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Saccharomyces cerevisiae from Brazilian kefir-fermented milk: An *in vitro* evaluation of probiotic properties

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A B S T R A C T

The therapeutic use of probiotics for supporting the antibiotic action against gastrointestinal disorders is a current trend and emerging applications have gained popularity because of their support for various microbiological activities in digestive processes. Microorganisms isolated from kefir with great probiotic properties, in addition to high resistance to harsh environmental conditions, have been widely researched. Administration of probiotic yeasts offers a number of advantages, when compared to bacteria, because of particular characteristics as their larger cell size. In the present study, 28 strains of *Saccharomyces cerevisiae* were isolated, after *in vitro* digestion of kefir-fermented milk, and identified by molecular based approaches. A screening was performed to determine important quality requirements for probiotics including: antagonistic and antioxidant activities, β-galactosidase synthesis, autoaggregation, surface hydrophobicity and adhesion to epithelial cells. The results showed strains: with antagonistic activity against microbial pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis;* able to produce β-galactosidase; with antioxidant activity levels higher than 90%; with hydrophobicity activity and autoaggregation ability (evaluated by adhesion test, where all the strains presented adhesion to mice ileal epithelial cells). These findings are relevant and the strains are recommended for further *in vivo* studies as well as for potential therapeutic applications.

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1. Introduction

Probiotics are live microorganisms which, when administered in suitable doses, provide benefits to human and animal health [1]. To act in a positive way, probiotics need to be alive to create a symbiotic balance in the host's digestive tract [2], what emphasizes the importance of the microbial viability during the gastrointestinal transit, mainly in case of oral administration.

Microbial survival rate depends on the administration regimen, thereby the use of synbiotic matrices for probiotic application and maintenance of microbial viability has become significant [3]. A well known matrix for distribution of probiotics is the kefir, a fermented milk obtained by incubation of milk with kefir grains (protein and polysaccharide matrix containing bacteria and yeasts) [4].

Microorganisms in the kefir grains proliferate and produce enzymes and other biogenic elements causing physicochemical changes in the environment. For this reason, kefir has been increasingly applied to medical benefits, since it represents a great source of natural probiotics and can be easily digested. Another particular characteristic of kefir is that its grains can be recovered after fermentation, making the process profitable [5].

Among the most common probiotics used, some species of lactic acid bacteria of the genus *Lactobacillus* and *Bifidobacterium* and the yeast *Saccharomyces boulardii* have been often reported [6]. Yeasts are particularly promising because they are not affected by antibacterial agents [7], and this property is relevant since some therapies combine the administration of probiotics with antibiotics in the treatment of gastrointestinal infections. The use of yeasts is also advantageous because their genetic material can not be transferred

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to commensal bacteria [8].

Yeasts have long been considered safe for applications [9], and their beneficial effects include competitiveness for nutrients, better cell adhesion ability (in consequence of its size), production of antagonistic compounds, immunomodulation, cholesterol assimilation, toxin elimination and then neutralization of pathogenic bacteria [10–13].

Saccharomyces boulardii has been the only yeast commercialized worldwide as probiotic for humans, but some authors have suggested other species or genera, based essentially on *in vitro* assays and on clinical trials [8,11,14]. Further, it has been discussed that there is a lack of concrete data about whether other yeast strains (particularly *S. cerevisiae*) possess probiotic properties [15,16].

Different *in vitro* systems [17] have been preferentially used to evaluate the gastrointestinal tolerance of probiotic strains [18] and the microbial viability along the digestive tract [19]. For this reason, Ötles et al. [20] enumerated generally required probiotic properties as following: adherence to cells; exclusion or reduction of pathogenic adherence; persistence and multiplicity; production of acids, peroxide, and bacteriocins antagonistic to pathogen growth; to be non-invasive, non-carcinogenic, and nonpathogenic; capability to form a normal and balanced intestinal flora.

To our best knowledge, only a small number of reports have focused on the potential of probiotic yeasts to survive the transit through the gastrointestinal tract [21,22]. Thus, this study aimed to evaluate probiotic characteristics of yeast strains obtained after *in vitro* simulation of digestion of kefir-fermented milk.

2. Material and methods

2.1. Fermented milk

Kefir grains were obtained from a private household located in Recife, Pernambuco, Brazil. They were washed with ultrapure water and inoculated in UHT milk. The grains were separated from the fermented milk by filtering them through a sieve, and then washed for later use. This fermented milk was made by adding 5% (w/w) kefir grains as inoculum. After incubation at 20 $\pm$ 2 °C for 45–48 h, aliquots were collected, at random intervals, and the pH was monitored until reaching the value of 4.5. The grains were then separated from the fermented milk by filtration through a plastic sieve and washed with ultrapure water before the next culture incubation.

2.2. In vitro digestion of kefir-fermented milk

The *in vitro* simulation of human digestion was based on the method of Saito et al. [23]. To simulate the digestion, 10 mL of the fermented milk was diluted 1:1 in a sterile electrolyte solution containing 16.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$; added of 0.6% (w/v) pepsin (Sigma-Aldrich, St. Louis, USA) and further incubated for 5 min at 37 °C. The pH was adjusted to 2.0 by adding different volumes of 1 mol/L HCl and 5 mol/L HCl solutions, for tight pH control. After 120 min of incubation, 1.5 mL were harvested by centrifugation at 14.560 $\times$ g for 5 min at 4 °C (Hermle Labortechnik, Wehingen, Germany) and then washed twice with sterile PBS buffer pH 7.4 and resuspended with 1.5 mL of 1% (w/v) bile salts (Merck, Darmstadt, Germany) in PBS buffer pH 8.

