%$$

where A_0 and A are the absorbance before and after extraction with organic solvents, respectively.

2.4.2. Autoaggregation

Autoaggregation was performed according to Ogunremi et al. (2015) with modifications. Yeasts were grown in YEPD broth for 24 h at 37 °C at 150 rpm, harvested by centrifugation at 3000 rpm at 4 °C for 15 min and washed twice with PBS. Cells were resuspended in PBS at a concentration of $5x10^7$ CFU/mL with a final volume of 30 mL in 50 mL-falcon tubes, subsequently vortexed for 10 s and incubated at 37 °C. Absorbance was measured at 600 nm after 0, 2, 4 and 24 h by extracting the sample and withdrawing it from the upper suspension layer (2.5 mL). Autoaggregation was calculated as follows:

$$\mathrm{Au}(\%) = 1 - \left(\frac{A_t}{A_i}\right) * 100\%$$

where A_t is the absorbance over time and A_i is the initial absorbance.

2.4.3. Biofilm formation

Biofilm formation was evaluated following the protocol by Růžička et al. (2007) with modifications. Yeasts previously activated were suspended in water until reaching a concentration of $1x10^6$ CFU/mL. A volume of 200 µl of each YEPD broth yeast suspension (37 °C, 28 h) was added to a 96-well microplate. YEPD broth without yeast inoculation was used as a negative control. After the incubation period, the medium was removed and the wells were rinsed 2 times with PBS and left to dry. The biofilm layer adhered to the walls of the well was stained with 200 µl of a 1 % crystal violet solution for 30 min, and rinsed twice with distilled water. The attached dye was eluted with 200 µl of 96 % ethanol. 100 µl of the solution was placed on a new microplate and the absorbance of each well was measured at 620 nm. Biofilm formation was confirmed when the absorbance was greater than the negative control.

2.5. Simulated gastrointestinal passage

Simulations of digestion under gastric (GD) and pancreatic (PD) conditions were performed in vitro using the method described by Zullo et al. (2019) with modifications. For GD, a synthetic gastric juice was prepared in a buffer solution at pH 2 containing (g/L): NaCl 2.05, KH_2PO_4 0.60, $CaCl_2$ 0.11 and KCl 0.37. After sterilization of the medium, 0.0133 g/L filter-sterilized (0.22 µm) pepsin was added to complete the gastric solution. Yeast cultures were activated in YEPD broth for 48 h at 30 °C. The supernatant was separated by centrifugation at 4600 rpm for 10 min and the pellet washed twice with a sterile PBS solution. Subsequently, yeast cells were resuspended in 7 mL of the gastric solution, and the cell concentration was adjusted to approximately $1x10^8$ CFU/mL. The gastric solution was incubated for 2.5 h at 37 °C under constant shaking at 100 rpm to simulate peristaltic movements. Samples were taken after 0, 75 and 150 min, diluted and then seeded onto a plate for subsequent viable cell counting (CFUs).

PD simulation was performed using a buffer solution with pH 7.0 (adjusted with 1 M HCl) with the following formula: bile salts (3.0 g/L), filter-sterilized pancreatin (0.1 g/L), Na_2HPO_4 (26.9 g/L) and NaCl (8.5 g/L). Cells harvested during the previous GD step were centrifuged at 4600 rpm for 5 min, and then resuspended in 7 mL of the same solution. The suspension was incubated for 3 h at 37 °C with shaking (100 rpm). Similarly, samples were taken after 0, 90 and 180 min, diluted and seeded onto a plate for subsequent viable cell counting (CFUs). Survival during the PD and GD for each sample point was determined as follows.

$$\%\mathrm{survival} = \left(\frac{log\ n^o\ final CFU/mL}{log\ n^o\ initial CFU/mL}\right) * 100$$

Where:

log n° initial CFU/mL is the natural logarithm of initial number colony forming units

log n° final CFU/mL is the natural logarithm of final number of colony forming units

2.6. Data analysis

Each assay was performed independently in triplicate and the results represent the average of the three determinations with the corresponding standard deviation (±SD). Experimental data were processed with analysis of variance (ANOVA), the Shapiro-Wilk test was used for assumption of normality and Levene's test for homogeneity of variance. Significant differences were determined using the Tukey test and the results were considered significant if the associated p value was < 0.5. To analyze non-parametric data, the Kruskal-Wallis test or the Friedman test equivalent to the ANOVA test for repeated measures were used. SPSS version 19.0 and Statistics R 3.6.2 were used for all tests.

3. Results and discussion

3.1. First screening: Biosafety assessment

None of the yeast strains evaluated in the present study were susceptible to antibacterials or showed hemolytic activity. As for urease activity, 9.3 % (PB 1, 4, 24, 25, 61, 70, 71, 73, 81) of the total isolates were positive and were therefore withdrawn from the study. Regarding antifungal susceptibility, 61.45 % of the isolates were sensitive to at least 3 agents. The most effective antifungal was nystatin, with 97.89 % of sensitive isolates, followed by miconazole, with 93.68 % of sensitive strains. Fluconazole was the least effective antifungal, with only 26.31 % of isolates showing sensitivity. Isolates with sensitivity to all azoles tested were PB 6, 9, 21, 31, 51, 58. while PB 13 and 77 isolates were the most resistant (to>4 azoles). In general, *M. pulcherrima* isolates were the most susceptible (to 5 or more of the antifungals). On the other hand, most *Saccharomyces* isolates were resistant to at least 4 antifungal agents. The results indicate that the tested yeasts showed a wide variability with respect to antifungal susceptibility and resistance, indicating a strain-dependent characteristic (Fig. 1).

Biosafety testing is crucial because any yeast isolate may be a potential pathogen, especially against immunocompromised individuals (Fleet and Balia, 2006). Most probiotic microorganisms are bacteria, so probiotic yeasts could present an advantage for use in patients treated with antibacterials, since their viability and probiotic properties would remain intact. Furthermore, transfer of genetic material between bacteria and yeasts is a fairly rare event, making yeasts safer for use during antibiotic treatment ((Czerucka et al., 2007; Lara-Hidalgo et al., 2017; Daniali et al., 2020). Several studies have focused on the probiotic potential of isolates of the genera Candida, Pichia, *Wickerhamomyces* and *Saccharomyces* (Chelliah et al., 2016; di Cagno et al., 2020). However, some authors have raised doubts about the safety of certain species of these genera because they could be opportunistic pathogens responsible for morbidity and mortality in immunocompromised individuals (de Llanos et al., 2006; Fleet and Balia, 2006). Therefore, in the present study we examined the susceptibility of yeasts to clinically relevant broad-spectrum antifungals to control a potential probiotic yeast infection with these antifungal agents. The genus *Saccharomyces* is one of the most favored genera for the search of possible probiotic candidates in many studies. However, our results show that it is the genus with the highest number of strains with a high number of antifungal resistance. Lim et al.,(2019) reported that reduced susceptibility to most azole agents, especially fluconazole, may be inherent to the genus *Saccharomyces*.

