Interpretative summary: Screening for potential probiotic properties of lactic acid bacteria isolated from kefir grains

Leite

Lactic acid bacteria and their metabolites may exert beneficial effects on human health from the consumption of food containing these microorganisms, known as probiotic. Kefir is a functional dairy beverage and can be an interesting source for the isolation of microorganisms with probiotic properties. In this study, the potential probiotic of lactic acid bacteria isolated from different kefir grains was investigated. A *Lactobacillus paracasei* strain was selected as appropriate probiotic candidate for potential use in new functional fermented products based on its in vitro tolerance to gastrointestinal conditions, epithelial adhesion, antimicrobial and antioxidative activities, besides the absence of undesirable properties.

PROBIOTIC PROPERTIES OF LAB FROM KEFIR GRAINS

Probiotic potential of selected lactic acid bacteria strains isolated from Brazilian kefir grains

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ABSTRACT

A total of 34 lactic acid bacteria (LAB) isolates from four different Brazilian kefir grains were identified and characterized among a group of 150 isolates, using the ability to grow at acidic pH and resistance to bile salts as restrictive criteria for probiotic potential. All isolates were identified by amplified ribosomal DNA restriction analysis (ARDRA) and 16S rDNA sequencing of representative amplicons. Eighteen isolates belonged to the species *Leuconostoc mesenteroides*, 11 to *Lactococcus lactis* (of which eight belonged to the subsp. *cremoris* and three to the subsp. *lactis*) and five to *Lactobacillus paracasei*. In order to exclude replicates, a molecular typing analysis was performed by combining rep-PCR and RAPD techniques. Considering a threshold of 90% similarity, 32 different strains were considered. All strains showed some antagonistic activity against four model food pathogens. In addition, three *Lc. lactis* strains and one *Lb. paracasei* produced bacteriocin-like inhibitory substances against at least two indicator organisms. Moreover, one *Lc. lactis* and two *Lb. paracasei* presented good total antioxidative activity. None of these strains showed undesirable enzymatic or haemolytic activities, while proving susceptible or intrinsically resistant to a series of clinically relevant antibiotics. The *Lb. paracasei* strain MRS59 showed a level of adhesion to human Caco-2 epithelial cells comparable to that observed for *Lactobacillus rhamnosus* GG.

Taken together, these properties allow the MRS59 strain to be considered a promising probiotic candidate.

Key Words: kefir grain, lactic acid bacteria, lactobacilli, probiotic property

INTRODUCTION

Kefir is a fermented milk product originating from the Northern Caucasus. The name kefir is derived from the Turkish language word "keyif", meaning "good feeling" after the feelings experienced after drinking it (Leite et al., 2013a). The fermented beverage is acidic, viscous, slightly carbonated and presents small amounts of alcohol (Leite et al., 2013b). Traditionally, kefir is made by using kefir grains as a starter (Leite et al., 2013a,b). Kefir grains are white to yellowish, cauliflower-like, grains, 0.3 - 3.5 cm in diameter, with a slimy but firm texture. The grains are composed of an inert matrix made up of polysaccharides and proteins. The matrix is densely populated by lactic acid bacteria (**LAB**) species, acetic acid bacteria (**AAB**) and yeasts (Leite et al., 2012; Leite et al., 2013a).

Kefir beverage has a long tradition of consumption in Eastern Europe, and it is nowadays spreading around the world due to its potential health-associated properties. Although for some of the health benefits reported there is not yet well documented scientific evidence or clinical demonstration, several *in vitro* and animal studies have associated kefir beverage with alleviation of lactose intolerance (Hertzler and Clancy, 2003), immunomodulation (Hong et al., 2009), antimicrobial activity against pathogenic microorganisms (Chifiriuc et al., 2011) and balance of the intestinal microbiota (Urdaneta et al., 2007). Traditionally, the functional properties of kefir have been attributed mainly to its bioactive peptide content and to kefiran, its main soluble exopolysaccharidic component (Santos et al., 2003; Rodrigues et al., 2005). However, the potential beneficial effects might also be mediated by the undefined

74 microbial composition of this fermented milk or by their secondary metabolites
75 (Nielsen et al., 2014).

76 Although there is a reasonable number of well-characterized probiotic strains
77 commercially available around the world, screening for novel strains is still of great
78 interest from the industrial points of view (Vinderola et al., 2008; Ayeni et al., 2011).
79 Additionally, strain expressing unique and particular characteristics that may enable
80 health benefit may arise in the characterization of natural fermented dairy products
81 such as kefir. This traditional product might be an interesting source of LAB strain
82 with specific functional properties. In fact, previous reports described the evaluation
83 and selection ok kefir LAB isolates for potential use as probiotics (Golowczyc et al.,
84 2008; Zanirati et al., 2014).

85 The aim of the present study was to identify and characterize LAB strains isolated
86 from traditional kefir grains, displaying *in vitro* properties related to their probiotic
87 potential according the guidelines recommended by FAO/WHO (2006). After a
88 complete characterization and the corresponding *in vivo* trials, these strains could be
89 ultimately included as probiotics in functional foods.