The solution was finally diluted in an artificial duodenal secretion (pH 8.0) consisting of: PBS buffer, 0.3% (w/v) bile salts and 0.1% (w/v) pancreatin (Sigma-Aldrich, St. Louis, USA). After 90 min of incubation at 37 °C, a 100 μL aliquot was pipetted out, serially diluted into 0.1% peptone water solution and spread-plate on MRS agar to determine the CFU/mL.

2.3. Purification and identification of yeasts resistant to in vitro digestion

After the growth period, 28 microorganism colonies, resistant to *in vitro* digestion procedures, were collected from each Petri dish, cultured by pour-plate method and incubated at 37 °C for 48 h in MRS medium. This procedure was done twice in order to purify the colonies, which were subjected to Gram staining and classified as yeasts. The culture media Sabouraud dextrose broth (Himedia, Mumbai, India) and Sabouraud dextrose agar (Merck, Darmstadt, Germany) were chosen for the subsequent assays.

The identification of yeast species was done at the Department of Mycology, Federal University of Pernambuco, Recife, Brazil, an affiliate member of the World Federation for Culture Collections (WFCC), where the yeasts were subjected to taxonomic characterization according to Barnett et al. [24].

The yeasts were identified as *Saccharomyces cerevisiae* and one of them was randomly chosen for molecular identification. The molecular analysis was based on the following steps: 1) From the Indicating FTA micro card (GE Healthcare, USA) containing the ScD sample, a 2 mm punch was collected for the purification of the nucleic acids, which were purified using the FTA Purification Reagent (GE Healthcare, USA). 2) After DNA purification, the PCR was performed using the universal forward primer (27F - AGAGTTTGATCMTGGCTCAG) and the universal reverse primer (1492R - TACGGYTACCTTGTTACGACTT). 3) The PCR products were purified and analyzed in agarose gel. 4) Sequencing was performed using the BigDye Terminator 3 kit (Applied Biosystems, USA) and the fragments were sequenced on the 3730XL DNA Analyzer (Applied Biosystems, USA). 5) The generated sequences were aligned using the Sequencher 5.0 software (GeneCodes, USA), to obtain the consensus sequence. The consensus sequences were aligned using the BLAST database. All these procedures were carried out by the company STAB VIDA (Oeiras, Portugal).

2.4. Antagonistic activity

The spectrum of antagonistic activity of yeasts was determined by the spot-on-the-lawn method, according to Tulini et al. [25]. The indicator strains used, obtained from our culture collection, were dangerous pathogenic bacteria: *Klebsiella pneumoniae* ATCC29665 (Gram negative, encapsulated, facultative anaerobic) and *Pseudomonas aeruginosa* ATCC27853 (Gram negative, aerobic); and opportunistic bacteria: *Enterococcus faecalis* ATCC 6057 (Gram positive, commensal in the digestive tract, aerobic), *Bacillus subtilis* ATCC 6633 (Gram positive, ability to form spore, aerobic), *Bacillus cereus* ATCC 33019, (Gram positive, ability to form spore, facultative aerobic), *Staphylococcus aureus* ATCC 6538 (Gram positive, present in mucosal membranes, aerobic) and *Listeria innocua* ATCC 33090 (Gram positive, facultative anaerobic). These strains were chosen for their wide variety of physiological characteristics (which differ each other), providing a broad estimate of the antagonistic action of the yeasts studied. Five microliters of an overnight culture of each yeast strain grown in Sabouraud dextrose broth was spotted on filter paper discs (6 mm) on Sabouraud dextrose agar plates. After incubation at 37 °C for 72 h under aerobic conditions, the plates were overlaid with 7 mL of soft BHI agar (0.8% w/v bacteriological agar, Himedia) seeded with the indicator strain (10^6 CFU/mL), followed by incubation at 37 °C for 24 h. Inhibition halos ($\geq$7 cm) were indicative of susceptibility of the indicator strain.

2.5. Antioxidant activity

To evaluate the antioxidant activity, the percentage rate of reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was

performed as described by Gil-Rodríguez et al. [16] with minor modifications as suggested by Sousa et al. [26]. Briefly, 1 mL of the yeast culture in Sabouraud dextrose was harvested by centrifugation (14.560 × g for 5 min at 4 °C), washed twice with a sterile solution of PBS pH 7.4 and the resulting pellet resuspended in 1 mL of the same solution. The cell suspension (800 μL) was transferred into a new tube, where 1 mL of a DPPH solution (0.2 mmol/L in methanol) was afterwards added. The mix was vortexed and then incubated for 30 min at room temperature in darkness. The reaction tubes were centrifuged (14.560 × g for 5 min at 4 °C) and 300 μL of the supernatant were transferred into 96-well plates in order to measure the absorbance at 517 nm (A517). The percentage of reduction of DPPH was calculated according to Equation (1):

$$\%AA = \{[Abs_i - (Abs_s - Abs_b)]/Abs_i\} \times 100 \quad (1)$$

where Abs_i is the initial absorbance (methanolic solution + DPPH), Abs_s is the absorbance of the mix (DPPH + sample) and Abs_b is the absorbance value of the blank sample.