A total of 66 isolates showed the desired biosafety characteristics: negative hemolytic activity, negative urease activity, antibacterial resistance and susceptibility to 3 or more antifungals. Therefore, these isolates were used for further screening.

3.2. Screening: Resistance to the gastrointestinal tract (GIT)

For initial screening, yeast tolerance to human body temperature and feverish state, low pH values and the presence of bile salts were

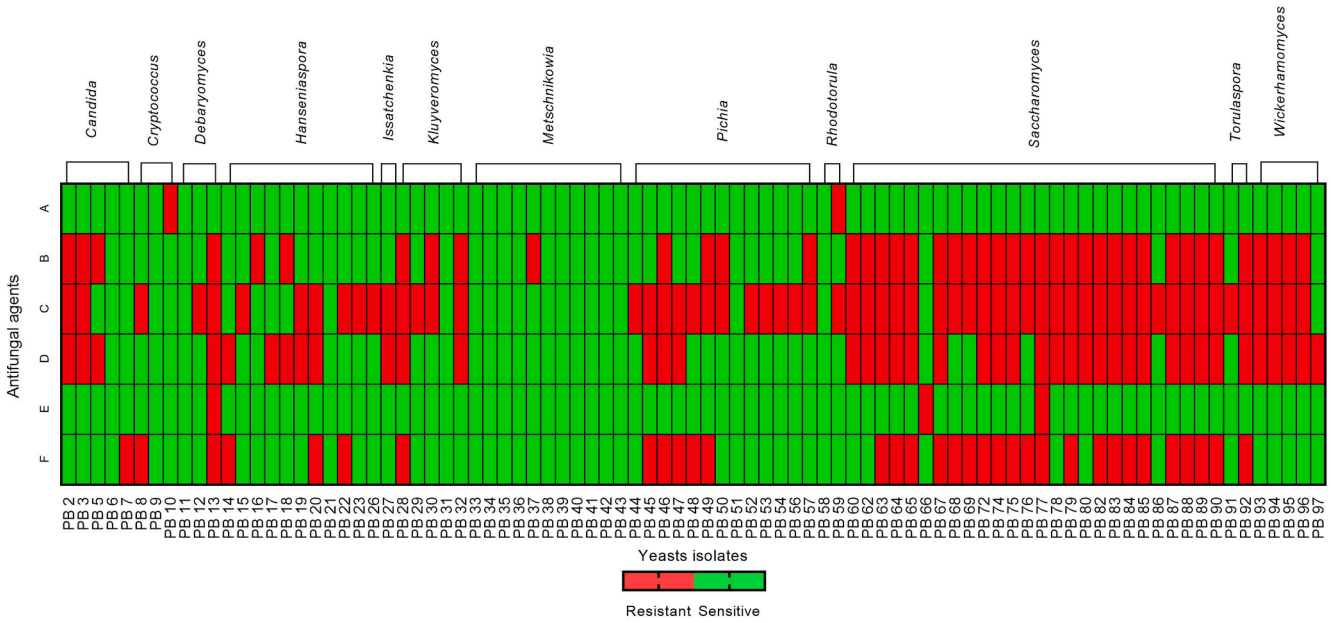

Fig. 1. Resistance and sensitivity of yeast isolates tested to different antifungals, Nystatin (A), Intraconazole (B), Fluconazole (C), Clotrimazole (D), Miconazole (E), Ketoconazole (F).

considered.

Of the 66 native yeasts tested, 49 isolates (74.24 %) showed favorable growth at 37 °C, 51 isolates (77.27 %) at 39 °C and 47 isolates (71.21 %), at 42 °C (Fig. 2). *Cryptococcus* and *Rhodotorula* isolates did not grow at any of the temperatures tested, while yeasts of the *Metschnikowia* genus showed increasing sensitivity with increasing temperature. Isolates of the genera *Pichia, Saccharomyces, Torulaspora* and *Wickerhamomyces* followed similar growth patterns within the same genus (Fig. 2).

It is well known that some wine-related non-*Saccharomyces* yeasts grow better at low temperatures, possibly due to the lipid composition of the membrane and the activity of transmembrane proteins (Torija et al., 2003; Maturano et al., 2019). Fortunately, several genera such as *Saccharomyces, Pichia, Debaryomyces, Wickerhamomyces* and *Candida* have a broader temperature spectrum (Fig. 2). During the tumultuous phase of red wine fermentation, temperatures can reach values of around 37–40 °C (García-Ríos et al., 2019). At these temperatures, yeast cells activate the heat shock response (HSR) and alter some other components of their physiology, including membrane composition and carbohydrate flux (Morano et al., 2012; Lindquist, 1986; Richter et al., 2010; Pereira et al., 2018). These changes, typical of oenological yeasts, added to the fact that the isolates come from warm areas where temperatures during grape ripening reach 46–47 °C, could respond to the good thermotolerance of the yeasts in this study. Psomas et al. (2001) evaluated the growth of *Saccharomyces, Candida, Issatchenkia, Debaryomyces, Kluyveromyces* and *Torulaspora* yeasts isolated from infant feces and feta cheese at different temperatures. All the isolates from infant feces grew at 25, 37 and 42 °C. Forty-four of the 50 yeasts isolated from feta cheese grew at 25 and 37 °C, and 43 of them even at 42 °C. Ogunremi et al.,(2015) conducted thermotolerance studies on strains of *Pichia kluyveri, P. kudriavzevii, Issatchenkia orientalis* and *Candida tropicalis* isolated from some Nigerian traditional foods, which showed

