YEASTS IN FERMENTED FOOD AND KEFIR: *IN VITRO* CHARACTERIZATION OF PROBIOTIC TRAITS

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ABSTRACT

The probiotics are live microorganisms that, when administered in adequate amounts, confer a beneficial effect on host's health. Although most probiotics are bacteria, however, one yeast species (*Saccharomyces boulardii*) has also been characterized as probiotic. In this study, yeasts have been very important in the food industry and have several beneficial properties that bacteria do not have. In this study, yeast strains were isolated from fermented food and beverage. Various physiological features of the candidate probiotic isolates were preliminarily investigated, including bile salt and acid tolerance, cell surface hydrophobicity, autoaggregation, antioxidant activity, and β -galactosidase activity. We demonstrated several yeast strains have potential as probiotics. For example, *Saccharomyces cerevisiae* JYC2508 adapted well in a bile salt and acid tolerance test and also had good autoaggregation ability. Moreover, *Kluyveromyces marxianus* JYC2528 grew well in the bile salt and acid tolerance test and also had good performance with antioxidant activity, cell surface hydrophobicity, and β -galactosidase activity. The overall findings of this study showed that these yeast strains may offer promising probiotic traits relevant for further study.

Key words: autoaggregation, antioxidant activity, β -galactosidase activity, bile salt and acid tolerance, cell surface hydrophobicity, probiotic, yeast.

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INTRODUCTION

The intestinal digestive system is responsible for digesting food and absorbing nutrients. Intestinal health can affect overall health because most symbiotic microorganisms colonize the intestines (Long-Smith *et al.*, 2020). Thus, numerous immunocytes are distributed in the intestinal wall. An unhealthy gut places considerable stress on the immune system, and may increase the body's susceptibility to infection from external pathogens (Kau *et al.*, 2011). Thousands of microbial species are present in the intestine, and their total cell number is considerably higher than that needed by the human body (Eckburg *et al.*, 2005). The behavior of these symbiotic microorganisms is closely related to the health of the intestines. Most microorganisms in a healthy human intestine are opportunistic; that is, they are typically non-pathogenic microorganisms that act as pathogens in certain circumstances. However, when pathogenic bacteria proliferate in the intestine and cause an imbalance in intestinal microflora, diseases may be triggered. Scientists have recently proposed that numerous physiological and mental diseases, such as irritable bowel syndrome, depression, anxiety disorder, autism, and chronic fatigue may be related to a patient's gut health (Cheung *et al.*, 2019; Czerucka *et al.*, 2007). The reason for this is that the number of nerve cells in the digestive system is second only to the number in the brain (Mayer, 2011). The concept of the intestinal digestive

system and the symbiotic microbes within it affecting the brain and even human behavior is called the microbiome-gut-brain axis (Heijtz *et al.*, 2011). The connections between the brain and nerve cells are incomplete in germ-free mice, which affects their behavioral patterns (Heijtz *et al.*, 2011). Although it is unclear whether intestinal microbes affect the nervous system, maintaining probiotic populations in the gut is vital for human health.

Probiotics are defined as live microorganisms that confer a beneficial effect on a host's health when present in adequate numbers (FAO/WHO, 2001, 2002). Several conditions are necessary for a microbe to be considered probiotic: it must be innocuous (not causing damage or disease to its host), its activity and population should remain high during the shelf life of the product it is in, and it must be able to survive gastrointestinal (GI) transit (De Vrese and Schrezenmeir, 2008; Hill *et al.*, 2014; Tripathi and Giri, 2014). Using multifunctional microorganisms as starter cultures with probiotic activity is a trend in food microbiology (Perricone *et al.*, 2014; Terpou *et al.*, 2019). People can improve their health by consuming foods containing probiotics as part of their daily diet. Therefore, evaluating the probiotic functions and properties of microorganisms in foods, such as the inhibition of pathogens (Binetti *et al.*, 2013; Silva *et al.*, 2011), antioxidant activity (Chen *et al.*, 2010b), and β -galactosidase activity (Kippert, 1995) is imperative. Most probiotics comprise *Lactobacillus* spp. and *Bifidobacterium* spp. (De Vrese and Schrezenmeir 2008;

Hill *et al.*, 2014). However, the probiotic or beneficial potential of yeasts is usually ignored, despite being imperative and widely distributed in foods and beverages (Chen *et al.*, 2010b; Diosma *et al.*, 2014; Gil-Rodríguez *et al.*, 2015). *Saccharomyces boulardii* is the only yeast species that has been recognized and characterized as a commercial probiotic (Czerucka and Rampal, 2002; Rima *et al.*, 2012). Whether other yeast species or strains have probiotic characteristics warrants exploration (Binetti *et al.*, 2013; Van der Aa Kühle *et al.*, 2005).

Yeasts are vital in the production of numerous foods and beverages, such as wine, bread, kefir, and beer (Antunes and Aguiar, 2012; Moreira *et al.*, 2011), and participate in the maturation process of some cheeses (Binetti *et al.*, 2013). Yeasts are useful in the food industry and usually used in traditional food fermentations are generally recognized as safe. It is worth exploring the probiotic potential of yeasts for the following reasons. First, they have excellent resistance to antibacterial agents. This feature is imperative because some medical treatments combine probiotics and antibiotics for treating GI infections. Second, the genetic material of yeasts is not transferred to other microorganisms through conjugation or transformation (Czerucka *et al.*, 2007). Antimicrobial resistance has become a critical public health problem. Thus, the major reason for using yeasts and antibiotics to treat patients is that, although yeasts exhibit antimicrobial resistance, they do not transfer drug resistance genes to other microorganisms. The transfer of genes between bacteria causes antimicrobial resistance. The mammalian GI tract produces a favorable environment for many bacterial species to transfer their genetic material (Salyers *et al.*, 2004). Resistance genes can be transferred not only between resident intestinal microflora, but also transferred from bacterial probiotics (Temmerman *et al.*, 2003; Mathur and Singh, 2005). Numerous researchers have suggested that commensal bacteria, including lactic bacteria, may act as reservoirs of antibiotics (Mathur and Singh, 2005). Yeasts have long been considered safe microbes (Diosma *et al.*, 2014), with beneficial characteristics including competitiveness for nutrition, better cell adherence (because of their size and autoaggregation ability), and genetic material that is not transferred to commensal bacteria (Czerucka *et al.*, 2007).