The percentage of scavenging of DPPH was experimentally confirmed as directly proportional to the OD_{600} of the cell solution used. The initial OD_{600} was measured for each culture and standardized to $OD_{600} = 1.00$.

The results are presented in accordance with the classification of Gil-Rodríguez et al. [16]: low activity (between 20 and 30%), good activity (between 30 and 40%), very good activity (between 40 and 50%) and excellent activity (percentage reduction of DPPH higher than 50%).

2.6. β-galactosidase activity

The o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate (Sigma-Aldrich, St. Louis, USA) was used to determine the β-galactosidase activity as described by Nagy et al. [27], with modifications. Cell-free extracts were prepared by sonication (sonicator Bandelin electronic, Berlin, Germany) to disrupt the cells. Briefly, 1 mL of overnight yeast cultures grown in Sabouraud broth were harvested by centrifugation (14.560 × g for 10 min at 4 °C) washed twice with 0.1 mol/L phosphate buffer, pH 7.0, and resuspended in the same buffer. The cell suspension was sonicated at approximately 30% amplitude during 4 min, in intervals of 30s on and 30s off. Cell debris was separated by centrifugation at 14.560 × g for 10 min at 4 °C. Cell extracts were kept on ice until incubation (for 30 min at 37 °C).

The reaction mixture was composed of 50 μL of the sample and 50 μL of ONPG at a concentration of 3 mmol/L dissolved in 0.1 mol/L phosphate buffer, pH 7.0. The reaction was stopped by addition of 200 μL of 0.1 mol/L sodium carbonate. The absorbance of the samples was measured at 405 nm. A standard curve of o-nitrophenol (ONP, Sigma-Aldrich, St. Louis, USA) was obtained using known concentrations of ONP, from 0.05 to 2 μmol/mL at intervals of 0.05 μmol/mL, calculated using an extinction coefficient of 4.0143 μmol/cm. One enzymatic unit was defined as specific activity (U/mg protein): 1 U is equivalent to 1 μmol of ONP produced per minute.

2.7. Autoaggregation assay

A preliminary screening of the autoaggregation ability and surface hydrophobicity was performed to identify potentially adherent strains. As described by Meira et al. [28], yeast strains were harvested by centrifugation (14.560 × g for 10 min at 4 °C), washed twice and resuspended in 0.1 mol/L phosphate buffer (pH 7.2). Aggregation assays were performed at 20–25 °C in 0.5 mol/L phosphate buffer (pH 7.2). In all assays, the initial concentration of microorganisms was standardized to OD_{600}nm = 1, approximately. The autoaggregation assay was performed as follows: yeast cell suspensions (2 mL) were incubated at 37 °C and absorbance values (at 600 nm) of the upper layer were measured at different time intervals (2, 4 and 24h) in a UV-Vis spectrophotometer 2000 UV (Bel Photonics, Osasco, Brazil). The percentage of aggregation (%A) was calculated according to Equation (2) [29]:

$$\%A_t = [1 - (OD_t/OD_i)] \times 100 \quad (2)$$

where OD_i is the initial optical density of the microbial suspension and OD_t is the optical density at the time *t*.

2.8. In vitro cell surface hydrophobicity

The isolates were screened for their cell surface hydrophobicity using the microbial adhesion to hydrocarbons (xylene) method, described by Rosenberg et al. [30]. Briefly, the yeasts isolates were grown overnight at 37 °C in Sabouraud dextrose broth and washed with sterile phosphate buffered saline solution (PBS, pH 7.2), harvested and resuspended in the same buffer. The suspension was then adjusted to approximately 10^8 CFU/mL (OD_{600}; A1). Aliquots (3 mL) of the yeast suspensions were added to 1 mL of xylene, mixed by vortexing for 60 s, and kept for 2 h at 37 °C, until the aqueous phase was carefully removed and the absorbance measured at 600 nm (A2) [28]. The Hydrophobicity Index (HPBI) was calculated according to Equation (3):

$$\%HPBI = [(A1 - A2)/A1] \times 100 \quad (3)$$

Isolates with a HPBI greater than 70% were arbitrarily classified by Pringsulaka et al. [31] as highly hydrophobic; isolates with HPBI between 50 and 70% were classified as moderate hydrophobic; and isolates with HPBI lower than 50% were classified as lowly hydrophobic. High hydrophobicity has been suggested as indicator of good adhesion capacity.

2.9. Epithelial cell adherence assay

The yeast adhesion to mice ileal epithelial cells was assessed by using eight-week-old male and female Swiss albino mice (30–40 g body weight). They were fed with a conventional balanced diet (16% protein, 56% carbohydrate, 2% fat, 5.3% cellulose, and 5% vitamins and minerals) and tap water *ad libitum* to improve their adaptation. Three to four mice were housed per polycarbonate cage, with softwood chips as bedding, in an air-conditioned room at 23–25 °C, air humidity of 50–60% and under a 12 h light/dark cycle. Intestines were collected immediately after euthanasia according to the ethical principles of animal experimentation of COBEA (Brazilian College of Animal Experimentation) and approved by the Animal Studies Committee of Federal University of Pernambuco (protocol number 23076.017009/2012-13).