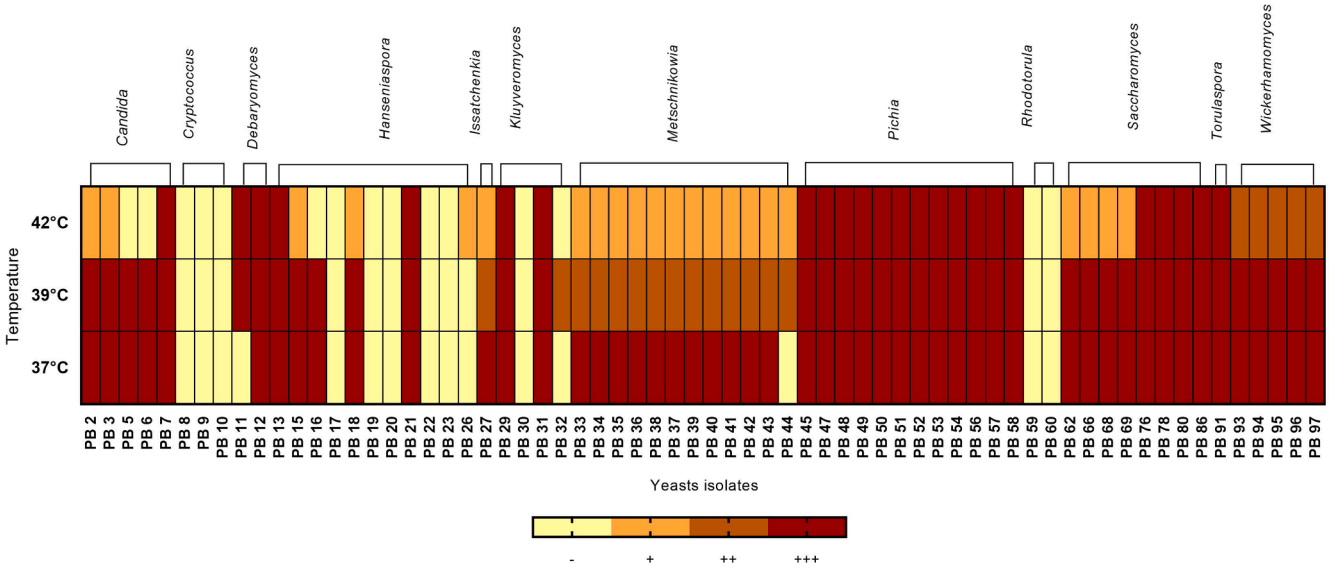

Fig. 2. Yeast isolates tolerant to different growth temperatures (37, 39 and 42 °C). No growth (-); with growth (+); with good growth (++); with very good growth as control (+++).

favorable growth at 37 °C. Menezes et al.,(2020) isolated yeasts from a fermentation of cocoa beans and Brazilian kefir belonging to the genera *Kluyveromyces, Saccharomyces, Pichia, Rhodotorula, Candida* and *Hanseniaspora,* which showed good growth at 37 °C. These genera showed comparable growth in the present study.

For a microorganism to be considered probiotic, it must reach the large intestine viable and functional. Therefore, strains must survive the natural barriers present in the gastrointestinal system. Existing records indicate that the ability of probiotic yeasts to survive the gastrointestinal tract is variable and depends on the strain ((Binetti et al., 2013; Fadda et al., 2017). Although many authors only evaluated growth at 37 °C (Syal & Vohra, 2012; Agarbati et al., 2020;) and taking into account that elevated temperatures can represent a limiting factor for yeast proliferation (Gil-Rodríguez et al., 2015; Romero-Gil et al., 2013), we believe it is important to select probiotic yeast candidates that are efficient, and tolerate temperatures between 39 (human high fever state) and 42 °C (temperature considered hyperpyrexia, associated with an increased risk of severe bacterial infection, especially in young infants) (Rosenfeld-Yehoshua, 2018), in order to perform under a febrile state, a common symptom of diseases associated with the gastrointestinal tract. At this stage, 47 yeasts were selected because they grew favorably at 3 temperatures.

After incubation at 37 °C for 4 h, viable yeast counts generally indicated that isolates had higher tolerance at pH 3 and higher sensitivity at pH 2. Isolates PB 52, 56 and 68 at pH 2 and isolates PB 7, 29, 45, 47–53, 56–58, 66, 69, 86, 91 and 97 at pH 3 had percentage tolerance exceeding 100 %. While strains PB 11–16, 52 and 56 at both pH had percentage tolerance exceeding 100 % (Table 2). It is well known that these species can grow at different external pH values and salt concentrations due to the fluidity and lipid composition of their plasma membranes (Turk et al., 2007). Isolates belonging to *P. kluyveri, P. kudriadzevii* and *W. anomalus* also showed good tolerance to acidic pH, as previously reported by García-Hernández et al., (2012). This was expected, as this acidity is common in winemaking. On the other hand, Menezes et al.,(2020) reported that *Kluyveromyces, Saccharomyces, Pichia, Rhodotorula, Candida* and *Hanseniaspora* yeasts isolated from Brazilian indigenous fermented foods, cocoa bean fermentation and kefir showed good growth at pH 2, which differs from our results as most isolates of these genera were sensitive to this condition.

An important barrier encountered by microorganisms after ingestion is gastric juice, where the inhibitory effect is strictly related to pH and hydrochloric acid concentration (Psomas et al., 2001). The pH of HCl excreted in the stomach is 0.9, but with the presence of food the pH increases to about 3, the average transit time of food in the stomach being 2 to 4 h (Erkkila and Petaja, 2000). The presence of food or other food components could buffer the ingested probiotics, which confers a certain protective effect on the microbial cells in the stomach (Conway et al., 1987; Prasad et al., 1998). The resistance of microorganisms to the acidity of gastric juice is fundamental for the selection of probiotics. Therefore, pH values of 2 and 3 were considered satisfactory for the selection of acid-resistant yeast strains.

