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Selection of potential non-*Sacharomyces* probiotic yeasts from food origin by a step-by-step approach

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ABSTRACT

Due to healthcare is increasing in nowadays, the use of the commercial probiotics is in progress and each day they are more demanded. The challenge of this study is to identify yeast species for using as probiotic organisms. Thus, the research applied a step-by-step approach, to study the probiotic potential of non-*Saccharomyces* yeast strains. The 215 yeasts were isolated from different environments such as wineries, oil mills, brines cheeses, fermented vegetables and distilleries in previous works and were identified to strain level by RAPD-PCR technique resulting 108 different strains. A general screening was carried out to know the probiotic capability of the yeasts, following the next steps: study of the ability to resist and grow of the yeasts when they exposed to simulated *in vitro* digestion conditions and influence of time, temperature, pH and the presence of enzymes on the kinetic growth parameters (lag phase (λ), generation time (G), maximum OD (OD_{max}) and the specific growth rate constant (μ_{max})). The results made possible the selection of the 23% of the strains and they were assayed for knowing their capability of self-aggregation and hydrophobicity. Biofilm formation capacity and viability after simulated sequential salivary-gastric-intestinal digestion were then studied for the 10 best strains. Statistical analyses were applied in each step to make the selection. The final results showed that two yeasts, *H. osmophila* and *P. kudriavzevii,* were the most promising strains.

1. Introduction

The Joint Food and Agricultural Organisation/World Health Organisation (2001) define probiotics as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host". However, Hill et al., 2014, published a document on the appropriate use and scope of the term probiotic for clinicians and consumers in differentiating the diverse products on the market. They conclude that this clarification is needed for facilitating the advances in probiotic research and for ensuring that their benefits, will be properly communicated to consumers and patients.

Due to healthcare is increasing in nowadays, the use of the commercial probiotics is in progress and each day are more demanded. Probiotics can be delivered as drugs or through food. However, they must survive the conditions of the upper gastrointestinal tract and then persist in the intestine, to provide beneficial effects to the host. Some of the potential mechanisms by which yeast protect the host against pathogens include immune system stimulation, the induction of growth of other probiotic organisms, degradation of bacterial toxins by the yeast's proteases and inhibition of pathogen adherence to gastrointestinal epithelial cells (Pérez-Sotelo et al., 2005; Arévalo-Villena, Fernández-Pacheco, Castillo, Bevilacqua, & Briones, 2018).

Most probiotics currently commercialised are of bacterial origin because the majority of yeasts are particularly sensitive to the gastrointestinal tract conditions (Czerucka & Rampal, 2002). The challenge is identifying yeast species that could be considered probiotic organisms. Some yeasts have shown resistance to the gastrointestinal conditions, which, together with their antibiotic resistance (Blehaut, Massot, Elmer, & Levy, 1989), has made these eukaryotic organisms possible candidates for the development of new probiotics (Kourelis et al., 2010). For instance, the probiotic character of *Saccharomyces cerevisiae* var. *boulardii*, in particular, is well documented (Blehaut, Massot, Elmer, & Levy, 1989; Czerucka & Rampal, 2002; Klein, Elmer, McFarland, Surawicz, & Levy, 1993). Perricone, Bevilacqua, Corbo, and Sinigaglia (2014) showed that this strain is able to survive at pH 2.5 in the presence of bile salts, displays a hydrophobic property and shows biofilm

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formation. Some authors have found that other *Saccharomyces* sp. also possess these properties (Pennacchia, Blaiotta, Pepe, & Villani, 2008; Pérez-Sotelo et al., 2005; Pizzolitto et al., 2012; Sourabh, Kanwar, & Sharma, 2012).

Besides *Saccharomyces* sp., recent studies have demonstrated the existence of other yeast species with probiotic potential, such as *Wickerhamomyces anomalus* (García-Hernández et al., 2012), *Candida krusei*, *Kluyveromyces marxianus*, *Candida rugosa* and *Trichosporon asahii* (Pedersen, Owusu-Kwarteng, Thorsen, & Jespersen, 2012) or *Pichia kudriavzevii* (Chelliah, Ramakrishnan, Prabhu, & Antony, 2016). Psani and Kotzekidou (2006) found that a large majority of *Torulaspora delbrueckii* and *Debaryomyces hansenii* strains tolerated high bile salt concentrations and inhibited the growth of several pathogens (Psani & Kotzekidou, 2006). Yeast strains *Pichia membranaefaciens* and *Candida oleophila* showed similar characteristics (Silva et al., 2011).

The aim of this research is to study the probiotic potential character of the non-*Saccharomyces* wild yeast strains isolated from different sources such as wineries, oil mills, brine cheeses, fermented vegetables and distilleries. For that tolerance to gastrointestinal conditions (pH, temperature, enzymes), auto-aggregation, hydrophobicity, biofilm formation and behavior after simulated sequential salivary-gastric-intestinal digestion were analysed. It is noted that each decision was made based on multifactorial statistical assay results.

2. Materials and methods

2.1. Yeast strains

A total of 215 yeasts belonging to various genera, such as *Candida* (10 species), *Debaryomyces* (3 species), *Hanseniaspora* (5 species), *Kazachstania* (2 species), *Kluyveromyces* (1 species), *Lachancea* (2 species), *Metschnikowia* (1 species), *Ogataea* (1 species), *Pichia* (9 species), *Rhodotorula* (1 species), *Torulaspora* (1 species), *Yarrowia* (1 species) and *Zygosaccharomyces* (3 species) were evaluated. The yeast strains are deposited in the culture collection of the Yeast Biotechnology Laboratory (University of Castilla-La Mancha, Spain) and maintained at −80 °C in a glycerol solution. All the strains were isolated in previous studies from food environment: winery, from white (Airen) or red (Cencibel) musts, at different stages of spontaneous fermentation (beginning or middle); oil mills from two different varieties (Arbequina and Cornicabra) being all of them from olive pastes and pomaces; brine chesses; different stages of vegetable fermentations and distillery plants sweet and fermented piquettes from ethanol production process (Barrajón, Arévalo-Villena, & Briones, 2009; Ortiz, Barrajón, Aalver-Baffi, Arévalo-Villena, & Briones, 2013; Romo Sánchez, Alves Baffi, Arévalo, Úbeda, & Briones, 2010; Úbeda, Maldonado Gil, Chiva, Guillamón, & Briones, 2014). As positive controls, a *S. cerevisiae* strain UCLM 3 with probiotic characteristics found by Arévalo-Villena et al. (2018) (control 1+) and a commercial probiotic *S. cerevisiae* var. *boulardii* strain (control 2+) were used.