90 **MATERIAL AND METHODS**

91 ***Isolation of bacteria from kefir grains***

92 LAB were isolated by dilution and plating from four kefir grains collected in
93 different regions of Brazil (AR - Niterói, RJ, AD - Lavras, MG, AV - Viçosa, RJ and
94 AF - Alfenas, MG). Briefly, 10 g of each kefir sample were homogenized in 90 mL of
95 sodium citrate (2%). Serial decimal dilutions were obtained and plated on lactobacilli
96 Man, Rogosa, and Sharpe (**MRS**) and M17 agar media (Difco, Sparks, MD, USA)
97 supplemented with 200 μg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO, USA),
98 and incubated in aerobic and anaerobic (Gaspak EZ; Difco) conditions at 30ºC for 72

h. Representative colonies of all morphologies were taken randomly and purified on the same media by subculturing. Gram-positive, catalase negative isolates were considered as presumptive LAB, which were stored in 15% glycerol at −80ºC.

For all subsequent assays, LAB were activated in the corresponding media at 30°C for 18-24 h, and subcultured in the same conditions.

Growth of the isolates at low pH

The ability of the isolates to grow at low pH was assayed as described by Nishida et al. (2008) in MRS-THIO broth (MRS supplemented with 0.2% sodium thioglycolate). In short, overnight cultures, corresponding with an initial bacterial population ranging from 10^7 to 10^9 cfu/mL, were harvested by centrifugation and cells were suspended in phosphate-saline-buffer (PBS, pH 6.5) to obtain an OD_{600} = 0.5. Cell suspensions were ten-fold diluted with MRS medium, adjusted to pH 3.0 with HCl and incubated at 37°C for 3 h. The pH tolerance of the cells was determined by enumerating the viable cells on MRS agar plates. Non-treated cultures used as controls were suspended in conventional, non-acidified MRS (pH 6.5).

Bile tolerance of the isolates

Tolerance to bovine bile (Oxgall; Difco) was assayed by growing the isolates in agar plates and broth, following the procedure reported by Delgado et al. (2007) and Guo et al. (2009), respectively. The growth rate of the strains was estimated in MRS-THIO broth in the absence (control) and in the presence (test) of 0.3% Oxgall. Overnight cultures were inoculated (1%) into the liquid medium, and cultured at 37°C for up to 9 h. Absorbance at 620 nm was measured every hour. The effect of the bile salts was scored as the time difference required to increase 0.3 units the absorbance of the culture at 620 nm (OD_{620}) in MRS-THIO broth with and without 0.3% bile salts.

The growth delay (hours) between the culture media was considered as the lag time (**LT**).

Additionally, tolerance of strains to different concentrations of bile salts was assayed by a plate assay. Individual colonies growing in MRS agar plates were suspended in 2-5 mL of sterile saline solution 0.85% at a density corresponding to McFarland standard 1. Aliquots of the suspensions (10 µL) were spotted onto bile-containing 0.3%, 0.5%, 1% and 2% (w/v) agar plates. The plates were incubated at 37°C under anaerobic conditions and growth was recorded after 24-48 h. A plate without bile was used as positive control. The experiments were performed in duplicate.

Identification of LAB isolates

Total genomic DNA of the isolates was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich), following the manufacturer's recommendations. Purified DNA was used as a template to amplify a segment of the 16S rRNA gene by the PCR technique using the universal prokaryotic primers S-D-Bact-0008-a-S-20 (27F) (5'-AGAGTTTGATCCTGGCTCAG-3') and S-*-Univ-1492R-b-A-21 (1492R) (5'-GGTTACCTTGTTACGACTT-3'). For the amplified ribosomal DNA restriction analysis (**ARDRA**), amplicons were purified through GenElute PCR Clean-Up columns (Sigma-Aldrich), digested with HaeIII and HhaI restriction enzymes (Invitrogen, Pasley, UK) and electrophoresed in agarose gels. Gels were stained with ethidium bromide (0.5 mg/mL) and photographed under UV light. Representative amplicons of the different ARDRA profiles were sequenced. Sequencing was accomplished in an ABI 373 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). On average, 850 bp of sequence were obtained, which were compared to those deposited in the GenBank database using the BLAST program

148 (http://www.ncbi.nlm.nih.gov/BLAST/). Following the criterion applied by Palys et
149 al. (1997), sequences with a percentage of identity of 98% or higher to those in
150 databases were allocated to the same species.

151 ***Molecular typing analyses***

152 In order to exclude replicates, LAB isolates were grouped by both repetitive
153 extragenic palyndromic PCR (**rep-PCR**), using the primer BOXA2R (5'-
154 ACGTGGTTTGAAGAGATTTTCG-3'), as reported by Koeuth et al. (1995), and
155 random amplification of polymorphic DNA (**RAPD**) with primer M13 (5'-
156 GAGGGTGGCGGTTCT-3'), as reported by Rossetti and Giraffa (2005). Banding
157 patterns were examined with the Bionumerics 6.5 software program (Applied Maths,
158 Belgium) using Dice's coefficient. Cluster analyses of composite data obtained with
159 rep-PCR and RAPD were achieved using the unweighted pair group method with
160 arithmetic averages (**UPGMA**).