The bile tolerance assays revealedthat 39.39 % (26/66) grew in the presence of 1 % bile salt with a tolerance index of $RI_{bile} = 15$ (corresponding to growth equal to 5 streaks in the treatment compared to the control). 19.64 % (13/66) presented a $RI_{bile} = 10$ (growth in 4 of the 5 striations in the treatment). The species with the highest number of resistant isolates ($RI_{bile} \geq 10$) were *W. anomalus* (5/5: 100 %), *P. kluyveri* (2/2: 100 %), *P. occidentalis* (3/3: 100 %), *P. kudriadzevii* (6/6: 100 %), *M. pulcherrima* (9/12: 75 %) and *Cryptococcus* (2/3: 66.6 %). In contrast, isolates of *Hanseniaspora* (5/12: 41.66 %), *Candida* (2/5: 40 %), *Saccharomyces cerevisiae* (1/8: 12.5 %) and *Kluyveromyces* (0/4: 0 %) showed little or no resistance ($RI_{bile} < 10$; Table 3). These results are in agreement with other authors who reported strains of *P. kudriavzevii, W. anomalus* and *P. kluyveri* resistant to 0.5 % ox bile (García-Hernández et al., 2012; Chelliah et al., 2016; Fernandez Pacheco et al., 2018). Diosma et al.,(2014) and Moradi et al.,(2018) reported isolates of

Table 2
Tolerance of isolates of autochthonous yeasts to pH 2 and pH 3.

Species	Isolate	% tolerance pH 2	pH3
Candida famata	PB 2	0.00 %	39.13 %
Candida intermedia	PB 3	27.77 %	95.41 %
Candida sake	PB 5	0.00 %	55.24 %
Candida sake	PB 6	1.12 %	11.24 %
Candida sake	PB 7	22.76 %	197.03 %
Cryptococcus albidus	PB 8	0.00 %	0.00 %
Cryptococcus magnus	PB 9	0.00 %	0.00 %
Cryptococcus magnus	PB 10	0.00 %	0.00 %
Debaryomyces hansenii	**PB 11**	**314.06 %**	**410.94 %**
Debaryomyces vanrijiae	**PB 12**	**116.48 %**	**169.23 %**
Hanseniaspora sp.	**PB 13**	**218.18 %**	**262.12 %**
Hanseniaspora guilliermondii	**PB 15**	**195.70 %**	**219.35 %**
Hanseniaspora guilliermondii	**PB 16**	**501.30 %**	**192.21 %**
Hanseniaspora uvarum	PB 17	0.00 %	0.00 %
Hanseniaspora uvarum	PB 18	44.54 %	73.11 %
Hanseniaspora uvarum	PB 19	1.79 %	0.00 %
Hanseniaspora uvarum	PB 20	0.00 %	0.00 %
Hanseniaspora uvarum	PB 21	0.00 %	5.00 %
Hanseniaspora uvarum	PB 22	0.00 %	0.00 %
Hanseniaspora uvarum	PB 23	0.00 %	0.00 %
Hanseniaspora vineae	PB 26	0.00 %	0.00 %
Issatchenkia orientalis	PB 27	13.16 %	10.53 %
Kluyveromyces sp.	PB 29	5.41 %	186.49 %
Kluyveromyces lactis	PB 30	0.00 %	0.00 %
Kluyveromyces lactis	PB 31	15.91 %	52.27 %
Kluyveromyces maxianus	PB 32	0.00 %	4.00 %
Metschnikowia pulcherrima	PB 33	0.00 %	0.00 %
Metschnikowia pulcherrima	PB 34	4.84 %	3.23 %
Metschnikowia pulcherrima	PB 35	3.17 %	3.17 %
Metschnikowia pulcherrima	PB 36	4.44 %	0.00 %
Metschnikowia pulcherrima	PB 37	1.33 %	5.33 %
Metschnikowia pulcherrima	PB 38	20.27 %	60.81 %
Metschnikowia pulcherrima	PB 39	2.25 %	8.99 %
Metschnikowia pulcherrima	PB 40	14.52 %	25.81 %
Metschnikowia pulcherrima	PB 41	1.43 %	13.57 %
Metschnikowia pulcherrima	PB 42	1.75 %	43.86 %
Metschnikowia pulcherrima	PB 43	1.30 %	71.43 %
Metschnikowia pulcherrima	PB 44	0.00 %	0.00 %
Pichia fabiani	PB 45	11.29 %	117.74 %
Pichia kluyveri	**PB 47**	**79.22 %**	**188.31 %**
Pichia kudriavzevii	**PB 48**	**53.74 %**	**284.62 %**
Pichia kudriavzevii	**PB 49**	**10.39 %**	**462.34 %**
Pichia kudriavzevii	**PB 50**	**49.38 %**	**642.22 %**
Pichia kudriavzevii	**PB 51**	**47.99 %**	**259.02 %**
Pichia kudriavzevii	**PB 52**	**363.49 %**	**323.08 %**
Pichia kudriavzevii	**PB 53**	**63.01 %**	**550.00 %**
Pichia masmurika	**PB 54**	**47.99 %**	**50.62 %**
Pichia occidentalis	PB 56	178.00 %	110.89 %
Pichia occidentalis	**PB 57**	**69.70 %**	**246.46 %**
Pichia occidentalis	**PB 58**	**96.10 %**	**320.00 %**
Rhodotorula glutinis	PB 59	0.00 %	0.00 %
Rhodotorula glutinis	PB 60	0.00 %	20.69 %
Saccharomyces cerevisiae	PB 62	10.98 %	71.34 %
Saccharomyces cerevisiae	PB 66	16.92 %	221.54 %
Saccharomyces cerevisiae	PB 68	184.43 %	43.44 %
Saccharomyces cerevisiae	PB 69	5.26 %	185.26 %
Saccharomyces cerevisiae	PB 76	5.48 %	57.53 %
Saccharomyces cerevisiae	PB 78	4.71 %	76.47 %
Saccharomyces cerevisiae	PB 80	0.00 %	26.63 %
Saccharomyces cerevisiae	PB 86	31.15 %	104.92 %
Torulaspora delbrueckii	PB 91	29.19 %	153.33 %
Wickerhamomyces anomalus	PB 93	0.00 %	38.46 %
Wickerhamomyces anomalus	PB 94	0.00 %	22.12 %
Wickerhamomyces anomalus	PB 95	0.00 %	65.88 %
Wickerhamomyces anomalus	PB 96	0.00 %	56.57 %
Wickerhamomyces anomalus	PB 97	65.12 %	80.58 %
Saccharomyces cerevisiae var. *boulardii* CNCM I-745	***Sb***	21.36 %	50.49 %

Reference: ***Sb*** is reference strain. Isolates with remarkable growth are highlighted in bold.

Table 3
Yeast isolates showing bile resistance (RI_{bile} = 10 or 15) in the presence of bile salts and the corresponding Bile Resistance Index (RI_{bile}).