For their use and before each assay, the strains were grown in YPD broth (Pronadisa-Conda) and incubated at 30°C for 48 h with a shaking incubator. The cell concentration was determined by enumeration of yeast cells, using a Thoma chamber. The cells were harvested by centrifugation at room temperature (5000 rpm for 5 min). After that, cells were washed with saline solution and the pellet was immediately use for the corresponding assay.

2.2. Differentiation of non-Saccharomyces strains by random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

Molecular identification included PCR reactions of 5.8S rRNA region gene with ITS1 and ITS2 primers followed by restriction pattern analysis (RFLP) were used to identify of yeasts at specie level. It is well known that some microbial properties are strain-dependent, so, to optimise the work, an initial identification of the 215 yeasts mentioned above was made at the strain level. A cell pellet of each strain was treated with a zymolyase solution (10 mg/mL zymolyase 20 T in 1.2 M sorbitol buffer, 40 mM sodium phosphate buffer, pH 7). After incubation at 37°C/30 min and 95°C/5 min, a cell lysate was obtained. RAPD-PCR reaction was accomplished using primer R3 (5`ATGCAGCCAC3`) and 1 μL of DNA in a final volume of 10 μL, according to the conditions established by Corte et al. (2005), and primer M13 (5`GAGGGTGGCG GTTCT3`), using 1.5 μL of DNA in a final volume of 15 μL, under the conditions detailed by Padilla, Manzanares, and Belloch (2014). PCR amplification was performed in a Perkin − Elmer GeneAmp PCR System 2400. The amplified DNA was separated by electrophoresis in 2% *w/v* agarose gels and visualised by the gel Green (6×).

2.3. Growth kinetics under gastrointestinal conditions: preliminary probiotic screening

All strains were exposed to the gastrointestinal conditions described by Arévalo-Villena et al. (2018). For the gastric conditions, 8 log CFU/mL were inoculated in gastric solution (YPD containing 3 mg/mL pepsin in phosphate buffered saline, pH 2) and maintained at 37°C/3 h, under static conditions. Then, 10 μL of this solution was transferred to an intestinal solution (YPD containing 0.5% bile salts and 1 mg/mL pancreatin in YPD broth, pH 8) and incubated at 37°C/22 h. Cell growth in the intestinal solution was evaluated at 600 nm, using an ELX808 Absorbance Microplate Reader (Bio-Tek Instruments, Vermont, USA). Measurements were taken every 20 min for 24 h, with an agitation period of 15 s before reading.

Growth curves were obtained by plotting optical density (OD) *versus* time. Kinetic parameters including lag phase (λ), generation time (G), maximum OD (OD_{max}) and the specific growth rate constant (μ_{max}) were calculated, using the model described by Warringer and Blomberg (2003).

2.4. Auto-aggregation capability

The method proposed by Bautista-Gallego et al. (2013), with slight modifications, was adopted to investigate the auto-aggregation. Briefly, the washed cell pellets from overnight cultures were resuspended in an equal volume of saline solution (0.9% NaCl) and incubated at 37°C/30 min. Absorbance at 600 nm (Jasco V-530 spectrophotometer) of an aliquot taken from the upper suspension, both at time zero (inoculation time) (A_0) and 30 min (A_F) were measured. The auto-aggregation percentage was calculated as follows:

$$[1 - (A_F/A_0)] \times 100\%$$

2.5. Cell surface hydrophobicity

Hydrophobicity assays were performed according to the method described by Bautista-Gallego et al. (2013), with modifications. Cell biomass was suspended in 10 mL KNO_3 (0.1 M) and absorbance at 600 nm was measured (A_0). Then, 3 mL toluene and xylene, respectively, were added to independent samples. After incubation at 37°C min/60 min, without shaking, the absorbance of the interphase was measured (A_F). The hydrophobicity percentage was calculated as follows:

$$[1 - (A_F/A_0)] \times 100\%$$

2.6. Biofilm formation

Biofilm formation was monitored as described by Speranza, Corbo, and Sinigaglia (2011), with modifications. A population of 6 log CFU/mL was inoculated in flasks with YPD and a sterile glass slide, and incubated at 37°C. After 24 h, each slide was aseptically removed, washed with sterile water and introduced into a flask containing 45 mL of

sterile saline solution. The samples were sonicated (Ultrasonic processor, Qsonica) for 3 min at a constant 20% power, to suspend the biofilm adhered to the surface slide. Cell viability was determined after seeding of the cells using an Eddy Jet 2 spiral seeding machine (IUL Instruments). Results were expressed as log CFU/cm^2.

2.7. Study of viability and biofilm formation of yeasts after sequential simulated digestion

Sequential simulated digestion was performed as reported by Armando et al. (2012), with the modifications proposed by Kos, Šušković, Goreta, and Matošić (2000) and Priya, Vijayalakshmi, and Raichur (2011). For the salivary conditions, a population of 8 log CFU/mL was suspended in a sterile electrolyte solution containing 0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$ (Sigma-Aldrich) and 100 mg/L lysozyme (pH 6.5), and incubated at 300 rpm/37 °C/5 min. After exposure to salivary conditions, cells were harvested by centrifugation (5000 rpm/25 °C/15 min), suspended in simulated gastric fluid (0.9% NaCl, buffered to pH 2.0 and containing 3 g/L pepsin [porcine gastric mucosal, Sigma-Aldrich]) and then, incubated at 37 °C/2 h with shaking (300 rpm). After gastric simulation, cells were harvested by centrifugation (5000 rpm/25 °C/15 min), and suspended in simulated intestinal fluid at 37 °C/4 h at 300 rpm, using an orbital shaker. The intestinal fluid was prepared by adding 1 g/L pancreatin (porcine pancreas, Sigma-Aldrich) and 3 g/L bile extract (porcine bile extract, Sigma-Aldrich) to a solution at pH 8, containing 6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$ and 1.386 g/L $NaHCO_3$.

Before and after sequential simulated digestion, the loss of viability was evaluated by plate counting, and biofilm formation by the procedure described in section 2.6.

Experiments detailed from between Sections 2.3 to 2.7 were carried out by quadruplicate.

2.8. Statistical analysis

Analysis of variance (ANOVA) and Duncan's test were applied to study the significant differences between the parameters found for each strain ($p < 0.05$). Correlations among the variables were identified by principal component analysis (PCA). All data analysis was performed with Excel 2013 (Microsoft Corporation) and SPSS (IBM SPSS Statistics 20).