161 ***Antimicrobial activity***

162 ***Pathogen inhibition.*** The capability of the strains to inhibit a group of food-borne
163 pathogens was determined using an agar spot test as described by Ripamonti et al.
164 (2011). Overnight test cultures were spotted (2 µL) on the surface of modified MRS
165 agar (without ammonium citrate and sodium acetate) and incubated anaerobically for
166 24 h at 30°C. Cells were then inactivated with chloroform for 30 min. *Escherichia*
167 *coli* ATCC 25922, *Salmonella enterica* var. Enteritidis ATCC 13076, *Staphylococcus*
168 *aureus* ATCC 25923, and *Listeria monocytogenes* ATCC 15313 were used as
169 indicators. A 100 µL volume of an overnight culture of each indicator was mixed with
170 10 mL of Brain Heart Infusion (**BHI**; Difco) soft agar (0.7%), and poured onto MRS
171 agar plates. These were incubated aerobically at 37°C for 24 h. *Lactobacillus*
172 *acidophilus* ATCC 4356 was used as a negative control. Inhibition but not clear-cut

halo or less than 1mm was recorded as (+/-), a clear zone of growth inhibition around spots bigger than 1 mm was scored as positive (+), meanwhile an inhibition zone between 2 and 5 mm surrounding the colony was recorded as (++).

Bacteriocin production. The production of bacteriocin-like inhibitory substances (**BLIS**) was successively examined using an agar spot test and a well-diffusion assay. *Lactobacillus sakei* CECT 906 and *Lactococcus lactis* IL 1403, two well-recognized bacteriocin-susceptible strains (Alegria et al., 2010), and *Listeria monocytogenes* ATCC 15313 were used as indicators. Overnight cultures were spotted on the surface of M17 and MRS agar plates (0.2% glucose), incubated for 24 h at 30°C for lactococci and leuconostoc strains, and at 37°C for lactobacilli, and inactivated as described previously with chloroform. Spots were covered with 10 mL of soft agar (0.75%) inoculated with the indicators and incubated under the required conditions.

Positive cultures were tested by a well-diffusion assay. Briefly, 20 mL of agar medium at 45°C were vigorously mixed with 200 µL of an overnight culture of each indicator and poured into Petri dishes. Supernatants from overnight cultures of the test strains were neutralized to pH 6.5-7.0 with NaOH 0.1 *M*, centrifuged at 12,000 *g* for 5 min, and filter-sterilized through a 0.20 µm pore membrane (Millipore, Bedford, MA, USA). 50 µL aliquots of each supernatant were placed in a well excavated in the agar. To allow the diffusion of the supernatant into the agar, plates were maintained at 4°C for 1 h before incubation. Inhibition of the indicators was evaluated after incubation at 37ºC for 24 h.

To investigate the proteinaceous nature of the BLIS, supernatants were tested in the vicinity of wells filled with 50 µL of a solution of either proteinase K or pronase (each at a concentration of 20 mg/mL). After incubation, plates were examined to judge whether the inhibitory substance was sensitive to proteolysis. *Lactococcus*

198 *lactis* 1A6, a nisin producer strain (Alegria et al., 2010) was used as control. Tests
199 were performed in duplicate.

200 ***Antioxidative activity***

201 ***Sample preparation for the antioxidative assays.*** Overnight cultures in MRS broth
202 were harvested by centrifugation at 4°C for 10 min, washing with isotonic saline
203 solution (0.85%) at 4°C and suspended in phosphate buffer with 1m*M* EDTA, pH 7.5.
204 The suspension was adjusted to an OD_{600} = 1.0. In order to obtain the cell extracts,
205 cells were disrupted in a Cell disruptor (Constant Systems, Daventry, UK) and
206 deposited immediately in an ice-bath. The extracts were then centrifuged at 10,000 x
207 *g* at 4°C for 10 min to eliminate cell debris.

208 ***Total antioxidative activity (TAA).*** In order to evaluate the TAA of the strains, the
209 linolenic acid test (**LA test**) was used as described by Kullisaar et al. (2002), using 45
210 µL of the samples (lysate or whole bacterial cells). The absorbance at 534 nm was
211 measured on a UV-Vis Spectrophotometer (Hitachi High-Technologies, Tokyo,
212 Japan) and the percentage of TAA of the samples was expressed as $[1 - (A_s/A_c)] \times$
213 100], where: A_s is the absorbance in the presence of the sample and A_c is the
214 absorbance of the control without sample. Intact cells and cell lysates were assayed in
215 triplicate.

216 ***Glutathione assay.*** Reduced and oxidized glutathione and the glutathione redox status
217 were evaluated using cell-free extracts and the GSH/GSSG Ratio Assay kit
218 (Millipore, Billerica, MA, USA) following the manufacturer's instructions. The
219 glutathione content was quantified on the basis of a standard curve generated with
220 known amounts of glutathione. The reduced glutathione (**GSH**) content was
221 calculated as the difference between the total glutathione (**tGSH**) and the oxidized

glutathione (**GSSG**). The glutathione redox ratio was expressed as GSH/GSSG. *Escherichia coli* CECT 515 was used as a positive control (Masip et al., 2006).

Adhesion assay

The epithelial intestinal cell line Caco-2 purchased from the European Collection of Cell Culture (ECACC 86010202) was used to assess the adhesion ability of selected strains. The culture and maintenance of the cell line were carried out following standard procedures (Sánchez et al., 2010) using DMEM medium supplemented with 20% fetal bovine serum, non-essential aminoacid solution and a mixture of antibiotics (50 µg/mL penicillin-streptomycin, 50 µg/mL gentamicin and 1.25 µg/mL amphotericin B) (all reagents from Sigma-Aldrich). The cell line was used after reaching the confluent-differentiated monolayer state (13 ± 1 day). The strain *Lactobacillus rhamnosus* LMG 18243 (also known as *Lb. rhamnosus* GG) was used as a reference control for adherence.