Especie	Isolate	RI_{bile}	Especie	Isolate	RI_{bile}
Candida famata	Pb 1	IR 15	*Candida intermedia*	Pb 3	IR 10
Candida sake	Pb 7	IR 15	*Candida membranefaciens*	Pb 4	IR 10
Hanseniaspora sp	Pb 14	IR 15	*Cryptococcus albidus*	Pb 8	IR 10
Hanseniaspora uvarum	Pb 22	IR 15	*Cryptococcus magnus*	Pb 10	IR 10
Hanseniaspora vineae	Pb 26	IR 15	*Hanseniaspora guilliermondii*	Pb 15	IR 10
Issatchenkia orientalis	Pb 27	IR 15	*Hanseniaspora uvarum*	Pb 18	IR 10
Kluyveromyces sp	Pb 28	IR 15	*Hanseniaspora uvarum*	Pb 19	IR 10
Metschnikowia pulcherrima	Pb 33	IR 15	*Metschnikowia pulcherrima*	Pb 37	IR 10
Metschnikowia pulcherrima	Pb 35	IR 15	*metschnikowia pulcherrima*	Pb 38	IR 10
Metschnikowia pulcherrima	Pb 36	IR 15	*metschnikowia pulcherrima*	Pb 40	IR 10
Metschnikowia pulcherrima	Pb 44	IR 15	*metschnikowia pulcherrima*	Pb 41	IR 10
Pichia kluyveri	Pb 46	IR 15	*Metschnikowia pulcherrima*	Pb 42	IR 10
Pichia kluyveri	Pb 47	IR 15	*Pichia masmurika*	Pb 54	IR 10
Pichia kudriavzevii	Pb 48	IR 15	*Saccharomyces cerevisiae*	Pb 65	IR 10
Pichia kudriavzevii	Pb 49	IR 15	*Saccharomyces cerevisiae*	Pb 72	IR 10
Pichia kudriavzevii	Pb 50	IR 15	*Saccharomyces cerevisiae*	Pb 87	IR 10
Pichia kudriavzevii	Pb 51	IR 15	*Saccharomyces cerevisiae*	Pb 88	IR 10
Pichia kudriavzevii	Pb 52	IR 15	*Wickerhamomyces anomalus*	Pb 97	IR 10
Pichia kudriavzevii	Pb 53	IR 15			
Pichia occidentalis	Pb 56	IR 15			
Pichia occidentalis	Pb 57	IR 15			
Pichia occidentalis	Pb 58	IR 15			
Rhodotorula glutinis	Pb 60	IR 15			
Rhodotorula mucilaginosa	Pb 61	IR 15			
Saccharomyces cerevisiae	Pb 80	IR 15			
Torulaspora delbrueckii	Pb 91	IR 15			
Wickerhamomyces anomalus	Pb 93	IR 15			
Wickerhamomyces anomalus	Pb 94	IR 15			
Wickerhamomyces anomalus	Pb 95	IR 15			
Wickerhamomyces anomalus	Pb 96	IR 15			
Saccharomyces cerevisiae var. *boulardii* CNCM I-745	Sb	IR 15			

K. marxianus, S. cerevisiae and *Issatchenkia occidentalis (anamorph of P. occidentalis)* that were resistant to 1 % bile salt, which disagrees with our results for the latter two strains. Bile resistance is another important characteristic to consider when selecting a probiotic, as it allows the growth of a microorganism in the intestinal tract (Suscovic et al., 1997). Bile salt, a lipid emulsifying agent released into the duodenum after food intake, has an antimicrobial effect and therefore probiotics must demonstrate bile tolerance or exclusion mechanisms to survive in the intestine (Kumar et al., 2012).

Table 4 shows the 15 isolates that showed tolerance to the GIT conditions tested: growth at 37, 39 and 42 °C, tolerance at pH 2 and 3 (growth ≥ control strain *S. cerevisiae* var. *boulardii* CNCM I-745), and bile resistance (RI_{bile} 15 or 10). Growth under these stressful assay conditions should allow sufficient viable cells to enter the small intestine and suggests their possible survival and adequate activity.

3.3. Third Screening: Epithelial adhesion capacity

In addition to tolerating the upper gastrointestinal transit, another challenge for an effective probiotic is to adhere, at least temporarily, to small intestinal cells. Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, as this prevents their immediate elimination by peristalsis and provides a competitive advantage in this ecosystem (Kos et al., 2003; Ouwehand et al., 1999). Similarly, these characteristics are not considered to be mutually exclusive requirements. Therefore, hydrophobicity, autoaggregation and biofilm formation capacity were evaluated.

Hydrophobicity in our study was highly variable, ranging from 1.05 % to 79.67 % for the selected yeasts. The affinity of *S. cerevisiae* var. *boulardii* CNCM I-745 (reference strain) towards xylene was 35 %. *P. kudriadzevii* (PB50, PB51, PB52 and PB53) and *W. anomalus* (PB97) strains showed the highest values (>43 %) (Fig. 3). Our results are comparable with those reported by Binetti et al.,(2013) as to strain *P. kudriadzevii* recorded around 80 % hydrophobicity for strains of this species. Adhesion is a complex trait that generally involves a multistep process with electrostatic interactions and hydrophobic forces, involving specific interactions between the physical and chemical

Table 4
Selected yeasts after the second screening: bilis resistance (RI_{bile}), acidity tolerance (%) and temperature growth (37, 39 and 42 °C).

Specie	Isolate	IR_{bilis}	% tolerance		Growth at three temperatures
			pH 2	pH 3	
Candida intermedia	PB3	10	27.77 %	95.41 %	+
Candida sake	PB7	15	22.76 %	197.03 %	+
Hanseniaspora guilliermondii	PB15	10	195.70 %	219.35 %	+
Hanseniaspora uvarum	PB18	10	44.54 %	73.11 %	+
Pichia kudriavzevii	PB48	15	53.74 %	284.62 %	+
Pichia kudriavzevii	PB50	15	49.38 %	642.22 %	+
Pichia kudriavzevii	PB51	15	47.99 %	259.02 %	+
Pichia kudriavzevii	PB52	15	363.49 %	323.08 %	+
Pichia kudriavzevii	PB53	15	63.01 %	550.00 %	+
Pichia masmurika	PB54	10	47.99 %	50.62 %	+
Pichia occidentalis	PB56	15	178 %	110.89 %	+
Pichia occidentalis	PB57	15	69.70 %	246.46 %	+
Pichia occidentalis	PB58	15	96.10 %	320.00 %	+
Torulaspora delbrueckii	PB91	15	29.19 %	153.33 %	+
Wickerhamomyces anomalus	PB97	10	65.12 %	80.58 %	+