3. Results and discussion

3.1. Differentiation of non-Saccharomyces strains by random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)

Molecular techniques have been successfully applied by several authors to identify the yeast biodiversity in diverse environments, not only for species identification, but also for strains identification (Andrighetto, Psomas, Tzanetakis, Suzzi, & Lombardi, 2000; Grando, Ubeda, & Briones, 1994; Guillamón & Barrio, 2017; Suzzi et al., 2000; Úbeda, Maldonado Gil, Chiva, Guillamón, & Briones, 2014), due to the fact that probiotic activity it is associated to strain-level.

In the current study, isolates from the same yeast species showed different genetic polymorphisms that were effectively differentiated by RAPD-PCR. Consequently, the 215 original isolates were differentiated into 108 strains. Table 1 shows the species, number of isolates and number of strains identified by RAPD-PCR, as well as the nomenclature assigned to each strain.

3.2. Survival in simulated gastrointestinal digestion

The 108 non-*Saccharomyces* strains were evaluated to verify their capacity to survive a simulated gastrointestinal process, and their growth kinetic parameters were obtained. For a microorganism to be considered as possessing potential probiotic character, it must arrive viable and functional in the large intestine. Hence, for initial discrimination, all yeasts with generation times ≥22 h were discarded (Dunne et al., 2001). Twenty-two hours was chosen as the cut-off value because this is the usual time required for the total gastrointestinal transit. In all, 25 strains (23% of the total) displayed a generation time < 22 h (Table 2). The remaining strains either did not grow at 37°C or did not tolerate acid pHs. Thus, the 25 possible candidate strains were further investigated by additional probiotic tests.

One factor ANOVA and Duncan's test was performed to identify the strains presenting the best experimental results. These tests indicated that for the lag phase (λ) ($\alpha = 0.05$; $F = 19.22$; $p = 0.00$), the best strain belonged to subset 1, *C. pararugosa* (1231), with λ of 0.15 h. This was followed by subset 2, corresponding to strains belonging to *H. valbyensis* (1094), *K. thermotolerans* (1039), *P. anomala* (1082, 1090), *M. pulcherrima* (1012), *P. kudriavzevii* (1003, 1075), *C. vini* (1063), *Lachancea* sp. (1146) that had λ by 4,40–6, 45 and, also, the commercial probiotic (control 2+). Moreover, in subset 3, strains *H. osmophila* (1056), *K. thermotolerans* (1167), *Lachancea* sp. (1148) and *P. kudriavzevii* (1200) proved relevant, with λ ranging from 6.98 − 7.30 h. All other stains showed λ > 7.30 h.

For the generation and rate parameters ($\alpha = 0.05$; $F = 8.34$; $p = 0.00$), the strains that presented the best values, were *K. thermotolerans* (1167), *Lachancea* sp. (1148), *H. osmophila* (1056), *Z. fermentati* (1134), *P. kudriavzevii* (1200), *Z. fermentati* (1142), *Z. bailii* (1213) and *Lachancea* (1146), besides control 1+. Moreover, these strains had higher growth rates than the commercial yeast. Finally, for the parameter OD_{max}, ($\alpha = 0.05$; $F = 9.41$; $p = 0.00$) the strains with the best values were *H. osmophila* (1056), *Lachancea* (1148), *T. delbrueckii* (1055), *Z. fermentati* (1134), *Lachancea* (1146), *P. kudriavzevii* (1200), *P. Caribbica* (1135), *P. kudriavzevii* (1003), *K. thermotolerans* (1167), *Z. fermentati* (1187), as well as the controls (Table 2).

Considering the various parameters evaluated, strain *Lachancea* (1146) was the only one within the first two subsets that revealed the best generation, being, therefore, the one with the best kinetic characteristics, followed by *H. osmophila* (1056), *Lachancea* (1148), *K. thermotolerans* (1167) and *P. kudriavzevii* (1200). PCA verified these results (Fig. 1 and Table 3), where the five strains with the better aptitudes were included in the indicated zone. Moreover, these strains performed better than the controls. As expected, the parameters μ_{max} and OD_{max} presented values directly proportional among the best strains.

Other authors showed good results for *W. anomalus* (García-Hernández et al., 2012), *P. kudriavzevii* that tolerated physiological concentrations of bile salts, pepsin and pancreatin (Chelliah, Ramakrishnan, Prabhu, & Antony, 2016) or a large number of *T. delbrueckii* and *D. hansenii* strains tolerated high bile salt concentrations (Psani & Kotzekidou, 2006).

3.3. Auto-aggregation assays

The ability to adhere to epithelial cells and mucosal surfaces and, thereby, decrease or prevent the colonisation of pathogens has been suggested to be an important property of many probiotic strains (Vine et al., 2004). In most instances, aggregation ability was associated with cell adherence properties (Boris, Suárez, & Barbés, 1997). Cell aggregation between microorganisms of the same strain (auto-aggregation) or between different strains (coaggregation) is of considerable importance in several ecological niches. Recent studies have demonstrated that there are many yeast strains with very high auto-aggregation rates (Arévalo-Villena et al., 2018; Binetti, Carrasco, Reinheimer, & Suárez, 2013; Chelliah, Ramakrishnan, Prabhu, & Antony, 2016).

In this research, the controls and the 25 strains identified from the initial screening tests as potential probiotic candidates were tested for their ability to self-aggregate (Table 4). The auto-aggregation percentages were highly variable, ranging from 3.85 − 64.43%. *H. osmophila*

Table 1
Strains of each specie, its nomenclature and source of isolation.