Lactobacilli strains, grown overnight in 10 mL MRS under standard conditions, were harvested by centrifugation, washed twice in Dulbecco's PBS solution (Sigma-Aldrich) and suspended in supplemented-DMEM media without antibiotics, at a concentration of approximately 10^8 cfu/mL. To remove antibiotics from the cells, monolayers were washed twice in Dulbecco's PBS. Subsequently, the bacterial suspensions were added at a bacteria:eukaryotic cell ratio of 10:1, and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. Afterwards, wells were gently washed three times with Dulbecco's PBS buffer to remove non-adhered bacteria. Monolayers were disrupted with an EDTA-trypsin solution (Sigma-Aldrich), and the attached bacteria were counted by plating in MRS agar. Adhesion was expressed as the percentage of bacteria adhered with respect to total number of bacteria added. Experiments were

carried out using two independent Caco-2 plates (two consecutive passes) and in each plate bacterial strains were analyzed by duplicate.

Safety assessment

Haemolysin production. Haemolysin production was analyzed on Columbia agar plates containing 5% sheep blood (bioMérieux, Montalieu-Vercieu, France). The presence of β- or α-haemolysis is indicated by the formation of clear or greenish zones around the colonies, respectively.

Antibiotic resistance. The minimum inhibitory concentration (**MIC**) of a series of antibiotics was assayed on the selected strains by microdilution in VetMIC plates for LAB (SVA, Uppsala, Sweden), following the manufacturer's recommendations. Colonies grown on LSM (Klare et al., 2005) agar plates were suspended in 5 mL of sterile saline solution (0.9%) to obtain a density corresponding to McFarland standard 1. Suspensions were further diluted 1:1000 in LSM. One hundred µL of this dilution were added to each well of the VetMIC plate. The plates were incubated at 37°C for 48 h. MICs were defined as the lowest antibiotic concentration at which no visual growth was observed.

Enzyme activities. Enzyme activities were measured by the commercial, semi quantitative API-ZYM system (bioMérieux) following the manufacturer's recommendations. In short, 65 µL of a cell suspension corresponding to McFarland standard 5 were inoculated in each well of the API-ZYM strips. Enzyme activities were evaluated after 4 h of incubation in anaerobiosis at 37°C. Enzyme activities were recorded from 0 (no activity) to 5 (≥ 40 nmols of product released) with the API-ZYM colour reaction chart.

Statistical analyses

Statistical comparisons for both the adhesion and antioxidative tests were performed using the Statistica software package for Windows version 7.0 (Statsoft, Tulsa, OK, USA). Significant differences between treatments were tested by the analysis of variance test (one-way ANOVA), followed by a comparison between means using Fisher's least significance difference (**LSD**) method, with levels of significance set at $P < 0.05$.

RESULTS

LAB screening for pH and bile-salt tolerance

Thirty seven out of the 150 tested LAB isolates were able to grow at pH 3.0 during 3 h of incubation. However, the growth in these conditions was not comparable to that in conventional MRS (pH 6.5) with reductions in counts between 2 to 3 logarithmic units as compared to controls. These 37 isolates were tested by a plate assay for resistance to different bile concentrations ranging from 0.3 to 2%, and by tolerance in liquid containing 0.3% of Oxgall. In the latter medium, a lag time (LT) ranging from 0.5 to 4 h was observed for 34 isolates (Table 1); these were considered to be bile tolerant. In contrast, three LAB isolates presented an LT > 9 h; these were considered bile susceptible.

As a result of the bile plate assay, 34 isolates grew in 0.3%, 25 grew in 0.5%, 13 in 1%, and eight in 2% (Table 1).

LAB identification

Among the 34 isolates four different representative ARDRA profiles were observed with the HaeIII and HhaI restriction enzymes (data not shown). Representative 16S rDNA amplicons of each of the different profiles were selected for sequencing. Sequence comparisons showed a homology higher than 98% to four different LAB species. Therefore, the 34 isolates were identified as follow;

295 *Leuconostoc mesenteroides* (18), *Lactococcus lactis* subsp. *cremoris* (8),
296 *Lactobacillus paracasei* (5), and *Lactococcus lactis* subsp. *lactis* (3).

297 ***LAB Typing***

298 LAB isolate typing was performed by a combination of rep-PCR and RAPD
299 techniques. Using a 90% similarity as threshold (Fig. 1), 32 different strains (four *Lb.*
300 *paracasei*, ten *Lc. lactis* and 18 *Leu. mesenteroides*) were considered. These were all
301 subjected to further analyses.

302 ***Antimicrobial properties***

303 ***Antagonistic activity against pathogens.*** Pathogens were inhibited by most strains in
304 the agar spot test (Table 2). *Listeria monocytogenes, Escherichia coli* and *Salmonella*
305 *enterica* were inhibited by 23, 22, and 20 strains, respectively. In contrast,
306 *Staphylococcus aureus* was inhibited by only seven strains. Pathogen inhibition,
307 however, was not confirmed by the agar well-diffusion assay, with the exception of *L.*
308 *monocytogenes*, that was inhibited by four strains after neutralization of the cell-free
309 supernatants (see below).