Epithelial cells were prepared as described by Annika et al. [32] and improved by Kumar and Kumar [33]. A small segment of ileum from female and male Swiss albino mice *Mus musculus*, was opened, washed twice with sterilized PBS (0.1 mol/L, pH 7.2) and incubated in PBS at 4 °C for 30 min to remove the surface mucus. Epithelial cells were scraped off with the edge of a microscope slide, suspended in PBS, and microscopically examined to ensure the removal of adherent bacteria. Further, 1 mL of each yeast inoculum (10^8 CFU/mL) and the epithelial cell suspension were mixed, incubated at 37 °C and at 40 rpm of agitation for 30 min. The attachment of yeast cells was studied microscopically after the Gram stained preparations.

2.10. Statistical analysis

Experimental data, microorganism counting and frequency (%) tables are shown as the mean of multiple assays (±standard deviation, when indicated). Analyses were performed by the software Microsoft Excel 2010 (Microsoft, Redmond, USA).

3. Results

3.1. Identification of yeasts resistant to in vitro digestion

The colony count of the fermented milk showed approximately 10 log_{10} CFU/mL and 2 log_{10} CFU/mL before and after the *in vitro* digestion process, respectively. Among the resistant strains, 28 were isolated (Fig. 1) to be further evaluated for their morphological and physiological characteristics. All the strains were identified as *Saccharomyces cerevisae* and named as: ScA, ScA1, ScB, ScB1, ScC, ScD, ScE, ScF, ScG,ScH, ScI, ScJ, ScK, ScL, ScM, ScN, ScO, ScP, ScQ, ScR, ScS, ScT, ScU, ScV, ScW, ScX, ScY, ScZ.

The strain ScD was randomly selected for molecular taxonomic identification, which confirmed the species identification based on 99% identity of ITS sequences, compared to database sequences (GenBank accession no. CP006424.1).

3.2. Antagonistic activity

Table 1 presents the antagonistic activities of the yeasts studied. All the *S. cerevisiae* species inhibited *K. pneumoniae* ATCC 29665 and 82.1%, 53.5% and 25% of them presented antagonism against *P. aeruginosa* ATCC27853, *S. aureus* ATCC 6538 and *B. subtilis* ATCC 6633, respectively. In total, 17 yeasts were able to inhibit at least three indicator strains, 8 yeasts presented antagonism against two indicator strains and 2 yeasts inhibited at least one of them. The antagonistic activity of the strain ScL was the most relevant because it inhibited 4 indicator strains, whilst the highest antagonism was found for the strain ScO, which produced a halo of inhibition of 47.7 ± 8.5 against *K. pneumoniae*. No antagonistic activity was observed against *E. faecalis* ATCC 6057, *B. cereus* ATCC 33019 and *L. innocua* ATCC 33090.

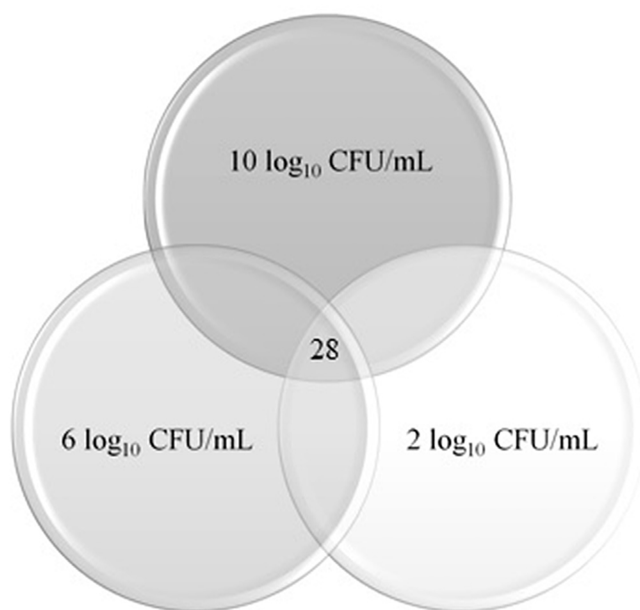

Fig. 1. Counting of microorganisms before (dark grey), during (grey) and after (white) the *in vitro* digestion. In the center of the diagram are represented the number of microorganisms isolated and evaluated in the present study.

3.3. Antioxidant activity

Different levels of antioxidant activity were found for the strains tested (Table 2). Among the 28 yeasts analyzed, 5 strains (17.85%) showed good activity (levels between 30 and 40%); 11 strains (39.3%) exhibited very good activity (levels between 40 and 50%) and 12 strains (42.85%) showed excellent activity (percentage reduction of DPPH higher than 50%); among the last group, ScJ and ScZ presented antioxidant activity higher than 90% (Table 2). The lowest and highest activities were observed for the strains ScN (34.83 ± 0.01%) and ScZ (114.16 ± 0.01%), respectively.

3.4. β-galactosidase activity

According to the methods used, all the yeasts, after cell breakage by sonication, exhibited intracellular β-galactosidase activity (Fig. 2). Lactose was hydrolyzed by the yeast extracts in different levels, and depending on the strain, whose values ranged from 1.25 to 5.32 U/mL, with the highest value obtained for the strain SCG.