Species	I	St	Nomenclature	Species	I	St	Nomenclature
Candida lactis-condensi	4	2	1113[D],1115[D]	*Kluyveromyces thermotolerans*	12	6	1007[W],1039 [W],1093 [D], 1109 [D],1167 [V],1186 [W]
C. ethanolica	4	3	1099 [D],1104 [D],1111[D]	*Lachancea sp.*	4	2	1146[M],1148[M]
C. apícola	8	8	1054[W],1064 [W],1065 [W], 1066[W],1067 [W],1068 [W], 1070 [W],1071 [W]	*L. thermotolerans*	1	1	1189[M]
C. diddensiae	1	1	1154[M]	*Metschnikowia pulcherrima*	5	4	1002[W],1004[W],1005[W], 1012[W]
C. pararugosa	5	3	1228[C],1231[C],1237[C]	*Ogataea polymorpha*	1	1	1087[D]
C. sake	1	1	1107[D]	*Pichia anomala*	6	3	1082 [D],1089 [D],1090 [D]
Candida sp.	2	2	1151[M],1152[M]	*P. galeiformes*	7	5	1081 [D],1101 [D],1102 [D], 1110 [D],1112 [D]
C. stellata	3	2	1016[W],1003[W]	*P. holstii*	6	3	1169[M],1176[M],1188[M]
C. thermophila	1	1	1179[M]	*P. caribbica*	14	3	1135[M],1162[M],1181[M]
C. vini	1	1	1063[W]	*P. galeiformes*	10	1	1081[D]
Debaryomyces hansenii	8	5	1001[C],1225[C],1235[C], 1236[C],1240[C]	*P. kudriavzevii*	12	5	1003 [W],1075 [D],1084 [D], 1085 [D],1200[W]
D. pseudopolymorphus	1	1	1072[W]	*P. membranaefaciens*	8	3	1006[W],1019 [W],1091[D]
D. polymorphus	1	1	1053[W]	*P. mississippiensis*	2	1	1091[D]
Hanseniaspora meyery	1	1	1079[D]	*P. occidentalis*	8	4	1203[V],1206[V],1208[V], 1212[V]
H. osmophila	15	5	1056 [D],1076 [D],1094 [D],1117 [D], 1118 [D]	*Rhodotorula mucilaginosa*	2	3	1017 [W],1047 [W],1229[C]
H. valbyensis	1	1	1077[D]	*Torulaspora delbrueckii*	17	4	1018 [W],1055 [W],1073 [D], 1192 [W]
H. guillermondii	4	2	1035[D],1199 [D]	*Yarrowia lipolitica*	2	1	1222[C]
H. uvarum	8	5	1032[D],1033[D],1034[D], 1083[D],1096 [D]	*Zygosaccharomyces fermentati*	18	7	1061 [W],1086 [D],1134 [W], 1142 [W],1157 [W],1171 [W], 1187 [W]
Kazachstania exigua	2	1	1220[W]	*Z. bailii*	4	3	1098 [D],1213 [V],1214 [V]
K. unispora	4	1	1216[V]	*Z. florentinus*	1	1	1183[M]

I: number of isolates; St: number of strains.
C: brine cheese; D: distillery; V: fermented vegetables; M: oil mill; W: Winery.

Table 2
Kinetic parameters obtained for strains with generation times (G) < 22 h.

Species	Strain nomenclature	λ (h)	μ (1/h)	G (h)	OD_{max} (nm)
Control 1+	**3**	**8.75 ± 1.25^{fghi}**	**0.16 ± 0.05^{hijkl}**	**1.99 ± 0.47^{a}**	**1.37 ± 0.13^{i}**
Control 2+	**24**	**5.40 ± 0.73^{bcd}**	**0.09 ± 0.03^{bcdef}**	**4.18 ± 2.84^{bc}**	**1.05 ± 0.47^{defghi}**
P. kudriavzevii	**1003**	**6.32 ± 0.16^{bcdef}**	**0.10 ± 0.03^{cdefgh}**	**3.26 ± 0.89^{ab}**	**1.07 ± 0.21^{defghi}**
M. pulcherrima	1012	5.55 ± 4.75^{bcd}	0.09 ± 0.00^{bcdefg}	3.23 ± 0.10^{ab}	0.82 ± 0.16^{bcde}
P. membranaefaciens	1019	14.30 ± 1.28^{lm}	0.08 ± 0.02^{abcde}	3.84 ± 1.00^{ab}	0.92 ± 0.28^{bcdef}
K. thermotolerans	1039	5.13 ± 1.04^{bc}	0.10 ± 0.01^{cdefgh}	2.89 ± 0.18^{ab}	1.00 ± 0.17^{cdefgh}
T. delbrueckii	**1055**	**11.68 ± 1.66^{jk}**	**0.15 ± 0.07^{fghijk}**	**2.38 ± 1.07^{ab}**	**1.31 ± 0.11^{ghi}**
H. osmophila	**1056**	**6.98 ± 0.35^{cdefg}**	**0.21 ± 0.02^{kl}**	**1.46 ± 0.17^{a}**	**1.38 ± 0.04^{i}**
C. vini	1063	6.20 ± 0.50^{bcde}	0.08 ± 0.02^{bcde}	3.78 ± 0.93^{ab}	0.72 ± 0.30^{bcd}
P. kudriavzevii	1075	6.15 ± 0.00^{bcde}	0.11 ± 0.01^{defghi}	2.64 ± 0.20^{ab}	0.97 ± 0.15^{bcdefg}
P. galeiforms	1081	12.02 ± 0.75^{k}	0.04 ± 0.01^{abc}	7.93 ± 2.02^{d}	0.80 ± 0.45^{bcde}
P. anomala	1082	5.15 ± 0.00^{bc}	0.09 ± 0.02^{bcdef}	3.44 ± 0.53^{ab}	0.93 ± 0.18^{bcdef}
O. polymorpha	1087	8.15 ± 0.00^{efgh}	0.05 ± 0.01^{abcd}	6.43 ± 1.34^{cd}	0.67 ± 0.45^{bc}
P. anomala	1090	5.30 ± 3.23^{bcd}	0.16 ± 0.04^{ghijk}	1.97 ± 0.53^{a}	0.93 ± 0.29^{bcdef}
H. osmophila	1094	4.40 ± 0.50^{b}	0.13 ± 0.04^{efghij}	2.59 ± 0.93^{ab}	0.83 ± 0.04^{bcde}
C. sake	1107	9.35 ± 3.01^{ghij}	0.03 ± 0.01^{ab}	12.64 ± 5.86^{e}	0.81 ± 0.44^{bcde}
Z. fermentati	**1134**	**10.88 ± 0.85^{ijk}**	**0.20 ± 0.01^{kl}**	**1.51 ± 0.11^{a}**	**1.29 ± 0.03^{fghi}**
P. caribbica	**1135**	**9.48 ± 0.83^{hij}**	**0.15 ± 0.01^{fghijk}**	**1.97 ± 0.07^{a}**	**1.16 ± 0.15^{efghi}**
Z. fermentati	**1142**	**9.48 ± 0.47^{hij}**	**0.17 ± 0.02^{ijkl}**	**1.74 ± 0.16^{a}**	**0.61 ± 0.04^{b}**
***Lachancea* sp.**	**1146**	**6.45 ± 0.00^{bcdef}**	**0.17 ± 0.13^{ijkl}**	**1.74 ± 0.14^{a}**	**1.20 ± 0.04^{fghi}**
***Lachancea* sp.**	**1148**	**7.30 ± 0.17^{cdefgh}**	**0.22 ± 0.01^{kl}**	**1.46 ± 0.45^{a}**	**1.35 ± 0.07^{hi}**
Candida sp.	1151	15.80 ± 4.74^{m}	0.02 ± 0.01^{a}	18.57 ± 6.01^{f}	0.03 ± 0.01^{a}
K. thermotolerans	**1167**	**7.15 ± 0.00^{cdefgh}**	**0.23 ± 0.01^{kl}**	**1.31 ± 0.03^{a}**	**1.04 ± 0.07^{defghi}**
Z. fermentati	**1187**	**7.80 ± 1.76^{defgh}**	**0.12 ± 0.02^{defghi}**	**2.67 ± 0.41^{ab}**	**1.06 ± 0.06^{defghi}**
P. kudriavzevii	**1200**	**7.33 ± 1.17^{cdefgh}**	**0.19 ± 0.11^{jkl}**	**2.43 ± 1.80^{ab}**	**1.12 ± 0.23^{efghi}**
Z. bailii	**1213**	**12.81 ± 0.58^{kl}**	**0.17 ± 0.01^{ijkl}**	**1.73 ± 0.12^{a}**	**0.94 ± 0.14^{bcdef}**
C. pararugosa	**1231**	**0.15 ± 0.00^{a}**	**0.04 ± 0.00^{abc}**	**8.21 ± 0.36^{d}**	**0.20 ± 0.05^{a}**