310 ***Bacteriocin-like inhibitory substances production.*** *Lb. sakei* CECT 906 was
311 inhibited by four strains and *Lc. lactis* IL 1403 by 12 strains (Table 2) in the agar spot
312 assay. The isolates demonstrating antibacterial activity against any of the indicators
313 were subsequently subjected to the well-diffusion assay. None of the *Leuconostoc*
314 strains showed inhibition against indicators in this assay. *Lc. lactis* IL 1403 was
315 inhibited by three lactococci strains (MRS26, M171 and MRS52). Both *L.*
316 *monocytogenes* ATCC 15313 and *Lb. sakei* CECT 906 was inhibited by the three
317 above mentioned lactococci strains and one *Lb. paracasei* (MRS55) strain. The
318 proteinaceous nature of the BLIS produced by these four strains was confirmed by
319 proteinase treatment of the cell-free supernatants.

320 ***Antioxidative activity***

321 According to Hutt et al. (2006) LAB strains with a TAA value > 20% are
322 considered to have antioxidative activity. Three of the LAB strains from kefir grains
323 (two *Lb. paracasei*; MRS 59 and M1743, and one lactococci strain; MRS 52) showed
324 percentages above this value in either intact cells or lysate supernatants (Table 3). All
325 other strains showed mean TAA values below 15% (data not shown), except for two
326 other lactobacilli strains (MRS55 and MRN4), which showed a TAA between 15 and
327 20% (Table 3).

328 The three LAB strains that showed higher values in the TAA test ($P < 0.05$) were
329 further analyzed by the glutathione assay (Table 3). The total gluthatione content
330 values (tGSH) found for the LAB strains were lower than the positive control (*E. coli*)
331 ($P < 0.05$). However the calculated glutathione redox ratio (GSSG/GSH), used to
332 investigate oxidative stress, was similar for all the strains, except for the *Lb.*
333 *paracasei* M1743 strain, in which the oxidized glutathione (GSSG) content was not
334 detectable and, consequently, its GSH/GSSG redox ratio could not be calculated.

335 Based on the results obtained in the antimicrobial and antioxidative tests, three *Lb.*
336 *paracasei* strains (MRS55, MRS59 and M1743) were considered as presenting
337 probiotic potential and were selected for further analyses.

338 ***Adhesion capability***

339 The results of the adhesion assay for the three selected strains are depicted in
340 Figure 2. Adhesion percentages ranged from 0.9 to 9%. The adhesion level of the
341 MRS59 strain was similar to that of the reference strain GG, whereas those of M1743
342 and MRS 55 were lower ($P < 0.05$).

343 ***Enzymatic activities and haemolysin production***

Undesirable activities, such as trypsin, α–chymotrypsin, and β-glucuronidase activities, were not detected in any of the selected strains. In addition, none of the three strains showed hemolytic activity under the assay conditions.

Antibiotic resistance

The selected lactobacilli strains were susceptible to all the analyzed antimicrobial agents (including tetracycline, erythromycin, clindamycin, ampicillin and aminoglycosides) with the exception of vancomycin, for which an intrinsic resistance was observed. The chloramphenicol MIC for MRS55 and M1743 strains was only one dilution higher (8 µg/mL) than the microbiological breakpoint defined by the EFSA (4 µg/mL), which is within the normal acceptable variation around the means (EFSA, 2012).

DISCUSSION

Beyond their technological function, there is currently an increasing demand for new LAB strain probiotic candidates (Ayeni et al., 2011; Argyri et al., 2013). The complex microbiota of kefir, a traditional beverage endowed with several health benefits (Leite et al., 2013a), could be a source for obtaining novel probiotic strains (Santos et al., 2003; Kumura et al., 2004).

In the present study, LAB were isolated from four kefir grain samples, identified and typed by molecular methods, and characterized *in vitro* for recognized probiosis properties, such as acidity and bile tolerance and antimicrobial and antioxidant activities, key features to consider bacterial strains as probiotics. The secretion of gastric acid and transit through stomach constitutes a primary defence mechanism that all ingested microorganisms must overcome, including probiotics (Gueimonde and Salminen, 2006). LAB isolates from kefir grains were screened and selected for their resistance and survival in an acidic environment, as well as for their growth in the

presence of 0.3% bile salts, a similar concentration to that present in the small intestine (Vinderola et al., 2008). Though there is no scientific consensus on the pH and bile concentration to which probiotic strains should be tolerant to (Zago et al., 2011), based on the results, the lactobacilli were able to grow in higher bile concentrations (1%). Similar results have been previously reported by other authors analyzing LAB strains from different environments (Delgado et al., 2007; Vinderola et al., 2008; Zago et al., 2011; Ramos et al., 2013).

Molecular typing showed a rather high genetic heterogeneity among the LAB isolates from the four kefir grains as judged by the large number of different profiles obtained.