Microorganisms are subjected to numerous harsh environments as they pass through the human GI tract. For example, the pH in the stomach ranges from 2 to 5, which is harmful to most microorganisms. While the pH increases toward the distal part of the GI tract, the presence of aggressive intestinal fluids (e.g., bile and pancreatic juices) and the short transit time in the duodenum, creates a harsh environment for most microorganisms in the duodenum. Thus, the duodenum contains relatively few microbes. The presence of yeasts

in this stressful environment can be explained by their resistance to pH variation (Czerucka *et al.*, 2007). Most yeasts can grow at a pH of 3.0, and some can tolerate highly acidic conditions (pH 1.5) (Czerucka *et al.*, 2007). Probiotics must adapt to the pressure of the GI tract caused by bile salts, organic acids, multiple digestive enzymes, and considerable variations in pH. Thus, yeasts are good candidates as probiotics and numerous studies have shown that oxidative damage produced by free radicals, especially reactive oxygen species (ROS), causes many chronic diseases (e.g., osteoarthritis, rheumatoid arthritis, cardiovascular disease, and some tumors) (Ciafardini and Zullo 2020; De Vrese and Schrezenmeir, 2008; Kawagishi and Finkel, 2014; Valko *et al.*, 2006). The risk of developing these diseases can be reduced by decreasing this oxidative damage (Serafini, 2006; Trotta *et al.*, 2012). Studies on natural antioxidants have reported that yeasts and yeast extracts have antioxidant activity due to a wide range of substances (e.g., superoxide dismutase, catalase, oxygenated carotenoids, resveratrol, and octacosanol), and that (1-3)- β -D-glucan and other β -glucans, which constitute the cell wall of yeasts, have imperative functions (Abbas, 2006; Jaehrig *et al.*, 2007). Moreover, cell surface hydrophobicity and autoaggregation are considered to be correlated to the retention capacity of microorganisms in the intestine (de Lima *et al.*, 2017). Because yeast species are larger than other microorganisms, they easily attach to the intestinal wall. Mannose is one of the components in the cell wall of yeasts, which has excellent viscosity and enables yeasts to aggregate quickly (Ofek and Beachey, 1978). Most food in the intestine is in a mobile phase and can remove the microorganisms on the intestinal wall through intestinal motility. Microorganisms with good cell surface hydrophobicity are less likely to be removed. Therefore, cell surface hydrophobicity is also vital for maintaining microbes in the intestine.

Probiotics are beneficial for promoting the gut absorption efficiency of their host because they secrete enzymes to improve the utilization and absorption of nutrients along the digestive tract. For example, lactose is hydrolyzed into glucose and galactose by β -galactosidase, which are then transported across the brush border membrane of epithelial cells into the cytosol (Saqib *et al.*, 2017). When undigested lactose is transported to the small intestine and is fermented by colonic bacteria, hydrogen gas is released and lactose indigestion symptoms occur (Hertzler and Clancy, 2003). Probiotics that have β -galactosidase activity are imperative because they can avoid discomfort during intestinal fermentation after consumption of dairy products (Sourabh *et al.*, 2011). This enzyme is also utilized to avoid lactose crystallization, increase the solubility of milk products, and solve the problems associated with whey utilization and whey disposal, which can cause pollution

(Rosenberg, 2006). In this study, we isolated yeast strains from fermented food and beverage and evaluated their probiotic potential. Probiotic potential was assessed using several experiments, namely the ability of the strains to grow at 37 °C and their survival rate in bile salt and acid, as well as their autoaggregation activity, cell surface hydrophobicity, antioxidant activity, and β -galactosidase activity.

MATERIALS AND METHODS

Yeast strain isolation, identification, and culture conditions: In this study, yeast strains were isolated from various fermented foods and beverages, milk kefir, water kefir, and kombucha. The samples were serially diluted with sterile deionized water and then spread uniformly on yeast extract peptone dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) supplemented with 0.3% lactic acid by using sterile glass beads. Yeast colonies of different morphologies were selected and purified by streaking them on YPD agar plates. Purified yeast cultures (1 mL) were mixed with 200 μ L of glycerol ($\geq 99.5\%$) and stored at -80 °C. The purified yeast was refreshed in YPD medium and incubated at 28 °C for 3 h. Yeast cultures (1 mL) were transferred to a 1.5-mL centrifuge tube and centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the cell pellet was suspended in 200 μ L of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM sodium chloride, and 10 mM Tris [pH 8.0], and 1 mM ethylenediaminetetraacetic acid), after which 200 μ L of phenol-chloroform-isoamyl alcohol (25:24:1; isoamyl alcohol is optional) and 0.3-g acid-washed glass beads (0.45–0.52 mm) were added and mixed gently. The samples were vortexed at 2500 rpm for 10 min to disrupt the cells and then were centrifuged at 13000 rpm for 5 min. The aqueous layer of each sample was transferred to a new tube with 400 μ L of 95% ethanol and 16 μ L of 3 M sodium acetate. The samples were vortexed again, stored at -20 °C for 15 min, and then centrifuged at 13000 rpm for 5 min at 4 °C. The pellets were washed with 300 μ L of 70% ethanol, and the tubes were aspirated with air for 3 h to dry the pellets. Finally, genomic deoxyribonucleic acid (DNA) from each sample was suspended in 50 μ L of sterile water.