3.5. Autoaggregation assay

Yeast cells are considered relatively large and heavy. For this reason, since the second hour of test, it was possible to observe high levels of precipitation. The percentage of autoaggregation showed high variability among the strains, with percentages higher than 60%, ranging from 61.4 ± 0.1% for ScA to 96.2 ± 0.2% for ScO. As shown in Fig. 3, since the fourth hour, the strains ScP and ScZ showed 100% of aggregation, whilst twenty-three strains (82.1%), among all studied, took 24 h to reach the same level of aggregation.

3.6. Cell surface hydrophobicity

All the tested strains showed moderate or high hydrophobicity. In Table 3, the strain ScR showed a moderate rate between 50 and 70%, while the other strains exhibited high hydrophobicity. Eight of them (28.5%) showed a percentage from 80 to 90%, and nineteen of them (67.9%) showed rates higher than 90%. The lowest value was obtained for the ScR (63.36 ± 1.91%), and the highest for the ScB (96.08 ± 1.48%).

3.7. Epithelial cell adherence assay

Fig. 4 shows a significant yeast adherence of all the strains to intestinal cells. A commercial *Saccharomyces cerevisiae* yeast was used as negative control for not being able to adhere to epithelial cells (data not shown). These results confirm what has been discussed in the previous sections, that hydrophobicity and autoaggregation all together support the yeast adherence to epithelial cells.

4. Discussion

The yeasts studied were suggested as probiotic because their source of kefir, which is already known as probiotic complex and their resistance to the harsh conditions of the gastrointestinal tract., One of the most desirable properties of probiotic yeasts is their antagonistic activity against adherence and translocation of pathogens [34]. The strains studied here showed antagonistic activity against Gram negative (*K. pneumoniae* and *P. aeruginosa*) and Gram positive (*S. aureus* and *B. subtilis*) bacteria. Binetti et al. [15] evaluated the antagonistic activity of yeasts from autochthonal cheese

Table 1
Antagonistic activity of *S. cerevisiae* strains against pathogens after *in vitro* digestion of kefir-fermented milk.

	Indicator strains with IZD[a]						
Yeast Strain	*Klebsiella pneumoniae* ATCC 29665	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus aureus* ATCC 6538	*Bacillus subtilis* ATCC 6633	*Enterococcus faecalis* ATCC 6057	*Listeria innocua* ATCC 33090	*Bacillus cereus* ATCC 3019
ScA	22.7 ± 2.6	28.9 ± 4.0	—	19.8 ± 0.1	—	—	—
ScA1	15.9 ± 0.0	33.8 ± 4.4	—	—	—	—	—
ScB	21.1 ± 2.3	25.41 ± 0.0	—	21.9 ± 1.8	—	—	—
ScB1	26.9 ± 5.8	31.5 ± 1.4	—	15.5 ± 0.0	—	—	—
ScC	21.2 ± 5.6	22.6 ± 3.8	—	—	—	—	—
ScD	22.8 ± 6.2	15.4 ± 5.3	—	17.4 ± 0.9	—	—	—
ScE	16.7 ± 2.1	16.9 ± 0.0	—	19.5 ± 1.3	—	—	—
ScF	15.7 ± 0.0	32.2 ± 0.0	—	20.4 ± 2.2	—	—	—
ScG	18.8 ± 0.0	—	—	—	—	—	—
ScH	20.8 ± 0.1	19.3 ± 2.9	—	—	—	—	—
ScI	28.9 ± 0.0	30.9 ± 0.0	23.8 ± 0.0	—	—	—	—
ScJ	23.8 ± 0.0	38.9 ± 0.0	—	—	—	—	—
ScK	19.1 ± 0.9	—	16.46 ± 0.0	—	—	—	—
ScL	27.6 ± 0.6	13.2 ± 0.1	17.2 ± 5.4	15.2 ± 1.7	—	—	—
ScM	34.9 ± 0.0	—	18.8 ± 8.0	—	—	—	—
ScN	29.5 ± 0.0	—	—	—	—	—	—
ScO	47.7 ± 8.5	20.0 ± 0.0	18.5 ± 5.3	—	—	—	—
ScP	21.5 ± 3.1	25.7 ± 9.6	26.4 ± 9.2	—	—	—	—
ScQ	19.3 ± 0.2	22.4 ± 9.8	23.1 ± 1.8	—	—	—	—
ScR	17.6 ± 2.0	21.6 ± 9.2	22.6 ± 3.4	—	—	—	—
ScS	27.1 ± 0.0	19.9 ± 6.0	18.8 ± 3.5	—	—	—	—
ScT	26.5 ± 2.2	29.9 ± 5.9	15.9 ± 1.1	—	—	—	—
ScU	30.7 ± 7.0	25.4 ± 1.4	35.0 ± 6.6	—	—	—	—
ScV	16.5 ± 1.9	14.5 ± 0.2	21.6 ± 1.4	—	—	—	—
ScW	18.5 ± 6.7	37.2 ± 2.2	29.9 ± 0.1	—	—	—	—
ScX	30.7 ± 2.7	27.7 ± 0.1	15.0 ± 0.1	—	—	—	—
ScY	24.0 ± 1.8	—	29.9 ± 0.1	—	—	—	—
ScZ	26.1 ± 2.7	35.7 ± 1.2	—	—	—	—	—

IZD = Inhibition Zone Diameter, — = no inhibition.
[a] Values expressed as mean (mm) ± S.D. (n = 3).