Some of these LAB strains exhibited antimicrobial activity against pathogens, and four of them produced BLIS, which might provide advantage in competing either in a food product or in the gut (Servin, 2004; Vinderola et al., 2008). Moreover, we observed one *Lactococcus* BLIS-producing strain, as well as, two *Lb. paracasei*, showed an antioxidative activity higher than those of other tested LAB strains ($P < 0.05$), indicating they may aid in protecting cells from oxidative damage (Lin and Chang, 2000; Zhang et al., 2010). In particular, the lactococcal strain showed higher TAA in intact cells, meanwhile in the *Lb. paracasei* strains, the antioxidative activity was more relevant in cell extracts indicating that this activity might be also relevant in case of bacterial lysis into the gastrointestinal tract and release of the intracellular content. Furthermore, reduced glutathione (GSH), an important component of the cell defence system against oxidative stress (Masip et al., 2006), was detected for all the LAB strains that showed TAA values > 20%. However the obtained values suggest that the antioxidative effect displayed by some of these strains could be attained by means of other mechanisms different from glutathione protection, such as enzymatic

antioxidants. The antioxidative activities of LAB strains have been under investigation in other studies (Kullisaar et al., 2002; Li et al., 2012; Chen et al., 2014). This protective property may be useful as a defence mechanism in the intestinal microbial ecosystem.

Strains belonging to the *Lactobacillus* genus are commonly used as probiotics (FAO/WHO, 2006; Gueimonde and Salminen, 2006; Zago et al., 2011). Three *Lb. paracasei* strains were considered as appropriate probiotic candidates and selected for further characterization of desirable and undesirable probiotic-related properties.

The capacity to adhere to the intestinal mucosa is an important property for probiotic strains, since they should, at least transiently, colonize, the host gut (FAO/WHO, 2006). The human colon adenocarcinoma cell line Caco-2 is widely accepted as a model for assessing the adhesion ability of probiotic candidates (Gueimonde and Salminen, 2006; Ayeni et al., 2011). One *Lb. paracasei* strain in this study (MRS59) showed similar adhesion values than the recognized probiotic strain *Lb. rhamnosus* GG, suggesting they may well be good *in vivo* colonizers.

None of these lactobacilli showed β-glucuronidase activity, which may have negative effects in the colon and has been considered a carcinogenic enzyme (Monteagudo-Mera et al., 2011). In contrast, all three strains produced β-galactosidase, a beneficial enzyme considering both probiotic and technological aspects, supporting the reduction of lactose intolerance as well as milk acidification (Monteagudo-Mera et al., 2011). These strains also presented α-glucosidase and β-glucosidase activity, which could contribute to polysaccharide digestion (Papamanoli et al., 2003). The presence of glycosidase activities in food cultures seems to have an impact on sensory properties, since flavour is often linked to sugar metabolism (Papamanoli et al., 2003; Mesas et al., 2011).

In summary, 32 different LAB strains isolated from kefir grains showing good survival under normal gastrointestinal conditions were evaluated *in vitro* for antimicrobial and antioxidative activities in this study. Among the analyzed strains, three *Lb. paracasei* strains were further selected. The presence of harmful enzymatic activities and atypical antibiotic resistances among the selected strains were discarded. Based on a long history of human consumption, *Lb. paracasei*, like many other LAB species, is granted a Qualified Presumption of Safety (**QPS**) status (EFSA, 2011), allowing unrestricted application in food and feed, provided there is an absence of antibiotic resistance. Among the three selected strains, *Lb. paracasei* MRS59 showed the highest number of *in vitro* probiosis properties; consequently, it was considered as the most appropriate kefir-derived candidate to be used as a probiotic. Further *in vivo* studies should be also performed to confirm its potential beneficial effects.

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581

582 **Table 1**. Bile resistance of 32 identified and typed LAB isolated from kefir grains

Strain	Identification	Origin	Bile assay				
			Lag time (h) 0.3% bile	Growth plate[1]			
				0.3% bile	0.5% bile	1% bile	2% bile
MRS17		AR	2	+	+	+	+
M1711B		AR	3	+	+	-	-
MRS26	*Lactococcus lactis* subsp. *cremoris*	AR	1.5	+	+	-	-
MRS47		AD	3	+	-	-	-
M1732		AR	1	+	-	-	-
M1734		AR	2	+	+	-	-
M171		AR	1	+	+	-	-
MR2	*Lactococcus lactis* subsp. *lactis*	AV	1	+	+	+	+
MRS52		AD	1	+	+	-	-
MRN3		AV	4	+	+	-	-
MRN4	*Lactobacillus paracasei*	AR	1	+	+	+	+
MRS59		AF	2	+	+	+	-
M1743		AF	2	+	+	+	+
MRS55		AF	3	+	+	+	-
MRS2	*Leuconostoc mesenteroides*	AR	2	+	+	-	-
GYP8		AR	3	+	+	+	-
MRS12		AR	1.5	+	+	-	-
MRS50		AD	2	+	-	-	-
MRS48		AF	3	+	-	-	-
MRS53		AF	2	+	-	-	-
MRS25		AR	2	+	+	+	+
GYP7		AR	1	+	+	+	+
MRS8		AR	2	+	+	-	-
MRS14		AR	1.5	+	+	-	-
GYP5		AR	2	+	-	-	-
MRS10		AR	4	+	+	-	-
GYP9		AR	1	+	+	+	+
GYP12		AR	0.5	+	+	+	-
M1721		AR	2.5	+	-	-	-
MRS19		AR	1.5	+	-	-	-
MRS24		AR	1	+	-	-	-
M1711A		AR	2	+	+	-	-

583
584 [1]Growth plate: – = no growth; + = positive growth
585

586 **Table 2**. Antimicrobial activity data of 32 identified and typed LAB isolated from
587 kefir grains