The large subunit (LSU) rDNA D1/D2 domain and the internal transcribed spacer (ITS) region were amplified were amplified with the universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') through polymerase chain reaction (White *et al.*, 1990; Kurtzman and Robnett, 1997). Samples were sent to Tri-I Biotech, Inc. for DNA sequencing. The sequences obtained in this study (ITS regions, and partial LSU) were identified by searching databases using the BLAST sequence analysis tool (<http://www.ncbi.nlm.nih.gov/BLAST/>) on the

National Center for Biotechnology Information's homepage. The sequences were compared with the default setting using nucleotide-nucleotide BLAST (blastn). Yeast identification was based on sequence differences; yeast strains with a $\leq 1\%$ sequence difference are considered conspecific species. Nucleotide sequences of the studied strains deposited in the GenBank are given in Table 1.

Bile salt and acid tolerance: The samples were cultured in YPD medium overnight at 28 °C and were refreshed in the same medium for 3 h to let the cells into the log phase. Yeast cultures were quantified using a spectrophotometer (Unico 1200 Spectrophotometer, USA) at 660 nm. The cell numbers were all adjusted to 1.28×10^6 /mL according to the optical density (OD) of the yeast strains. The samples were washed twice with 0.9% NaCl solution and then were transferred to YPD medium at pH 3 (adjusted with 6 M HCl) and incubated at 37 °C for 2 h. Next, 1 mL of culture was harvested (13000 rpm, 1 min), washed twice with 0.9% NaCl solution, and resuspended in YPD medium with 3.0 g/L bile salt (Sigma-Aldrich). The samples were incubated at 37 °C. The optical density at a wavelength of 600 nm (OD₆₆₀) was measured after 3 h and 24 h of incubation (Ogunremi *et al.*, 2015).

Autoaggregation and settlement assay: The screening of the autoaggregation ability and surface hydrophobicity was performed to identify the adherent ability of the yeast strains. The samples were cultured in YPD medium overnight at 28 °C and were refreshed in the same medium for 3 h. The cell numbers were adjusted to 1.85×10^7 /mL according to the OD₆₆₀. The samples were washed twice with phosphate buffered saline solution (PBS, pH 7.4, Sigma-Aldrich), and the cells were harvested by centrifugation and resuspended in the same solution. In autoaggregation (sometimes also called autoagglutination or flocculation), yeast cells of the same type form multicellular clumps that easily settle at the bottom of culture tubes. Thus, the OD₆₆₀ was measured after incubation at 37 °C after 0, 2, 4, and 24 h to analyze the autoaggregation and settlement ability. The percentage of aggregation (%A) was calculated according to the following equation:

$\%A_t = [1 - (OD_t - OD_i)] \times 100$, where OD_i is the initial optical density of the microbial suspension and OD_t is the optical density at time t (Ogunremi *et al.*, 2015; Romero-Luna *et al.*, 2019).

Cell surface hydrophobicity assay: The hydrophobicity of the yeast strains was evaluated following the classification in Rosenberg *et al.* (1980). Cell surface hydrophobicity was assessed using microbial adhesion to hydrocarbons. Thus, the microbial adhesion to hydrocarbon (xylene) was used to analyze the cell surface hydrophobicity of the yeast strains. Yeast strains were

refreshed in YPD medium overnight at 28 °C and incubated at 37 °C for 8 h. The cell numbers were adjusted to $1.85 \times 10^7/\text{mL}$ according to the OD_{660} . The samples were washed with PBS buffer, and the cells were harvested by centrifugation and resuspended in the same buffer. The OD_{660} was measured using a spectrophotometer to confirm the cell numbers (OD_1). Next, 0.8 mL of xylene was added to 1.2 mL of the yeast suspensions, and then the mix was vortexed for 60 s and rested for 2 h at 37 °C. Next, draw the aqueous phase carefully and the OD_{660} (OD_2) was measured. The hydrophobicity index (HPBI) was calculated according to the following equation: $\% \text{HPBI} = [(\text{OD}_1 - \text{OD}_2) / \text{OD}_1] \times 100$ (de Lima *et al.*, 2017).

Antioxidant activity: The percentage reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich) radical was used to evaluate the antioxidant activity of the yeast strains (Chen *et al.*, 2010b). Yeast strains were cultured in YPD medium overnight at 28 °C and refreshed in the same medium for 3 h. The cell numbers were adjusted to $1.85 \times 10^7/\text{mL}$ according to the OD_{660} . The samples were washed once with 0.9% NaCl solution and then the cells were transferred to 400 μL of PBS solution. The cell suspension (400 μL) was mixed with 500 μL of 0.2 mM DPPH solution (dissolved in methanol). The mixture was vortexed and then incubated for 30 min at room temperature in darkness. The reaction tubes were centrifuged (13000 rpm, 1 min) and 200 μL of the supernatant was transferred to 96-well plates to measure the optical density at 517 nm by using a microplate reader (Thermo Scientific Multiskan GO, USA). The radical scavenging activity was calculated as a percentage of DPPH discoloration using the following equation:

$$[(1 - \text{OD}_{517(\text{sample})} / \text{OD}_{517(\text{blank})}) \times 100\%]$$

To quantify the antioxidant activity, we also assessed the Trolox equivalent antioxidant capacity of each strain by using a solution with 1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich) dissolved in methanol (Ogunremi *et al.*, 2015). Trolox was diluted to different concentrations (0–80 μM) by using methanol, and 400 μL of each concentration of Trolox was mixed with 500 μL of 0.2 mM DPPH and incubated for 30 min at room temperature in darkness. Finally, 200 μL of the mixture was transferred to 96-well plates to measure the optical density at 517 nm. In doing so, the scavenging ratio of the sample and Trolox on DPPH at the same time was tested, and then a suitable range of concentration of the Trolox and its scavenging percentage was found. Thus, a linear regression equation between the Trolox concentration and its scavenging percentage was built, and the Trolox equivalent antioxidant capacity was calculated through the equation, according to the

scavenging percentage of the sample solution to the DPPH radical solution.