Indicate in bold those strains that present the best results for at least one of the parameters. Different letter indicate significantly differences between strains for each parameter.

(1056) and *C. pararugosa* (1231) provided the best results, with a self-aggregation of 64.43 and 63.34%, respectively, which were statistically similar ($\alpha = 0.05$; $F = 17.53$; $p = 0.00$). Thus, ten strains, including 1134, 1090, 1003, 1094, 1213, 1019, 1063, 1039 and 1231 exhibited a higher auto-aggregation than the controls, with values ranging from 26.05−64.43%. There were no significant differences among the strains despite the majority of the strains having 5 to 20% auto-aggregation.

Gil-Rodríguez, Carrascosa, and Requena (2015) found that the auto-aggregation for one strain of *T. delbrueckii* was 21.4%, a result comparable with our *T. delbrueckii* strain (1055) (19.06%). *S. cerevisiae* obtained from traditional fermented foods of the Western Himalayas (Sourabh, Kanwar, & Sharma, 2011, 2012) showed an auto-aggregation ability of about 67.54% in 20 h. Some studies observed auto-aggregation values of around 70% in 2 h (Binetti, Carrasco, Reinheimer, & Suárez, 2013; Gil-Rodríguez, Carrascosa, & Requena, 2015). In contrast, results of the present study showed certain strains that achieved between 41 and 64% auto-aggregation, at only 30 min of incubation. Auto-aggregation is dependent on the strain and its cell wall composition because this property is mediated by cell-surface molecules, which

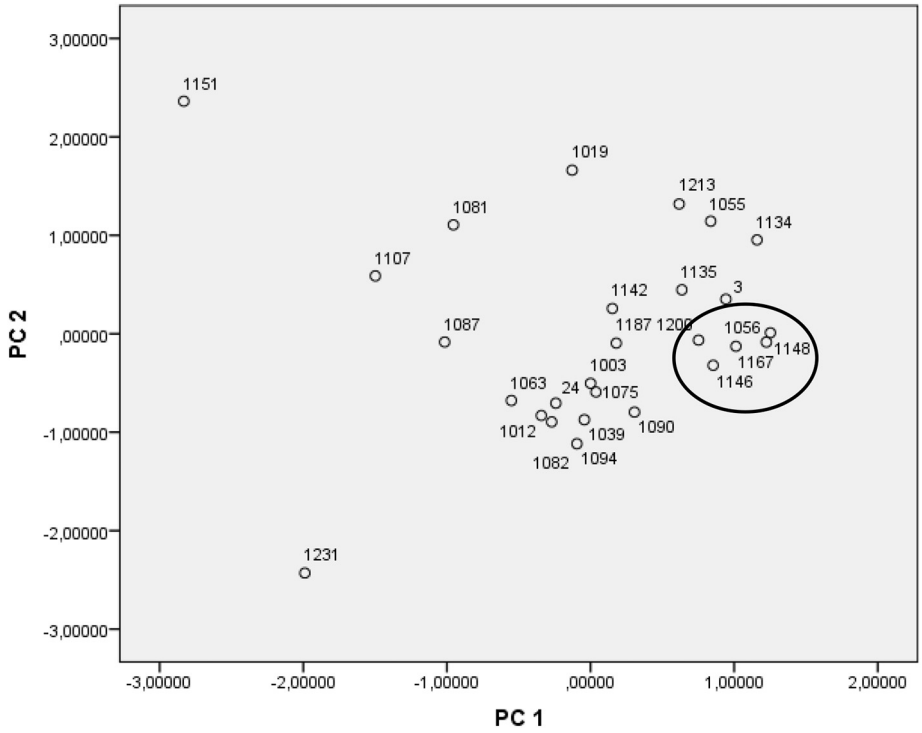

Fig. 1. Principal Component Analysis run on the fitting parameters of Warringer and Blomberg (2003) recovered for all the strains.

Table 3
Contribution of the highly correlated variables and their loadings in principal components 1 and 2.

Principal component	Variance explained (%)	Total variance (%)	Most highly correlated	Loading
1	63.08	63.08	G	−0.89
			λ	−0.05
2	25.84	88.92	G	0.31
			μ	0.02
			OD_{max}	0.04

could explain the differences in the values found among the yeasts. The ability to self-aggregate within 30 min, as demonstrated by certain strains in this study, could be an advantage, considering that during their passage through the gastrointestinal tract they may rapidly adhere to the mucosa before being expelled.

3.4. Cell surface hydrophobicity

The 25 selected strains and the controls were tested for their hydrophobicity, using two solvents, toluene and xylene. These solvents have an apolar character, and the greater the solubility of the cells in these solvents, the greater the degree of hydrophobicity.

For xylene (Table 5), the hydrophobicity values ranged from 3.16 − 54.16%. The strains that presented the best results were *Z. bailii* (1213) and *C. pararugosa* (1231), with > 50% hydrophobicity. Control 1+ showed 38.74% hydrophobicity, followed by strains *P. kudriavzevii* (1003) and *T. delbrueckii* (1055), possessing about 37% hydrophobicity.