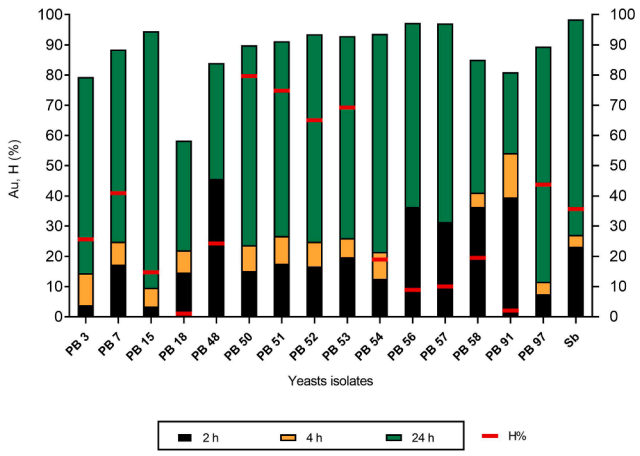

Fig. 3. Percentage of autoaggregation (Au%) at 2, 4, 24 h of incubation; percentage of hydrophobicity (H%) of selected and reference probiotic strains *Saccharomyces cerevisiae* var. *boullardii* CNCM I-745 (Sb).

characteristics of the microbial surface and the intestinal mucosa (Menezes et al., 2020). Cell surface hydrophobicity is considered an important factor in the adhesion and proliferation of microorganisms on intestinal epithelial cells (Sourabh et al., 2011). Many authors have reported that a hydrophobicity of 30–40 % would allow probiotics to interact with mucus and perform at least temporary adhesion (Abdulla et al., 2014; Ilavenil et al., 2016; Sidira et al., 2015). Therefore, the mentioned strains with higher values could be considered as hydrophobic.

In the present study, significant differences were found between autoaggregation (Au %) at the beginning (after 2 and 4 h) and at the end of the trial (24 h) ($p = 0.05$). While there were no significant differences after 2 h and 4 h ($p < 0.05$). Four yeasts after 2 h, (PB 48, PB56, PB58 and PB91) demonstrated values higher than 35 %, while the rest obtained values lower than 20 %, with no significant changes after 4 h. An increase between 68 % and 94 % was observed for all isolates after 24 h of incubation (Fig. 3). Our results show that Au % between 2 and 4 h was variable and strain dependent, but the strains reached similar stable values after 24 h. It is noteworthy that all strains of the genus *Pichia* obtained percentages higher than 90 % together with *Candida sake* Pb7 and the reference strain (Fig. 3). According to Menezes et al.,(2020), Au % values < 30 are considered low, 30–60 intermediate and > 60 high, therefore it could be said that all yeast strains tested presented high Au % values.

Amorim et al.,(2018) evaluated yeast isolates of *Saccharomyces, Kluyveromyces* and *Debaryomyces* and reported similar behavior to the yeasts in our study: an Au % value < 16 after 2 h of incubation that increased to > 96 after 24 h. On the other hand, Bonatsou et al.,(2018) evaluated autoaggregation in *Rhodotorula, Pichia, Candida, Metschnikowia* and *Saccharomyces* strains among others, which were isolated from Kalamata table olive fermentation, and reported that 20/38 strains showed a high Au % value with mean values ranging from 72 to 91 % within the first 4 h of incubation; these percentages are higher than those of our study. After 24 h their results agreed with ours and 34/38 of the yeast strains showed Au % >90.

Autoaggregation is defined as the ability of (yeast) cells to aggregate within the same species to form flocs (Stevens et al., 2015). This property is associated with cell adhesion (Boris et al., 1997), and may provide yeast with a competitive advantage over other microorganisms by preventing colonization to epithelial cells and mucosal surfaces (Vine et al., 2004) (including enteric bacterial pathogens) or allowing their maintenance in a hostile environment such as human GIT (Naidu et al., 1999; Pennacchia et al., 2008; Brückner et al., 2012, García-Cayuela et al., 2014; Porru et al., 2018).

As expected, yeasts of oenological origin usually show certain evolutionary adaptations to the stressful environment of wine. Flocculation (related to the presence of FLO genes) is a type of cell–cell interaction that results in cell aggregates (Teunissen and Steensma, 1995; Conacher et al., 2019), being a central mechanism in the structural organization of yeast communities (Honigberg 2011), as well as a protective mechanism in stress situations in which internal cells are protected by external ones (Smukalla et al., 2008; Conacher et al., 2019). It can be inferred that the high Au % values of the strains in our study are due to the presence of this adaptive feature in wine communities.

Considering both parameters evaluated, no correlation was observed between Au % and hydrophobicity ($r = 0.15$; $p > 0.05$), although *P. kudriadzevii* PB50, PB51, PB52 and *P. masmurika* PB54 strains showed high values of hydrophobicity and Au %. It should be noted that a good correlation between hydrophobicity (in vitro assays) and intestinal adherence (in vivo assays) has not always been detected (Martins et al., 2009; Kourelis et al., 2010; Binetti et al., 2013; Perricone et al., 2014) and that Au % has been more associated with intestinal adherence. Cell wall composition affects hydrophobicity and Au % properties, which explains why certain yeast strains have relatively slower elimination kinetics in the gastrointestinal tract ((Rodríguez et al., 2018; Van Mulders et al., 2010). Therefore, good Au ability despite low hydrophobicity values may predict the adhesion property of isolates (Sourabh et al., 2011).

In our assays, the threshold value for biofilm formation was set at $Abs_{620} = 0.102$, based on the absorbance obtained for the negative control (Růžička et al., 2007). Significant differences in biofilm production were observed between the different strains ($p < 0.05$). The reference probiotic strain, *S. cerevisiae* var. *boulardii* CNCM I-745, could be considered a poor biofilm producer (as reported for strains of the same species by Zullo et al, 2019) along with strains PB 3, PB15, PB18 and PB91) and while PB 7, PB 50 and PB53 showed the highest biofilm formation capacity. The remaining strains showed moderate production capacity (Fig. 4). As for the correlation between adhesion-related parameters, no correlation was observed between biofilm formation and Au % ($r = 0.44$; $p > 0.05$), but a correlation was observed between biofilm and hydrophobicity ($r = 0.66$; $p < 0.05$). Experiments conducted by Lutz et al., (2013) on biofilm formation by epiphytic yeast strains from pears after storage in two different cold rooms in northern Patagonia, Argentina, showed that isolates of the genus Pichia were poor biofilm builders which differs from our results.