β -galactosidase activity: To evaluate the β -galactosidase activity, the protocol described by Kippert (1995) was used with some modifications. Yeast strains were cultured in YPD medium overnight at 28 °C and were refreshed in the same medium for 3 h. The cell numbers were adjusted to $1.85 \times 10^7/\text{mL}$ according to the OD_{660} . The samples were washed with PBS buffer and centrifuged, and then 400 μL of Z buffer (0.06 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.01 M KCl, 0.001 M MgSO_4 , and 0.05 M β -mercaptoethanol) and 200 μL of lysis buffer were added. The samples were mixed and stored at -80 °C for 90 min. After the samples were thawed at room temperature for 20 min, 200 μL was transferred to a new tube and the same volume of 3 mM o-nitrophenyl- β -D-galactopyranoside (Sigma-Aldrich) was added. The tube was briefly shaken by hand and was incubated for 30 min at 37 °C. The reaction was stopped by adding 800 μL of 0.1 M Na_2CO_3 . The mixture was then centrifuged (13000 rpm, 1 min) and 200 μL of the supernatant were transferred to 96-well plates to measure the optical density at 420 nm. A standard curve of o-nitro-phenol (ONP, Acros-Organics) was obtained using known concentrations of ONP, from 0.05 to 2 mmol/mL at intervals of 0.05 mmol/mL. The concentration of ONP was calculated using a standard curve: $y = 1.3997x + 0.0449$ ($R^2 = 0.998$). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μM of ONP per min at 37 °C. Enzyme activity was computed in Miller units according to the following equation:

$$\text{Miller units of galactosidase activity} = 1000 \times \text{OD}_{420} / \text{time (min)} \times \text{volume (mL)} \times \text{OD}_{660} \text{ (Miller, 1972; Miller et al., 1992).}$$

Statistical analysis: Two biological replicates (three technical replicates per biological replicate) were designed and conducted of each assay abovementioned. A significant association between the biological replicates by calculating Spearman's rank correlation coefficient (rs) is indicated by $P < 0.05$. The figures shown here are for only one of the two biological replicates used in this work. Data are expressed as mean $\pm$ standard deviation (SD). The significance of differences between groups was determined using Student t tests and analyses of variance. $P < 0.05$ was considered statistically significant. * $P < 0.05$; ** $P < 0.01$.

RESULTS

Yeast strain isolation: In this study, 59 yeast strains were isolated from fermented foods and beverages, milk kefir, water kefir, and kombucha (26 strains from fermented foods and beverages, 3 strains from water kefir, 27 strains from milk kefir, and 3 strains from kombucha). These yeast strains represent 18 species

(Table 1) in the genera *Dekkera* (1 strain), *Hanseniaspora* (2 strains), *Kazachstania* (14 strains), *Kluyveromyces* (9 strains), *Kwoniella* (1 strain), *Pichia* (1 strain), *Saccharomycopsis* (6 strains), *Saccharomyces* (19 strains), *Torulaspora* (1 strain), *Wickerhamomyces* (1 strain), and *Zygosaccharomyces* (4 strains). The details of their pathogenic or nonpathogenic nature were confirmed through a literature search. Even though further research on the pathogenicity and virulence of these candidates is needed. *Saccharomyces boulardii* was purchased and isolated from a probiotic supplement (Now Foods, USA) and was used as the control group of each experiment.

Bile salt and acid tolerance: All the yeast strains survived and grew well in the YPD medium at pH 3 at 37 °C for 2 h (Fig. 1). Of the 60 strains analyzed, 9 strains (15%) exhibited growth 24 h after they were transferred to YPD with 0.3% bile salt (Fig. 2). The yeast strains that adapted well to the YPD medium containing bile salt were *Saccharomyces cerevisiae* JYC2507 and JYC2508, *Saccharomycopsis fibuligera* JYC2511 and JYC2523, and *Pichia kudriavzevii* JYC2527. These strains showed obvious cell growth after 24 h ($OD > 1$). The strains that survived but where the growth rate was inhibited in the YPD medium containing bile salt were *S. fibuligera* JYC2516, *Kluyveromyces marxianus* JYC2528, *S. cerevisiae* JYC2542, and *Wickerhamomyces anomalus* JYC2560. These strains showed a small amount of growth after 24 h ($OD > 0.1$). Overall, 30 strains (50%) endured the YPD medium containing bile salt for at least 3 h. Cell growth was observed in these strains for 3 h, but growth slowed or stopped at 24 h. Of the remaining strains that could not endure the YPD medium containing bile salt, their OD_{660} gradually decreased; thus, the OD values of most of these strains decreased to a negative value. The reason for these cases was that the bile salt is consumed to emulsifying lipid and cause cell lysis. In this experiment, 39 strains (66%) exhibited a better survival rate than that of *S. boulardii*.