Table 2
Evaluation of the antioxidant activity of *S. cerevisae* strains observed after *in vitro* digestion of kefir-fermented milk, according to the classification of Gil-Rodrígues et al. [16].

DPPH reduction activity	
Percentage	Yeast Strain
30–40%	ScN, ScP, ScS,ScT, ScU
40–50%	ScD, ScF, ScG, ScH, ScK, ScL, ScM, ScO, ScQ, ScR, ScY
50–60%	ScA1, ScB, ScB1, ScI, ScV, ScX
60–90%	ScE, ScW, ScA, ScC
>90%	ScJ, ScZ

starters, including strains of *S. cerevisiae* against *E. coli* V517, *Salmonella enteritidis* OMS-Ca and *Staphylococcus aureus* 76, although they were unable to inhibit the growth of pathogenic microorganisms. The same was verified by Perricone et al. [35] when studied yeasts isolated from Altamura sourdough as functional starter cultures for cereal based foods No antagonistic activity was observed when 14 different strains of *S. cerevisiae* were tested against *Listeria monocytogenes, Yersinia enterocolitica*, *Escherichia coli* O157:H7 and *Staphylococcus aureus*.

Kourelis et al. [36] tested the antimicrobial activity of sterilized supernatants from 4, 8, 16 and 24-h cultures obtained from dairy and human yeast strains and verified that none of the 20 tested strains were able to inhibit the growth of *Clostridium tyrobutyricum*, *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Yersinia enterocolitica*. The antagonistic activity and the capacity to produce different antimicrobial compounds are considered critical characteristics for effective competitive exclusion of pathogens and for probiotic benefits [37], which emphasize the relevance of the probiotic properties found in the present study.

The antioxidant activity observed in this study was higher than previously reported. Gil-Rodriguez et al. [16], for example, evaluated the probiotic properties of 59 yeast strains isolated from food and beverage products, and found that only two of them (3.4%) showed a percentage reduction of DPPH higher than 50%. Chen et al. [38] evaluated the DPPH radical scavenging (expressed as percentage of absorbance decrease) by using intact cells of 12 yeasts and observed antioxidant activity in the range of 4.25–46.78%. Food antioxidant activity promoted by probiotics has been increasingly used in healthcare contexts since their presence contribute to free radical scavenging. Since the strains reported in this study showed considerable antioxidant activity, they are suggested to improve the effectiveness of pharmacological and nutritional compounds.

As source of enzymes, probiotics are beneficial to the health of the host for improving the utilization and absorption of nutrients along the digestive tract. For example, β-galactosidase hydrolyzes lactose into glucose and galactose, facilitating the transport of milk components across the intestinal epithelium [39]. These findings are significant when compared to previous studies. Sourabh et al. [40] performed a qualitative screening for β-galactosidase of 23 *S. cerevisiae* strains obtained from various traditional fermented foods (chilra, babru, and bhaturu), alcoholic beverages (aara, chhang, chuli, faasur, lugari) and traditional inocula of western Himalayas (phab, dhaeli, khameer), however, none of the strains was positive. Pennacchia et al. [41] isolated *S. cerevisiae* strains from different food matrices (traditional salame 'Tipo Napoli'; wine; traditional Sopressata; natural starter culture for the production of mozzarella di Bufala Campana; caciotta cheese and sour-dough) to preliminary selection of a potential probiotic. The β-galactosidase activity of five selected strains were evaluated by the API-ZYM test