Strain	Identification	Origin	Inhibition profile						
			Spot test						
			Pathogen inhibition				Bacteriocin production		
			I[1]	II	III	IV	IV	V	VI
MRS17		AR	±[2]	++[3]	-[4]	-	-	-	-
M1711B		AR	±	±	-	-	-	-	-
MRS26*	*Lactococcus lactis* subsp. *cremoris*	AR	++	-	±	++	++	++	++
MRS47		AD	++	-	-	-	-	-	-
M1732		AR	-	++	-	±	±	-	-
M1734		AR	-	++	-	±	±	-	±
M171		AR	++	-	±	++	++	++	++
MR2	*Lactococcus lactis* subsp. *lactis*	AV	++	-	-	++	++	-	-
MRS52		AD	-	-	-	++	++	++	++
MRN3		AV	++	±	-	++	++	-	-
MRN4	*Lactobacillus paracasei*	AR	±	++	-	-	-	-	++
MRS59		AF	++	++	-	++	++	-	++
M1743		AF	++	++	±	±	±	-	±
MRS55		AF	++	++	-	++	++	++	++
MRS2	*Leuconostoc mesenteroides*	AR	-	-	-	±	±	-	±
GYP8		AR	±	±	±	-	-	-	-
MRS12		AR	+	±	±	±	±	-	-
MRS50		AD	-	-	±	±	±	-	±
MRS48		AF	-	-	+	±	±	-	±
MRS53		AF	-	-	-	-	-	-	-
MRS25		AR	±	-	-	±	±	-	±
GYP7		AR	±	-	-	-	-	-	-
MRS8		AR	-	±	-	±	±	-	-
MRS14		AR	-	±	-	±	±	-	-
GYP5		AR	+	±	-	±	±	-	-
MRS10		AR	±	±	-	±	±	-	-
GYP9		AR	±	±	-	±	±	-	-
GYP12		AR	-	-	-	-	-	-	-
M1721		AR	±	±	-	±	±	-	-
MRS19		AR	±	±	-	±	±	-	-
MRS24		AR	±	±	-	±	±	-	-
M1711A		AR	±	±	-	-	-	-	-

588 [1]Indicators: I = *Escherichia coli* ATCC 25922; II = *Salmonella enterica* serovar Enteritidis ATCC 13076; III =
589 *Staphylococcus aureus* ATCC 25923; IV = *Listeria monocytogenes* ATCC 15313; V = *Lactobacillus sakei* CECT
590 906; VI = *Lactococcus lactis* IL1403.
591 [2]±: inhibition but no clear halo.
592 [3]++ : presence of a clearly defined inhibition zone surrounding the colony in the spot test or the wells containing
593 neutralized, cell-free supernatant.
594 [4] –: no inhibition.
595 *strains in bold: producing bacteriocin-like inhibitory substances (BLIS)
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Table 3. Data on total antioxidative activity (TAA) and glutathione determinations (total, reduced and oxidized glutathione and glutathione redox ratio) of representative LAB strains from kefir grains showing antioxidative activity

Strain	Identification	TAA test[1] (%)		Glutathione test[2] (µM)			
		Intact cells	Cell lysate	Cell lysate			
				tGSH	GSSG	GSH	GSH/GSSG
MRS52	*Lc. lactis* subsp. *lactis*	20.3 ± 3.5^{a}	11.8 ± 3.9^{b}	0.586±0.23^{b}	0.062±0.03^{b}	0.461±0.14^{b}	8.75±3.19^{a}
MRS59	*Lb. paracasei*	18.4 ± 4.1^{a,c}	34.1 ± 9.9^{a}	0.157±0.21^{b}	0.024±0.01^{b}	0.110±0.19^{b}	5.72 ±3.09^{a}
MRN4	*Lb. paracasei*	3.5 ± 2.9^{b}	14.7 ± 12.7^{b}	n.d.	n.d	n.d	n.d.
M1743	*Lb. paracasei*	22.3 ± 9.0^{a}	26.0 ± 4.2^{a,b}	0.106±0.13^{b}	0	0.106±0.13^{b}	0
MRS55	*Lb. paracasei*	10.4 ± 2.3^{b,c}	18.2 ± 2.1^{b}	n.d..	n.d.	n.d.	n.d.
CECT515[3]	*E. coli*	n.d.	n.d.	2.313±0.48^{a}	0.31±0.04^{a}	1.686±0.40^{a}	5.33±0.67^{a}

[1]TAA test – Total antioxidative activitiy;

[2]Glutathione test: tGSH = total glutathione; GSSG = oxidized glutathione; GSH = reduced glutathione; n.d. = not determined;

[3] *E. coli* CECT 515 was used as positive control

The data are expressed as the means ± standard deviation, based on three replicates.

[a-c]Columns that do not share the same letter are statistically different according to the least significant differences (LSD) mean comparison test ($P < 0.05$)