Autoaggregation: The autoaggregation percentages obtained for all the yeast strains were highest at 24 h (Fig. 3). At 2 h, the autoaggregation percentages showed a high level of variability. Most of the strains displayed fast autoaggregation within the first 4 h of incubation. As shown in Figure 3, high aggregation percentages usually occurred between 2 and 4 h (in 57 of the 60 strains, 95%). The remaining three strains showed a negative value between 0 and 2 h and 2 and 4 h, which became positive between 4 h and 24 h. The strains with excessively low autoaggregation performance in the first 4 h belonged to *S. fibuligera*. Therefore, using the spectrophotometer, we observed that the density of cellular aggregates and the

optical density were higher than that in the previous time point.

Cell surface hydrophobicity: Most of the tested strains showed moderate to high hydrophobicity (Fig. 4): 23 strains (38.3%) showed a moderate hydrophobicity rate between 25% and 50% and 21 strains (35%) showed a high hydrophobicity rate over 50%. *K. marxianus* JYC2557 showed the highest hydrophobicity ($79.4\% \pm 4.9\%$), whereas *S. cerevisiae* JYC2535 showed the lowest ($6.61\% \pm 2.68\%$). Overall, 52 strains (86.4%) had a cell surface hydrophobicity higher than that of *S. boulardii*.

Antioxidant activity: The antioxidant capacities of the strains varied (Fig. 5). Among the 60 yeast strains analyzed, 3 strains (5%) showed low antioxidant activity (with a DPPH scavenging percentage under 20%), 6 strains (10%) showed moderate activity (between 20% and 30%), 12 strains (20%) showed good activity (between 30% and 40%), 20 strains (33.3%) exhibited very good activity (between 40% and 50%), and 19 strains (31.6%) displayed excellent activity (over 50%) (Table 2). *Hanseniaspora osmophila* JYC2504 produced the lowest value ($18.41\% \pm 5.81\%$), whereas *S. fibuligera* JYC2512 ($70.76\% \pm 2.07\%$) and JYC2519 ($70.59\% \pm 0.98\%$) produced the two highest values. The antioxidant activity was also measured as Trolox equivalents (TE, μM), and the results showed a range between 30.4 ± 5.4 TE (for *H. osmophila* JYC2504) and 158.2 ± 5.4 TE (for *S. fibuligera* JYC2512). Overall, 55 strains (91.5%) had a better antioxidant ability than that of *S. boulardii* ($25.3\% \pm 3.58\%$, 40.6 ± 4.8 TE).

β -galactosidase activity: Most of the strains in this study did not produce β -galactosidase activity. Of the 60 strains analyzed, only 10 strains (16.7%) showed β -galactosidase activity (Fig. 6). The strains that showed high to low β -galactosidase activities, *Kazachstania turicensis* JYC2529 (17.97 ± 0.08 U/mL; 113.24 ± 0.53 Miller units), *K. marxianus* JYC2528 (15.97 ± 0.53 U/mL; 119.23 ± 3.72 Miller units), *K. marxianus* JYC2557 (14.43 ± 0.7 U/mL; 108.47 ± 4.87 Miller units), *Kazachstania servazzii* JYC2565 (9.33 ± 0.87 U/mL; 72.76 ± 6.07 Miller units), *K. marxianus* JYC2569 (8.37 ± 0.56 U/mL; 66.09 ± 3.92 Miller units), *K. marxianus* JYC2575 (6.88 ± 1.23 U/mL; 55.64 ± 8.64 Miller units), *K. marxianus* JYC2578 (6.43 ± 0.4 U/mL; 52.45 ± 2.78 Miller units), *K. marxianus* JYC2563 (5.36 ± 1.01 U/mL; 44.99 ± 7.05 Miller units), *K. marxianus* JYC2567 (0.85 ± 0.38 U/mL; 13.46 ± 2.66 Miller units), and *K. marxianus* JYC2576 (0.58 ± 0.14 U/mL; 11.54 ± 1.00 Miller units).

Fig. 1. Growth of yeast strains in YPD medium (pH 3) after 0 h (white) and 2 h (grey). The significance of differences between groups (0 h and 2 h) was determined using Student t tests and analyses of variance. $P < 0.05$ was considered statistically significant. ** $P < 0.01$. The experiments were repeated twice with similar results.

Fig. 2. Growth of yeast strains in YPD medium (0.3% bile salt) after 0 h (white), 3 h (grey), and 24 h (black). The significance of differences between groups (0 h and 3 h, above the strain name; 3 h and 24 h, above the columns) was determined using Student t tests and analyses of variance. $P < 0.05$ was considered statistically significant. ** $P < 0.01$. The experiments were repeated twice with similar results.

Fig. 3. Autoaggregation percentages of yeasts after 2 h (black), 4 h (grey), and 24 h (white).

Fig. 4. Cell surface hydrophobicity percentage of yeast strains. The significance of differences between groups (*Saccharomyces boulardii* and each yeast isolated) was determined using Student t tests and analyses of variance. $P < 0.05$ was considered statistically significant. $**P < 0.01$. The experiments were repeated twice with similar results.

Fig. 5. Antioxidant activity percentage of yeast strains. The significance of differences between groups (*Saccharomyces boulardii* and each yeast isolated) was determined using Student t tests and analyses of variance. $P < 0.05$ was considered statistically significant. $**P < 0.01$. The experiments were repeated twice with similar results.

Fig. 6. β -galactosidase activity of yeast strains. 1 U is equivalent to ONP production of 1 μ M/min.

Table 1. Yeast species studied, number of strains belonging to each species, source of isolation, and origin.