For toluene (Table 5), the results ranged between 3.12 and 35.11%. Five strains (1063, 1094, 1146, 1167 and 1213) showed values higher than control 2+ (32.41%). Yeasts 1056, 1081, 1082, and 1090 showed about 30% hydrophobicity, which was greater than control 1+ (26.68%) although there were not significantly differences. These results are superior to that found for *W. anomalus*, of 25% hydrophobicity, using toluene as the solvent (García-Hernández et al., 2012).

Table 4
Auto-aggregation of each strain.

Species	Strain	Auto-aggregation (%)	Species	Strain	Auto-aggregation (%)
Control 1+	3	19.81 ± 5.86 ^{d,e,f,g}	*H. osmophila*	1094	32.05 ± 3.25 ^{g,h,i}
Control 2+	4	19.41 ± 6.40 ^{c,d,e,f,g}	*C. sake*	1107	15.80 ± 3.85 ^{a,b,c,d,e,f}
P. kudriavzevii	1003	30.36 ± 4.90 ^{g,h,i}	*Z. fermentati*	1134	26.05 ± 4.50 ^{e,f,g,h}
M. pulcherrima	1012	12.77 ± 7.55 ^{a,b,c,d}	*P. caribbica*	1135	7.34 ± 5.19 ^{a,b,c,d}
P. membranaefaciens	1019	41.38 ± 8.02 ^{i}	*Z. fermentati*	1142	4.09 ± 2.83 ^{a}
K. thermotolerans	1039	43.11 ± 13.57 ^{i}	*Lachancea sp.*	1146	6.06 ± 1.50 ^{a,b,c}
T. delbrueckii	1055	19.06 ± 0.10 ^{b,c,d,e,f,g}	*Lachancea sp.*	1148	13.97 ± 5.39 ^{a,b,c,d,e}
H. osmophila	1056	64.43 ± 12.70 ^{j}	*Candida sp.*	1151	5.70 ± 3.63 ^{a,b}
C. vini	1063	41.62 ± 2.05 ^{i}	*K. thermotolerans*	1167	13.83 ± 9.39 ^{a,b,c,d,e}
P. kudriavzevii	1075	4.84 ± 0.85 ^{a}	*Z. fermentati*	1187	9.40 ± 2.09 ^{a,b,c,d}
P. galeiforms	1081	12.07 ± 0.00 ^{a,b,c,d}	*P. kudriavzevii*	1200	27.84 ± 0.78 ^{f,g,h}
P. anomala	1082	11.05 ± 9.87 ^{a,b,c,d}	*Z. bailii*	1213	34.70 ± 2.53 ^{h,i}
O. polymorpha	1087	3.85 ± 0.71 ^{a}	*C. pararugosa*	1231	63.34 ± 11.18 ^{j}
P. anomala	1090	28.12 ± 5.83 ^{f,g,h}			

Different letter indicate significantly differences between strains.

Table 5
Hydrophobicity of each strain with xylene and toluene.

Species	Strains	Hydrophobicity (%)	
		Xylene	Toluene
Control 1 +	3	38.74 ± 8.37 ^{h}	26.68 ± 4.86 ^{c,d,e,f,g,h}
Control 2 +	24	37.15 ± 3.07^{h}	32.41 ± 8.36 ^{g,h}
P. kudriavzevii	1003	37.90 ± 0.92 ^{h}	10.55 ± 7.37 ^{a,b,c}
M. pulcherrima	1012	15.22 ± 4.20 ^{b,c,d}	15.05 ± 5.92 ^{a,b,c,d,e,f}
P. membranaefaciens	1019	33.04 ± 5.57 ^{g,h}	15.66 ± 4.37 ^{a,b,c,d,e,f,g}
K. thermotolerans	1039	31.12 ± 2.29 ^{f,g,h}	25.25 ± 10.85 ^{b,c,d,e,f,g,h}
T. delbrueckii	1055	37.41 ± 5.39 ^{h}	19.34 ± 2.66^{a,} ^{b,c,d,e,f,g,h}
H. osmophila	1056	31.51 ± 7.53 ^{g,h}	27.64 ± 12.77 ^{c,d,e,f,g,h}
C. vini	1063	30.81 ± 1,56 ^{f,g,h}	33.09 ± 19.57^{h}
P. kudriavzevii	1075	22.09 ± 3.11 ^{d,e,f}	11.03 ± 0.52 ^{a,b,c}
P. galeiforms	1081	21.49 ± 0.97 ^{d,e}	29.60 ± 1.80 ^{d,e,f,g,h}
P. anomala	1082	23.84 ± 1.70 ^{d,e,f,g}	31.45 ± 10.37 ^{e,f,g,h}
O. polymorpha	1087	10.55 ± 2.67 ^{a,b,c}	5.44 ± 0.60 ^{a,b}
P. anomala	1090	30.47 ± 3.28 ^{e,f,g,h}	31.97 ± 7.44 ^{f,g,h}
H. osmophila	1094	32.52 ± 0.03 ^{g,h}	32.80 ± 7.50 ^{g,h}
C. sake	1107	6.64 ± 4.26 ^{a,b,c}	13.35 ± 2.63 ^{a,b,c,d}
Z. fermentati	1134	10.33 ± 0.64 ^{a,b,c}	8.02 ± 2.22 ^{a}
P. caribbica	1135	3.16 ± 2.63^{a}	10.76 ± 9.45 ^{a,b,c}
Z. fermentati	1142	8.18 ± 2.62 ^{a,b,c}	3.12 ± 2.84 ^{a}
Lachancea sp.	1146	6.38 ± 1.95 ^{a,b,c}	33.24 ± 5.58 ^{h}
Lachancea sp.	1148	15.75 ± 4.58 ^{c,d}	5.82 ± 0.05 ^{a}
Candida sp.	1151	6.01 ± 2.53 ^{a,b}	14.71 ± 4.27 ^{a,b,c,d,e}
K. thermotolerans	1167	10.41 ± 0.95 ^{a,b,c}	3.67 ± 15.03 ^{g,h}
Z. fermentati	1187	10.26 ± 2.56 ^{a,b,c}	8.28 ± 1.85 ^{a}
P. kudriavzevii	1200	32.99 ± 9.92 ^{g,h}	11.22 ± 2.03 ^{a,b,c}
Z. bailii	1213	54.16 ± 2.23 ^{i}	35.11 ± 12.75 ^{h}
C. pararugosa	1231	51.09 ± 2.52 ^{i}	25.87 ± 6.36 ^{c,d,e,f,g,h}

Different letter indicate significantly differences between strains for each variable.