Although selection criteria for probiotic microorganisms remain controversial, three widely accepted conditions were considered in the present study: biosafety for consumption, ability to survive in the gastrointestinal environment, and adherence to epithelial cells.

Intestinal colonization by microorganisms is frequently preceded by adhesion to the intestinal mucosa leading to biofilm formation and these phenomena (adhesion and biofilm formation) are positively correlated (Arevalo-Villena et al., 2017). In a review on the establishment and development of intestinal microbiota, Macfarlane and Dillon (2007) describe the mechanism and importance of biofilm formation on both the surface and in the intestinal lumen. Microorganisms need to resist the forces produced by the entrainment of material flow in the intestinal lumen to avoid physical removal from epithelial surfaces by intestinal peristaltic movements (Iñiguez-Palomares et al., 2010). The ability to form biofilms is widespread in several yeast species related to winemaking. It is a good strategy to colonize and survive on grapes and in the winery, providing resistance to commonly used antimicrobial agents and disinfectants, extreme temperatures, low pH and to differences in osmolarity (Perpetuini et al., 2018; Eldarov et al., 2021). Therefore, in vitro assays on biofilm development can provide information on the ability of yeasts to colonize the human gut (di Cagno et al., 2020).

Although epithelial adherence-related properties are considered a selection criterion for potential probiotic strains, they are not eliminatory traits. For example, enteropathogenic bacteria, including *Escherichia coli* species, *Salmonella Typhimurium, S. Typhi* and yeast as

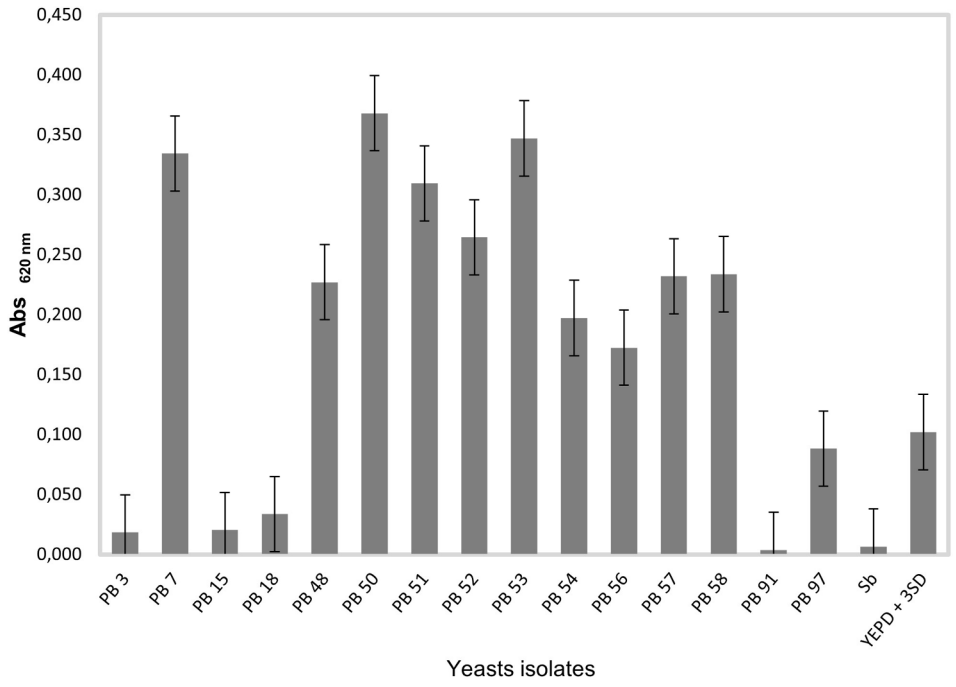

Fig. 4. Biofilm formation capacity (Abs $_{620\ nm}$) of native yeast isolates, negative control (YEPD + 3SD) and probiotic reference strain *Saccharomyces cerevisiae* var. *boullardii* CNCM I-745 (Sb).

Candida albicans, have been shown to preferentially and irreversibly bind to *S. boulardii* surfaces (Rajkowska et al., 2012; Tiago et al., 2012; Posadas et al., 2017). The binding of these pathogenic bacteria to the yeast cell wall limits their infectivity, as *S. boulardii* does not bind to the GIT and, consequently, the adherent bacteria transiently pass through the gastrointestinal tract and are subsequently excreted in the feces (Czerucka et al., 2007). It has been reported that, in addition to the property of adhering and eliminating pathogens, probiotic yeasts can also strongly adhere certain toxins such as cholera toxin, or compounds produced by Clostridium difficile, with the same elimination effect (Tiago et al., 2012). In relation to the results of Au %, hydrophobicity and biofilm formation (properties to be studied but not exclusive) in the