Species	Strains	Accession no.	Origin
<i>Dekkera bruxellensis</i>	JYC2537	MK044031	kombucha; Taiwan
<i>Hanseniaspora osmophila</i>	JYC2504	MK044002	fermented vinegar; Taiwan
<i>Hanseniaspora valbyensis</i>	JYC2522	MK044019	water kefir; Germany
<i>Kazachstania exigua</i>	JYC2539	MK044033	rice wine; Taiwan
<i>Kazachstania servazzii</i>	JYC2564	MK044044	milk kefir; Taiwan
	JYC2579	MK044059	milk kefir; Taiwan
	JYC2565	MK044045	milk kefir; Germany
	JYC2568	MK044048	milk kefir; Germany
	JYC2570	MK044050	milk kefir; Germany
	JYC2571	MK044051	milk kefir; Germany
	JYC2572	MK044052	milk kefir; Germany
	JYC2573	MK044053	milk kefir; America
	JYC2574	MK044054	milk kefir; America
	JYC2580	MK044060	milk kefir; Caucasus
	JYC2583	MK044063	milk kefir; Tibet
<i>Kazachstania turicensis</i>	JYC2529	MK044026	milk kefir; Taiwan
	JYC2559	MK044040	milk kefir; Taiwan
<i>Kluyveromyces marxianus</i>	JYC2528	MK044025	milk kefir; Taiwan
	JYC2557	MK044038	milk kefir; Taiwan
	JYC2563	MK044043	milk kefir; Taiwan
	JYC2578	MK044058	milk kefir; Taiwan
	JYC2566	MK044046	milk kefir; Germany
	JYC2567	MK044047	milk kefir; Germany
	JYC2569	MK044049	milk kefir; Germany
	JYC2575	MK044055	milk kefir; Israel
	JYC2576	MK044056	milk kefir; Israel
<i>Kwoniella shandongensis</i>	JYC2515	MK044012	wine lees; China
<i>Pichia kudriavzevii</i>	JYC2527	MK044024	milk kefir; Taiwan
<i>Saccharomyces boulardii</i>	I-3799		(purchased from New Foods, USA)
<i>Saccharomyces cerevisiae</i>	JYC2503	MK044001	fermented vinegar; Taiwan
	JYC2517	MK044014	wine lees; China
	JYC2506	MK044003	wine; Taiwan
	JYC2507	MK044004	wine; Taiwan
	JYC2508	MK044005	wine; Taiwan
	JYC2509	MK044006	wine; Taiwan
	JYC2532	MK079214	W303-derivative strain*
	JYC2510	MK044007	rice wine; Taiwan
	JYC2540	MK044034	rice wine; Taiwan
	JYC2541	MK044035	rice wine; Taiwan
	JYC2542	MK044036	rice wine; Taiwan
	JYC2521	MK044018	water kefir; Taiwan
	JYC2530	MK044027	wine; China
	JYC2531	MK044028	wine; China
	JYC2535	MK044029	kombucha; Taiwan
	JYC2558	MK044039	milk kefir; Taiwan
	JYC2577	MK044057	milk kefir; Israel
	JYC2581	MK044061	milk kefir; Caucasus
	JYC2582	MK044062	milk kefir; Caucasus
<i>Saccharomycopsis fibuligera</i>	JYC2511	MK044008	wine lees; China
	JYC2512	MK044009	wine lees; China
	JYC2516	MK044013	wine lees; China
	JYC2518	MK044015	wine lees; China
	JYC2519	MK044016	wine lees; China
	JYC2523	MK044020	wine lees; China
<i>Torulaspora delbrueckii</i>	JYC2520	MK044017	water kefir; Taiwan

<i>Wickerhamomyces anomalus</i>	JYC2560	MK044041	fermented vinegar; Tawian
<i>Zygosaccharomyces bisporus</i>	JYC2526	MK044023	coffee beans; Taiwan
	JYC2543	MK044037	chinese-herbal medicine; Taiwan
	JYC2536	MK044030	kombucha; Taiwan
<i>Zygosaccharomyces rouxii</i>	JYC2561	MK044042	fermented vinegar; Taiwan

*W303 is one laboratory strain that is closely related to the first completely sequenced yeast strain, S288C.

Table 1. Antioxidant activity and Trolox equivalents of yeast strains.

Percentage	Trolox equivalents	Species	Strain
<20%	From 30.4 ± 5.4 to 31.3 ± 7.9	<i>H. osmophila</i> <i>S. cerevisiae</i>	JYC2504 JYC2506, JYC2535
Between 20 and 30%	From 34.1 ± 18.5 to 43 ± 10.4	<i>K. servazzii</i> <i>S. boulardii</i> <i>S. cerevisiae</i> <i>W. anomalus</i>	JYC2570 I-3799 (New Foods, USA) JYC2508, JYC2509, JYC2510 JYC2560
Between 30 and 40%	From 50.2 ± 11.2 to 59.8 ± 10.9	<i>K. exigua</i> <i>K. marxianus</i> <i>K. servazzii</i> <i>K. shandongensis</i> <i>P. kudriavzevii</i> <i>S. cerevisiae</i> <i>S. fibuligera</i> <i>K. marxianus</i>	JYC2539, JYC2566, JYC2569 JYC2564, JYC2565, JYC2572 JYC2515 JYC2527 JYC2503, JYC2540, JYC2541 JYC2518 JYC2528, JYC2557, JYC2563, JYC2575, JYC2578
Between 40 and 50%	From 62.6 ± 6.2 to 79 ± 5.5	<i>K. servazzii</i> <i>K. turicensis</i> <i>S. cerevisiae</i> <i>S. fibuligera</i> <i>T. delbrueckii</i> <i>Z. bisporus</i>	JYC2568, JYC2571, JYC2574, JYC2583 JYC2529 JYC2507, JYC2517, JYC2521, JYC2530, JYC2542, JYC2558, JYC2531 JYC2516 JYC2520 JYC2543
>50%	From 80.6 ± 3.1 to 158.2 ± 5.4	<i>D. bruxellensis</i> <i>H. valbyensis</i> <i>K. marxianus</i> <i>K. servazzii</i> <i>K. turicensis</i> <i>S. cerevisiae</i> <i>S. fibuligera</i> <i>Z. bisporus</i> <i>Z. rouxii</i>	JYC2537 JYC2522 JYC2567, JYC2576 JYC2573, JYC2579, JYC2580 JYC2559 JYC2532, JYC2577, JYC2581, JYC2582 JYC2511, JYC2512, JYC2519, JYC2523 JYC2526, JYC2536 JYC2561