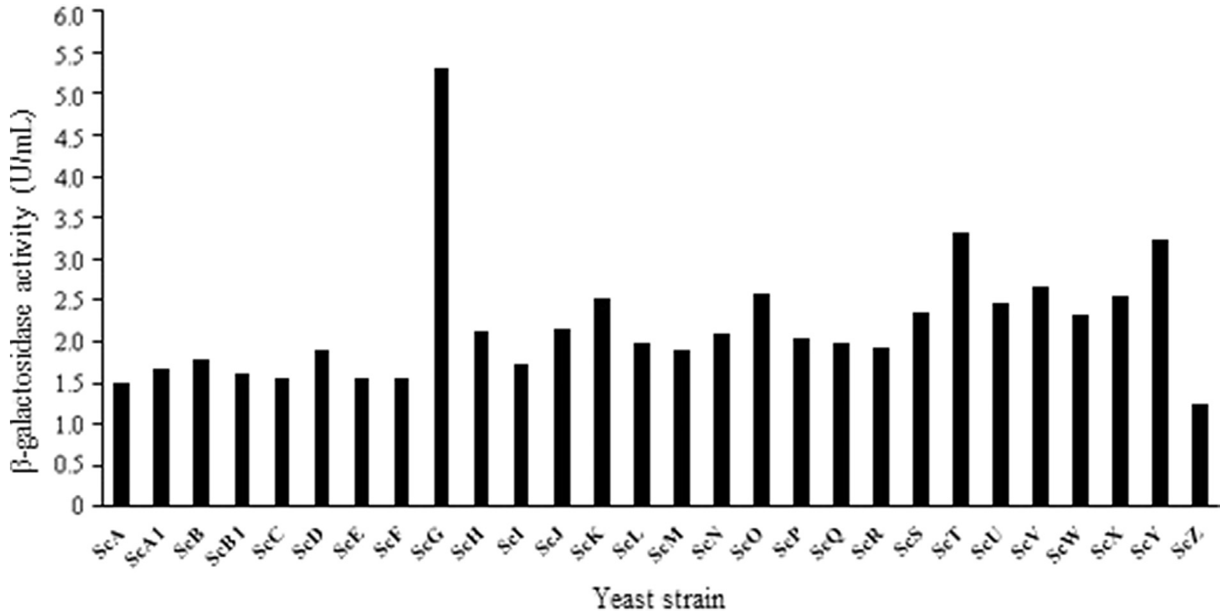

Fig. 2. β-galactosidase activity of the cell extract (without cell debris) of *S. cerevisae* strains obtained after *in vitro* digestion of kefir-fermented milk.

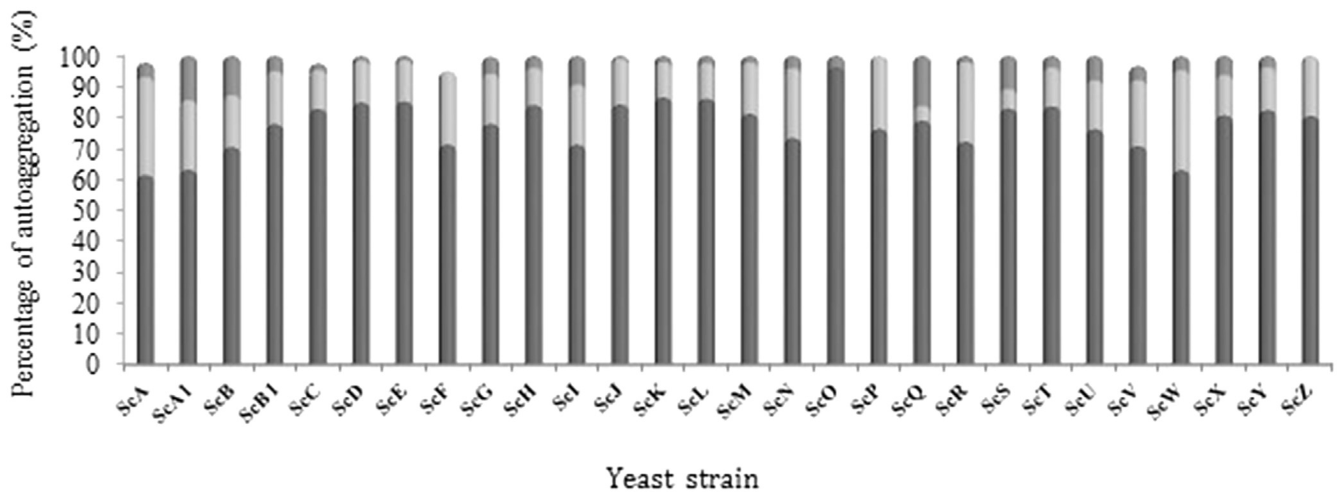

Fig. 3. Autoaggregation percentage of *S. cerevisae* strains obtained after *in vitro* digestion of kefir-fermented milk, percentage after 2 h (dark grey), 4 h (light grey) and 24 h (grey).

Table 3
Evaluation of Hydrophobicity of *S. cerevisae* strains obtained after *in vitro* digestion of kefir-fermented milk, according to the classification of Pringsulaka et al. [30].

In vitro cell surface hydrophobicity	
Percentage	Strain
60–80%	ScR
80–90%	ScA, ScH, ScI, ScJ, ScP, ScT, ScV, ScX
>90%	ScA1, ScB, ScB1, ScC, ScD, ScE, ScF, ScG, ScK, ScL, ScM, ScN, ScO, ScQ, ScS, ScU, ScW, ScY, ScZ

(bio-Mérieux, Marcy I'Etoile, France), but no activity was found. Our findings are relevant because they present many probiotic strains with intracellular β-galactosidase, able to digest lactose. Lactose indigestion symptoms occur when undigested lactose passes through the small intestine and is fermented by colonic bacteria, resulting in the generation of hydrogen gas [39]. Probiotics with β-galactosidase activity are important because they prevent discomfort arising from intestinal fermentation [40].

Adherence ability is essential to probiotic efficacy, and for this reason the analysis of autoaggregation and hydrophobicity are necessary to evaluate potential probiotic yeasts. In our assays, after 2 h, the strains presented higher percentage of aggregation than previously reported by Gil-Rodríguez et al. [16] when they investigated the autoaggregation ability of 53 *S. cerevisae* strains isolated from varieties of grape, wine, apple must, grape must, Juglans regia seeds, milk, mistela and quince jelly. Binetti et al. [15] isolated 4