Generally, as proven in the literature and in agreement with our data, more satisfactory hydrophobicity results are obtained, when xylene is employed as the solvent. Xylene is a more effective uncoupling agent than toluene and as such, is also more efficacious in depleting adenosine triphosphate. Xylene and toluene are aromatic hydrocarbons lacking proton-releasing groups. Thus, instead of a protonophoretic mechanism, attachment to specific hydrophobic sites, such as proteins, seems more likely to be responsible for the uncoupling effect of these solvents (Revilla et al., 2007).

The auto-aggregation and hydrophobicity properties are strongly linked to the ability of the microbe to adhere to the intestine. From the probiotic perspective, a high hydrophobicity of the cell surface explains why, among the yeasts, certain strains have relatively slower elimination kinetics from the gastrointestinal tract and exert different health effects. It has been observed that the hydrophobic property could be responsible for a better ability to colonize the gastrointestinal tract of germ-free mice (Martins et al., 2009).

From Fig. 2, it can be observed that yeasts with > 30% in both properties (hydrophobicity with xilene and auto-aggregation) are 1019, 1039, 1056, 1063, 1094, 1213 and 1231 and so they are suitable candidates.

3.5. Strains selected as best according to the preliminary tests

In order to select the strains that presented the best characteristics up to this point, PCA was applied (Fig. 3 and Table 6). Twenty-seven strains and six discriminating variables (rate, high OD, generation time, lag phase, auto-aggregation and hydrophobicity), were introduced in the analysis. It was noted that the rate and generation time were inversely proportional. Namely, a shorter doubling time resulted in a higher growth rate and, therefore, a more pronounced slope in the exponential phase. Thus, the strains of most interest were closest to the rate parameter. Again, one can see the same tendency between hydrophobicity and self-aggregation, noting greater apolarity of xylene than toluene.

Considering all of the above, the best yeasts were *H. osmophila* (1056 and 1094), *Z. bailii* (1213), *K. thermotolerans* (1039), *P. anomala* (1082, 1090), *P. kudriavzevii* (1003, 1200), *C. vini* (1063) and *P. membranaefaciens* (1019).

3.6. Biofilm formation

Biofilm formation is divided into four stages. In the initial stage, the yeasts adhere to the substrate that forms the base of the biofilm. This is followed by cell co-aggregation and colonisation. Then, the cells grow and proliferate, ultimately forming a basal layer that secures the cells (Ramage, Mowat, Jones, Williams, & López-Ribot, 2009). Generally, adhesion is favoured where the surfaces appear to be rougher, more hydrophobic and possess a coating.

For the 10 strains selected as the best, cell viability was observed after each sample was sonicated, to separate the possible biofilms formed from the slide contained in the Falcon flasks. For all 10 strains, the viable cell count was very high, around 6 log CFU/cm^2, with 1063 presenting the highest (6.23 ± 0.17 log CFU/cm^2). Strain 1039 had the lowest viability (5.30 ± 0.17 log CFU/cm^2). However, there are no intergroup differences ($\alpha = 0.05$; $p = 0.02$; $F = 3.26$). In comparison to these data, Perricone, Bevilacqua, Corbo, and Sinigaglia (2014) presented relatively lower viabilities (2.96 − 4.63 log CFU/cm^2) for yeasts from Altamura sourdough.

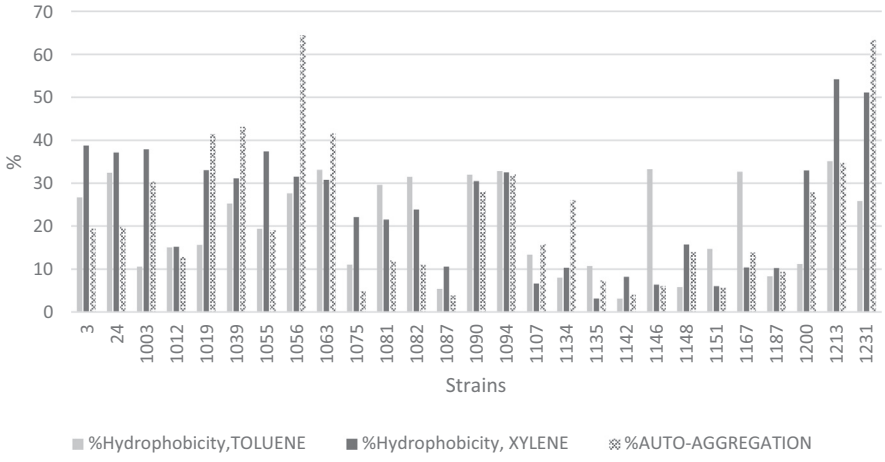

Fig. 2. Auto-aggregation and hydrophobicity percentages for the selected study strains.

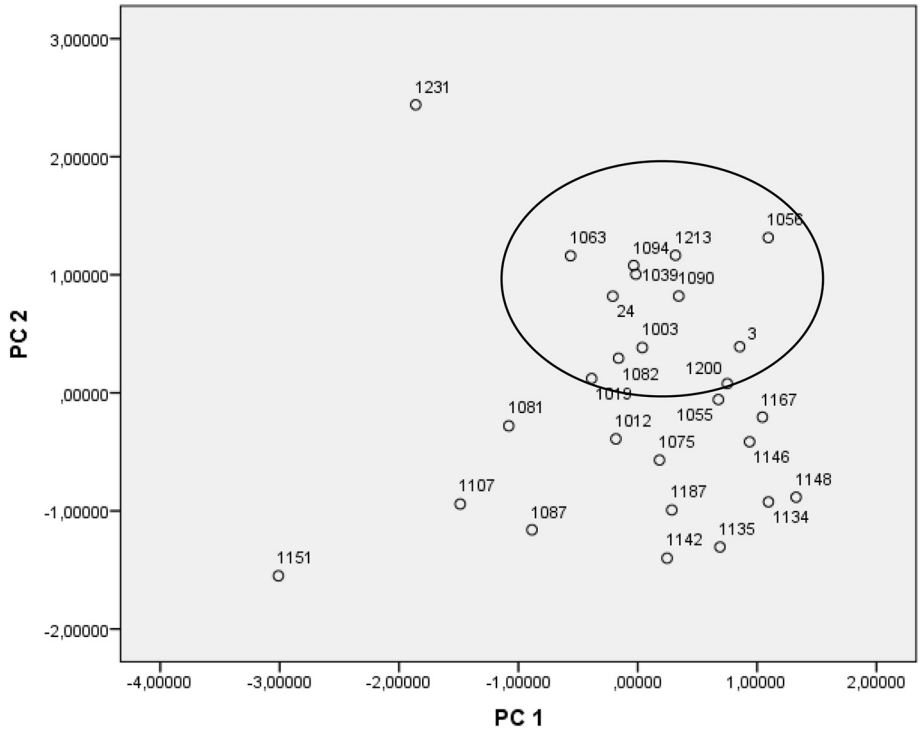

Fig. 3. Principal Component Analysis run on the fitting parameters of Warringer and Blomberg (2003) in addition to the auto-aggregation and hydrophobicity variables for the study strains.