DISCUSSION

Bile salt and acid tolerance: The most essential criterion of any probiotic is the ability to tolerate high acid levels, which are present in the stomach. The hydrochloric acid (HCl) that is found in the human stomach is a strong oxidizer and kills bacteria, protecting the human body from pathogenic microbes that can enter the body through food. HCl can oxidize fatty acids, proteins, cholesterol,

and DNA in cells and disrupt them while it will undergo reduction (Pan *et al.*, 2009). In this *in vitro* study, the threshold point for determining acid resistance was set at pH 3.0 with an incubation period of 2 h to simulate the residence time in the stomach (Prasad *et al.*, 1998). This method followed the experimental results from Liong and Shah (Liong and Shah, 2005), who suggested that resistance at pH 3 is a suitable standard for testing the acid tolerance of a probiotic culture. The survival of

commercial probiotic strains when subjected to low pH and bile conditions, similar to those present in the human GI tract, should be assessed and substantiated accordingly. Although the concentration of bile in the human GI tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v (Gilliland and Speck, 1977). Upon exposure to bile acids, cellular homeostasis disruptions cause the dissociation of lipid bilayers and the integral proteins in their cell membranes, resulting in the leakage of microbial content and ultimately cell death (Kumura *et al.*, 2004). Of the yeast strains analyzed in the current study, we found that 39 (65%) could adapt well to the environment that simulated the human GI tract. Furthermore, we found that five strains adapted well for more than 24 h to the environment containing bile salt. The strains in this study were subjected to an environment with 0.3% w/v bile salt, which is similar to that in the human gut. Our results showed that most of the strains had better adaptability in this harsh environment than that of the commercialized probiotic yeast *S. boulardii*. Therefore, the yeast strains in this study are worthy of further investigation. The method used in this study only provided partial information about the outcomes of bile exposure (gastric acid). The *in vitro* conditions that the yeasts were exposed to in this study were not identical to an *in situ* situation (Guantario *et al.*, 2018). Therefore, the results may not truly reflect how the yeast strains would perform *in situ*, because many other physiological conditions might affect the survival of the yeast strains. Furthermore, in the human gut, the food matrix protects microorganisms from the deleterious effects of gastric and small intestinal secretions (Begley *et al.*, 2005; Huang and Adams, 2004). Nevertheless, the *in vitro* bile salt and acid tolerance assay used in this study provides useful information about species and strain differences.

Autoaggregation and cell surface hydrophobicity:

Analyses of surface properties, including autoaggregation and cell surface hydrophobicity, both support a probiotic's adherence to epithelial cells (de Lima *et al.*, 2017). The autoaggregation ability of probiotic bacteria correlates with adhesion, which is a prerequisite for colonizing and protecting the GI tract (Kos *et al.*, 2003). Autoaggregation is the reversible accumulation of cells, which causes them to spontaneously precipitate in the medium. Autoaggregation is also affected by different cell wall compositions and the presence of appendages (Touhami *et al.*, 2003) or the amount of adhesins. Cellular aggregates should also be considered when examining the potential of a probiotic, because aggregates can increase a microbe's ability to adhere to the intestine, which is a known prerequisite for colonization and enhanced persistence in the GI system (García-Cayuela *et al.*, 2014). The autoaggregation ability of yeast is better than that of bacteria because their

cells are larger and heavier, enabling them to precipitate quicker and in higher proportions. Here we considered an autoaggregation percentage of 80% before 4 h at 37 °C a good threshold for autoaggregation. The results showed that most strains complied with this threshold value, which is consistent with related studies (de Lima *et al.*, 2017; Gil-Rodríguez *et al.*, 2015). Hydrophobicity is likely due to a complex interplay between negatively charged, positively charged, hydrophobic, and hydrophilic components on the surface of microbes. Herein, we report that the yeast isolates showed high cell surface hydrophobicity. This result is consistent with related studies (Syal and Vohra, 2013). The variation in hydrophobicity to solvents has been explained by the fact that adhesion depends on the origin of the strains as well as their surface properties. Cell surface hydrophobicity is affected by the affinity between the cell wall and water and also by the environmental temperature. Blanco *et al.* (1997) reported that *Candida albicans* was hydrophobic at 22 °C and hydrophilic at 37 °C. Probiotics with good autoaggregation ability together with good cell surface hydrophobicity can be strongly related to the adhesion ability of the microorganisms they contain, although these two characteristics are independent (Boirivant and Strober, 2007; Krausova *et al.*, 2019).