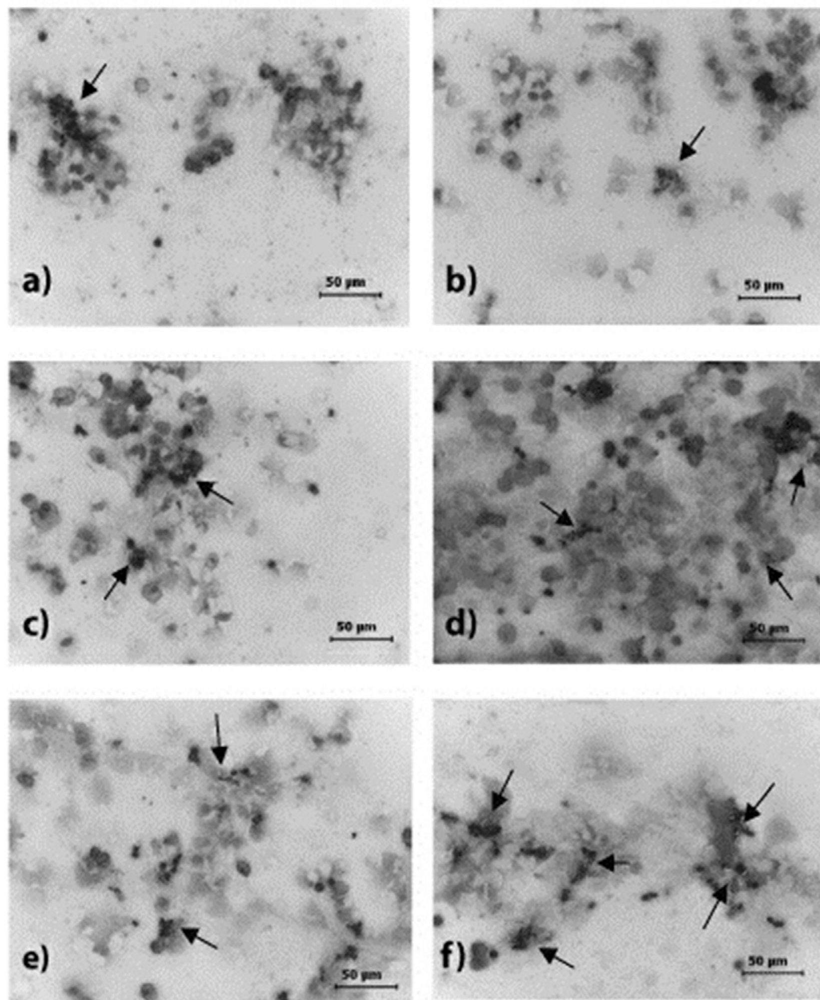

Fig. 4. Microscopic view of the adhesion of *S. cerevisae* strains obtained after *in vitro* digestion of kefir-fermented milk with intestinal epithelial cells from Swiss albino mice, stained by Gram's method. The arrow indicates the position of the adhered yeast: a) ScB, b) ScD, c) ScE, d) ScQ, e) ScV and f) ScZ.

S. cerevisae strains (from a total of 20 other strains) from cheese starters and observed percentages of 37.9 ± 6.7%, 44.1 ± 2.4%, 45.3 ± 1.0% and 48.6 ± 17.5%, showing intermediate autoaggregation ability, following the classification proposed by the authors. Syal and Vohra [42] reported that autoaggregation ability above 80% is considered strong, and this property has been related to the ability to adhere to intestinal epithelial cells [15].

The surface hydrophobicity of the evaluated strains was significant when compared to previous reports. Binetti et al. [15] studied the hydrophobicity of 20 autochthone strains isolated from milk and cheese whey starters, and reported percentages ranging from 45 ± 3% to 85 ± 3%, with the highest value for the *S. cerevisiae* L1 (80 ± 1%). Syal and Vohra [42] evaluated the hydrophobicity of 7 yeast strains isolated from fermented Indian foods and found percentages between 32% and 68%. Low levels of hydrophobicity were also found by Sourabh et al. [40] when they screened 23 yeast strains and found only 9 of them with percentage above 50% (59.65% was the highest). High hydrophobicity of the cell surface is supposed to explain why certain strains exhibit slower elimination kinetics from the gastrointestinal tract and also have different health effects [43]. Nevertheless, a good correlation between hydrophobicity (*in vitro* assays) and intestinal adhesion (*in vivo* assays) was not always found, as previously reported [15].

At the same time, adhesion to intestinal epithelial cells is also an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive environmental advantage [34,44]. Cell adhesion is due to non-specific physical interactions between two surfaces, which enable specific interactions between adhesins and complementary receptors [42]. Yeasts from kefir grains were shown to enhance aggregation and adhesion of lactic acid bacteria to the epithelial cells [18,45]. Verstrepen and Klis [46] reported that the ability of the yeasts to adhere to intestinal epithelial cells is probably due to their capacity to rapidly adapt to new habitats and form biofilms. Kourelis et al. [36] concluded, after studying the yeasts *Candida* sp. and *Saccharomyces* sp. from Feta cheese, that most of the yeasts able to adhere to the intestinal epithelium are pathogenic, such as *Candida* sp. These facts contradict our findings, since we have found that non-pathogenic *Saccharomyces cerevisiae* strains, isolated from milk fermented by kefir, presented good levels of adherence to epithelial cells.

5. Conclusion

Saccharomyces cerevisiae strains obtained after *in vitro* digestion of kefir-fermented milk showed interesting probiotic properties, resistance to harsh conditions of digestion and to antimicrobial agents. The strains studied should be considered potential

probiotics because of their properties not yet reported for *S. cerevisiae* as: antagonism to pathogenic microorganisms, antioxidant activity, adhesion to epithelial cells and ability to digest nutrients. Our findings should encourage further researches on the application of these strains as food and feed supplements.

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