Table 6
Contribution of the highly correlated variables and their loadings in principal components 1 and 2.

Principal component	Variance explained (%)	Total variance (%)	Most highly correlated	Loading
1	37.86	37.86	λ	−0.14
			OD_{max}	0.90
			Auto-aggregation	−0.01
			Xylene	0.01
			Toluene	
2	30.08	67.94	λ	−0.53
			G	−0.21
			μ	−0.02
			OD_{max}	−0.02
			Auto-aggregation	0.84
			Xylene	0.86
			Toluene	0.70

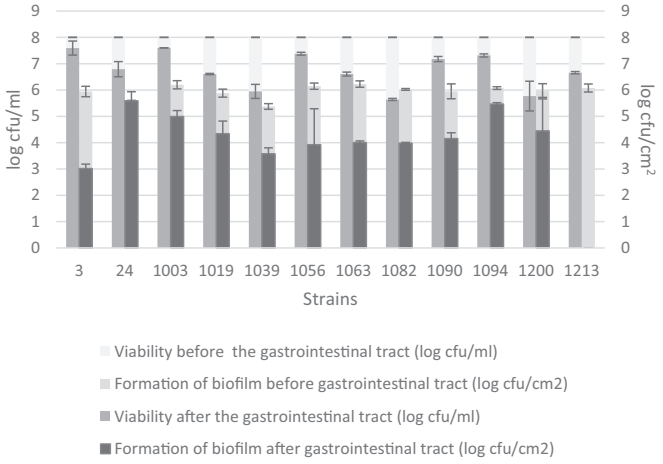

Fig. 4. Viability and biofilm formation before and after gastrointestinal tract by each of the strains selected as best according to gastrointestinal resistance, autoaggregation and hydrophobicity tests.

3.7. Sequential simulated digestion and its effect on viability and biofilm formation

As shown in Fig. 4, Table 7, the cell viability and biofilm formation of the 10 most relevant strains were investigated before and after exposure to sequential simulated digestion conditions (salivary-gastric-intestinal). The initial cell concentration was 8 log CFU/mL. After sequential simulated digestion, some strains, such as *P. kudriavzevii* (1003), *H. osmophila* (1056, 1094) and *P. anomala* (1090) exhibited better viability (> 7 log CFU/ml) than the controls. The remaining seven strains had a viability around 6 log CFU/mL. No significant intergroup differences were observed ($\alpha = 0.05$; $p = 0.005$; $F = 4.90$). Regarding biofilm formation, strains 1003, 1019 and 1094 showed the best results but the values were slightly lower than control 1+ (*S. cerevisiae* strain UCLM 3).

It is noted that in contrast to other strains, the biofilm forming ability of the commercial yeast (*S. cerevisiae* var. *boulardii*) was not affected by the digestive conditions. Nevertheless, 1003 and 1094, lost just 19 and 10% biofilm-forming ability, respectively, and two other strains (1019 and 1200) showed a loss of < 30%. Besides control 1+, none of the strains exceeded a viability loss of 40%, except strain 1213, which after simulated digestion, was not able to form a biofilm.

4. Conclusions

In this paper, a step-by-step approach was used to characterise the probiotic potential of certain non-*Saccharomyces* yeasts isolated from food ecosystems. From 215 isolates, 108 were identified as different strains by RAPD analysis. Some strains were distinguished for their optimal growth parameters, such as *Lachancea* sp. (1146 and 1148), *H. osmophila* (1056), *K. thermotolerans* (1167) and *P. kudriavzevii* (1200),

Table 7
Loss of viability and biofilm formation capability after simulation of gastrointestinal conditions.

Species	Strain	Loss viability after tract (%)	Loss biofilm formation capability (%)
Control 1 +	3	5 ± 0.66 [a]	48 ± 0.26 [d]
Control 2 +	24	15 ± 5.07 [bcd]	0 ± 0.27 [a]
P. kudriavzevii	1003	5 ± 0.10 [a]	19 ± 0.22 [bc]
P. membranaefaciens	1019	17 ± 0.42 [d]	26 ± 0.35 [bcd]
K. thermotolerans	1039	26 ± 4.72 [e]	33 ± 0.19 [cd]
H. osmophila	1056	8 ± 0.96 [ab]	36 ± 0.77 [cd]
C. vini	1063	17 ± 1.32 [d]	36 ± 0.12 [cd]
P. anomala	1082	29 ± 0.70 [e]	34 ± 0.04 [cd]
P. anomala	1090	10 ± 1.72 [abcd]	30 ± 0.31 [bcd]
H. osmophila	1094	9 ± 1.04 [abc]	10 ± 0.06 [ab]
P. kudriavzevii	1200	28 ± 9.99 [e]	26 ± 0.79b [cd]
Z. bailii	1213	17 ± 0.77 [cd]	100 ± 0.11 [e]

Different letter indicate significantly differences between strains for each variable.

which offered better results than the reference probiotics strains used as the controls. Furthermore, many strains were tolerant to simulated digestive conditions, an essential property for a probiotic. In addition, strain 1056 was noted for its aggregation capacity, followed by *C. pararugosa* (1231), which, in turn, was noted for its hydrophobic capacity. In terms of biofilm formation, *H. osmophila* (1094), 1200, *P. kudriavzevii* (1003) and *P. membranaefaciens* (1019) were the most promising.

These results suggest that various non-*Saccharomyces* yeast species harbour strains with probiotic potential, although there is no one with optimal results together. Thus, future studies will be done for the final selection that include the GRASS character of the selected strains among others. Moreover, the strains with multifunctional potentials isolated in this study, could be used to produce probiotic products (foods and drugs). This might be a solution to the steadily increasing demands for these products.

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