Antioxidant activity: Here, the ability of whole cells rather than cell-free extracts from a culture based on DPPH scavenging was assayed. Most of the isolated strains showed good antioxidant activity; 39 strains (66%) had $\geq 40\%$ antioxidant activity in 0.2 mM DPPH. Chen *et al.* (2010a) reported that the antioxidant activity of intact cells is significantly higher than that of their corresponding intracellular cell-free extracts, which was contrary to the properties of lactic acid bacteria (LAB). The antioxidant activity of yeasts is believed to be mainly due to the high amount of β -glucans found in their cell walls (Abbas, 2006; Jaehrig *et al.*, 2007), as well as other cellular compounds (e.g., superoxide dismutase, glutathione peroxidase, and catalase) (Chen *et al.*, 2010b). Because intact cells show conventionally higher activity than do cell extracts, we propose that other mechanisms may be involved (Naylin *et al.*, 2005). Nevertheless, the antioxidant activity of yeasts seems higher than that of LAB. In our study, the antioxidant activity in TE was generally higher than that reported by Amaretti *et al.* (2013) for different LAB strains. The mean value of TE obtained for the yeast strains was 68.19 μM , whereas a maximum mean value of 158.2 μM was obtained for *S. fibuligera* JYC2512. These results are similar to those reported by Gil-Rodríguez *et al.* who analyzed 130 yeast strains from wine and various foods and found that the activity ranged between 73.39 ± 0.73 and $177.60 \pm 0.85 \mu\text{M}$ (Gil-Rodríguez *et al.*, 2015). However, our results (mean = 44.83%) are higher than those reported by Chen *et al.* (2010b) who analyzed intact

yeast cells collected from raw milk and found that the antioxidant activity ranged from 4.25% to 46.78%. We found that the strains of *S. fibuligera* had higher antioxidant ability than the other yeast species we isolated. *S. fibuligera* is usually found worldwide in starchy substrates and is a major amylolytic yeast in indigenous food fermentation. It has recently drawn attention because of its ability to accumulate trehalose when grown on starch (Chi *et al.*, 2009). Trehalose functions as an antioxidant, although its underlying molecular mechanisms remain unclear. The results from the current study suggest that trehalose is able to protect enzymes such as superoxide dismutase, catalase, and ascorbate peroxidase. Trehalose itself plays a key role in directly scavenging ROS, including O_2^- and H_2O_2 and it suggests that trehalose accumulation may play a vital role in the antioxidant activity of *S. fibuligera* and other yeasts (Luo *et al.*, 2008; Zhang *et al.*, 2020).

β -galactosidase activity: In the current study, the yeast strains that produced β -galactosidase were all isolated from milk kefir. Most of the isolated strains had higher enzyme activity than that of *S. cerevisiae*, which was isolated from Brazilian kefir-fermented milk (de Lima *et al.*, 2017). The β -galactosidase activity of the yeast strains in this study ranged from 0.58 U/mL (11.54 Miller units) to 17.97 U/mL (113.24 Miller units), but these values were less than those of bifidobacteria (147 to 860 Miller units) and *Lactobacillus acidophilus* (675 to 1301 Miller units) reported by Vinderola and Reinheimer (2003). These variations observed among yeasts and probiotic bacteria emphasize the importance of selecting appropriate strains for use as dietary adjuvants. Most of the strains with β -galactosidase activity (8 out of 10 strains) in this study belong to *K. marxianus*. Commercial β -galactosidases are produced from *K. marxianus* and *K. lactis* and molds such as *Aspergillus niger* and *A. oryzae* (Rosenberg, 2006). Thus, numerous studies have investigated the optimization of β -galactosidase production and extraction using *K. marxianus* (Bansal *et al.*, 2008; Brady *et al.*, 1995; Martins *et al.*, 2002; Pinheiro *et al.*, 2003; Stred'anský *et al.*, 1993). However, the yeast strains with β -galactosidase activity in the current study showed a very low tolerance to bile salts. Marteau *et al.* (1997) stated that a low bile resistance should not be considered a disadvantage for probiotic strains, because intracellular β -galactosidase or other bioactive compounds might be released from the cells through lysis. Lin *et al.* (1991) used *L. acidophilus* strains with varying degrees of resistance to bile and total β -galactosidase-producing potential to monitor lactose maldigestion by measuring breath hydrogen excretion. They demonstrated that only one strain with low bile resistance and intermediate β -galactosidase activity was capable of significantly reducing breath hydrogen values. Moreover, Mc Donough *et al.* (1987) showed that

unfermented milk prepared with disrupted cells of *L. acidophilus* improved lactose digestion more effectively than did that prepared with intact cells. By contrast, Zárate *et al.* (2000) found that the most bile-tolerant propionibacteria strains significantly reduced lactose intolerance symptoms. Noh and Gilliland (1993; 1994) suggested that bile does not cause cell lysis but that bile salts increase cellular permeability and permit more substrates to enter cells to be hydrolyzed. Thus, yeasts with low gastric and small intestine transit tolerance may be essential for *in vivo* lactose digestion. Because microbial lysis in the small intestine depends on bile salts, intracellular β -galactosidase might be released from cells through lysis during the passage through the GI tract or, at least, permeabilized cells might be necessary for an efficient lactose hydrolysis to occur in the small intestine (Mustapha *et al.*, 1997). Furthermore, thermostable β -galactosidases have presently attracted considerable attention because of their significant advantages in processing (Liu *et al.*, 2019). It would be worth considering whether we could use benchtop molecular biology and computer-assisted strategies to improve the thermostability of β -galactosidases from these candidate yeasts in future investigations.

Conclusions: Probiotics occupy a considerable share in the food and drug industries. The present study focused on expanding the selection of probiotic yeasts from fermented food and beverage. The results showed that the probiotic characteristics and resistance to biological barriers of the isolated yeast strains varied considerable, even among strains belonging to the same species. For example, most strains of *S. fibuligera* had good antioxidant activity and most strains of *K. marxianus* had β -galactosidase activity and cell surface hydrophobicity. In particular, *S. cerevisiae* JYC2508 had good results in the assays but not in β -galactosidase activity, whereas *K. marxianus* JYC2528 had good performance in all the tests and demonstrated good β -galactosidase activity. Therefore, adequate strain selection should be conducted in future studies to combine different yeasts in foods to evaluate the total "probiotic value" of the starter strains involved. Our findings should encourage further studies on the application of the strains in this study as food and feed supplements. Nevertheless, further investigation is required to confirm their safety.

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