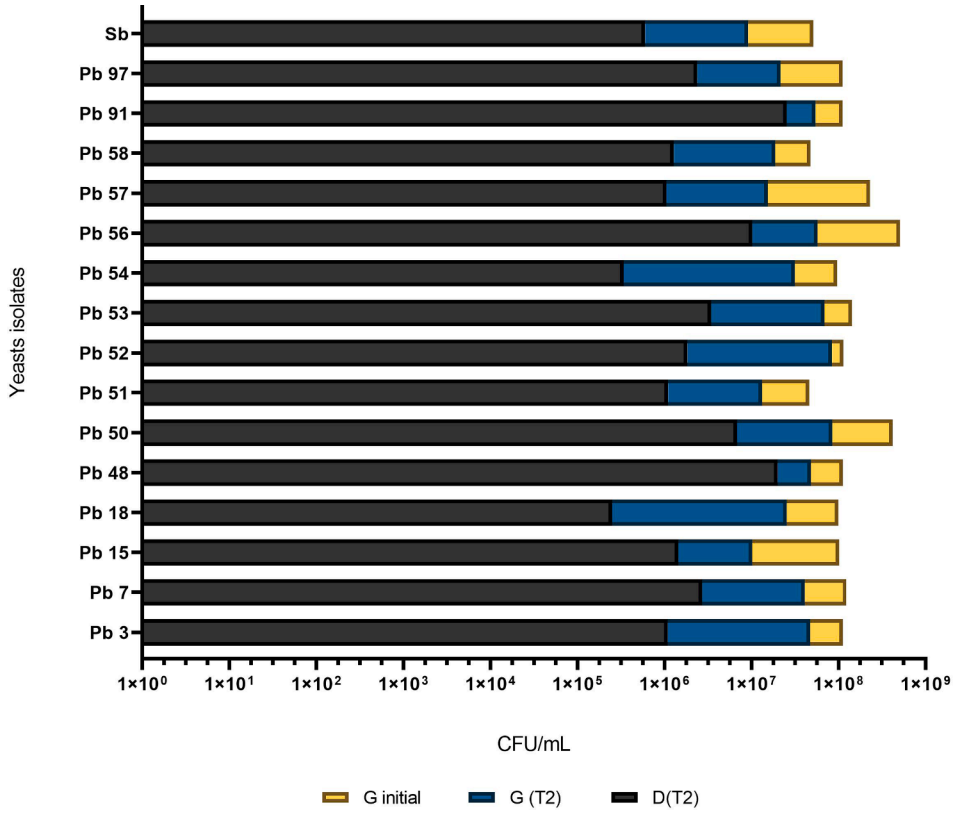

Fig. 5. Survival (CFU/mL) with respect to the initial inoculated number of the end of the gastric phase (G(T2)) and fine of the duodenal phase (D(T2)).

present work we consider that the 15 native yeast isolates should be further studied as probiotic candidates.

3.4. Yeast survival rate under simulated GIT conditions

As shown in Fig. 5, overall the initial population reduction did not exceed 30 % at the end of the trial. In our study, the isolates showed sensitivity to pancreatic conditions, with a decrease in live cells between 1 log10 − 2 log10. The isolates with the highest survival rates at the end of the duodenal phase were *T. delbrueckii* PB91 (94.76 %); *P. kudriadzevii* PB48, PB51 and PB53 (85.64 %, 89.63 % and 83.01 %, respectively); *P. occidentalis* PB56, PB58 (89.66 %, 81.75 %, respectively); *C. sake* PB7 (80.77 %) and *W. anomalus* PB97 (80.30 %). In our study, the isolates showed sensitivity to pancreatic conditions, with a decrease in live cells between 1 log10 − 2 log10. In particular, the reference probiotic strain *S. cerevisiae* var. *boulardii* CNCM I-745 showed a survival rate of 76.78 %, lower than most of the native yeast strains tested.

Comparable results were reported by Syal and Vohra (2012), where *Candida* and *Saccharomyces cerevisiae* showed high tolerance at pH 2 (≥93 %) while *P. masnhurika* isolates were more sensitive (61 %) after exposure for up to 3 h during the gastric phase. Piraine et al.,(2021) worked with isolates obtained from flowers, fruits, leaves and mixed fermentation beers, belonging to the genera *Pichia, Hanseniaspora, Saccharomyces*, *Issatchenkia, Cryptococcus and Candida* and did not observe a significant decrease in viability during passage through the gastric phase, where all isolates presented ≥ 93 % after gastric digestion. The authors observed a decrease in viable cell count after pancreatic digestion of *H. uvarum* and *C. intermedia* of 1 log10, whereas *P. kluyveri* demonstrated a decrease in final concentration of 2 log10. After pancreatic digestion, these rates decreased to approximately 88 % for *H. uvarum* and *C. intermedia* and 75 % for *P. kluyveri*. On the other hand, Merchán et al., (2020) observed with isolates of the genera *Debaryomyces, Kluyveromyces, Pichia, Yarrowia* and *Candida,* that the most restrictive phase was the gastric phase due to the low pH, rather than the duodenal phase. Although in our study a decrease of no>8 % was recorded in the gastric phase and greater sensitivity was evidenced in the duodenal phase, this could be due to the fact that their optimal response capacity to successive stresses is decreasing.

The selected native yeasts were subjected to a simulated digestion assay (gastric and pancreatic) to analyze survival along the path that the yeasts have to travel to reach their site of action. Some of the stress factors were previously assayed separately (projection 2), but it is necessary to evaluate yeasts subjected to continuous stress at successive stages.

The analysis of cell viability during in vitro simulation of the gastrointestinal tract is important, because probiotic-related effects are believed to be dose-dependent, suggesting an effective dose between 10^7-10^9 CFU/mL per day (Minelli and Benini, 2008). Furthermore, based on data published by Pennacchia et al. (2008), a survival rate of 70 % was considered for the selection of yeast strains as potential probiotics. Therefore, our results suggest that the concentration of viable cells after passage through the gastrointestinal tract of the tested yeasts is sufficient for the probiotic potential to exert a positive effect on the organism.

4. Conclusion

Due to the growing interest of the scientific community in finding new strains with probiotic capacity, the present study was designed to select native yeasts with probiotic potential, under the assumption that wine yeasts are promising candidates since they are adapted to wine environments which are highly selective and stressful.

Taking into account their biosafety characteristics, GIT tolerance and adhesion properties, out of 29 Saccharomyces and 67 non-Saccharomyces yeast isolates, it is important to highlight that 15 non-*Saccharomyces* yeasts were selected for their properties as probiotic candidates. In turn, the best results were recorded by yeasts of the genus Pichia. In fact, the wine strains performed better in several tests than the reference probiotic strain S. *cerevisiae* var. *boulardii* CNCM I-745, which reaffirms that these strains are well adapted to stress conditions.

It can be stated that the results of the present study are relevant and that These 15 yeast strains (Table 4) can be recommended for future trials on health-promoting properties and in vivo studies, as well as for possible therapeutic applications in the future. Furthermore, the study record of yeasts with *non-Saccharomyces* probiotic abilities within the probiotic area is increased.

CRediT authorship contribution statement

Silvia Cristina Vergara Alvarez: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision. **María José Leiva Alaniz:** Conceptualization, Validation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision. **María Victoria Mestre Furlani:** Investigation, Resources, Writing – review & editing, Visualization, Supervision. **Fabio Vazquez:** Resources, Writing – review & editing, Visualization. **Pamela Mancha Agresti:** Resources, Writing – review & editing. **María Cristina Nally:** Resources, Writing – review & editing, Visualization, Visualization, Supervision. **Yolanda Paola Maturano:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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