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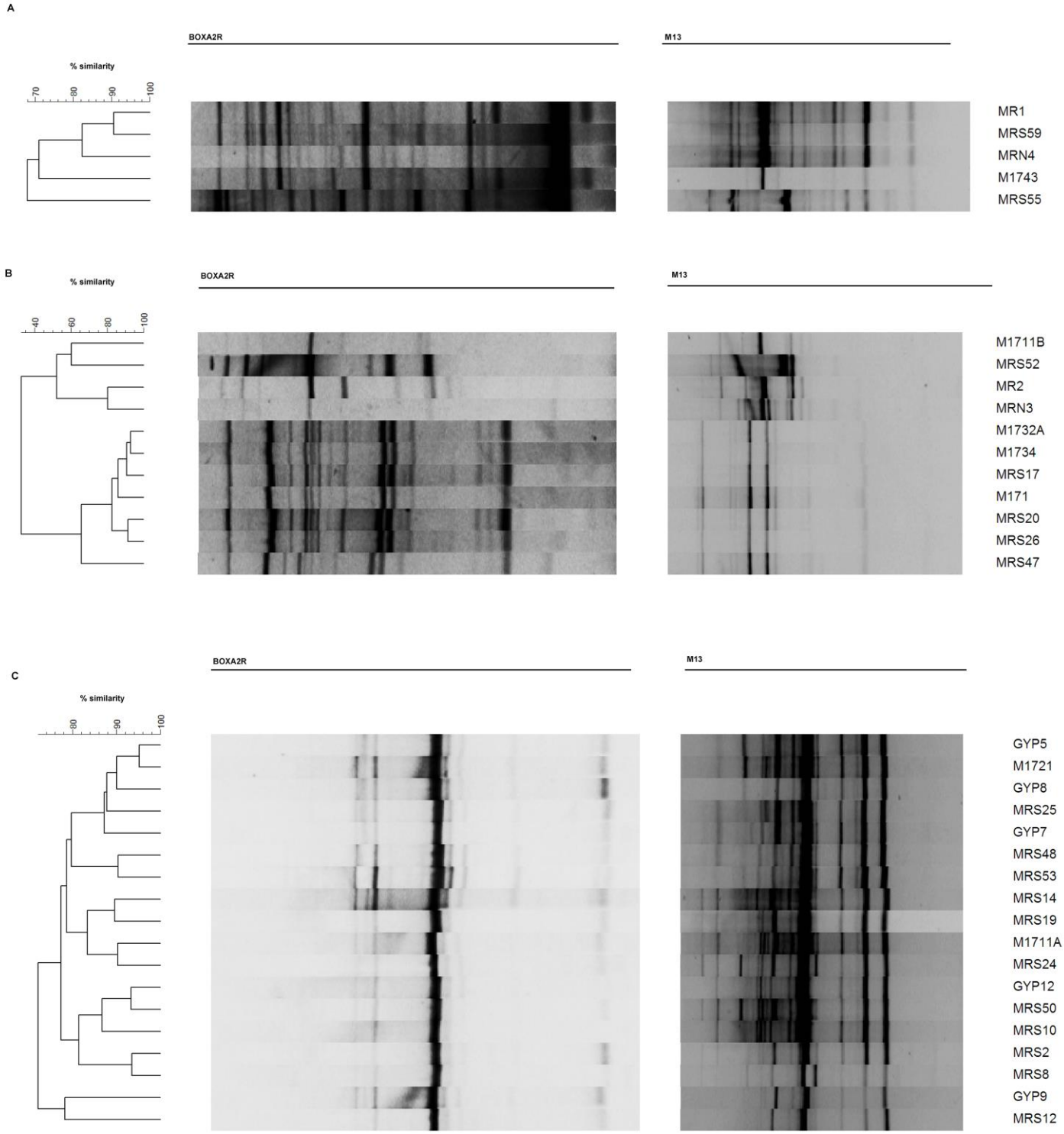

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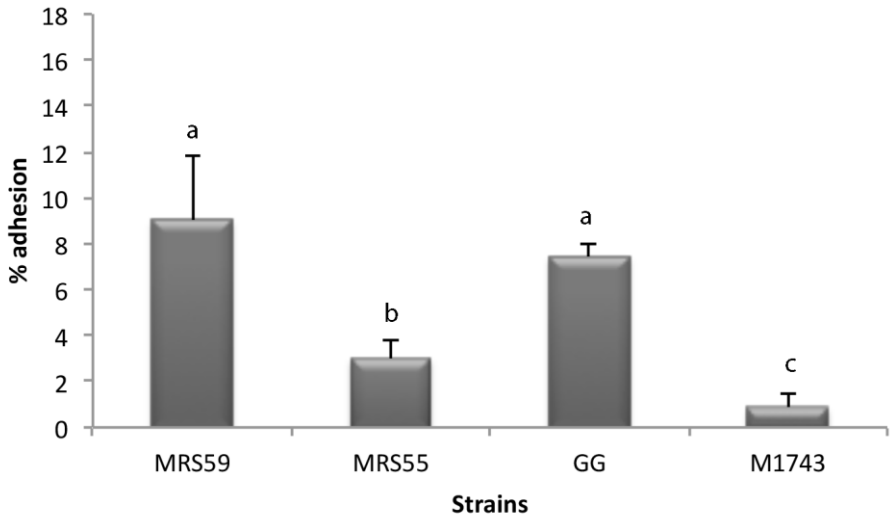

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Figure 1. Combined dendrogram obtained from rep-PCR and RAPD-PCR profiles, using BOXA2R and M13 primers corresponding to five *Lactobacillus paracasei* strains (Panel A); eleven *Lactococcus lactis* strains (Panel B) and eighteen *Leuconostoc mesenteroides* strains (Panel C).

Figure 2. Percentage of adhesion measured as the percentage of cfu/mL adhered bacteria with respect to cfu/mL added bacteria of the three *Lactobacillus paracasei* strains to the intestinal epithelial cell line Caco-2. Columns that do not share the same letter are statistically different according to the least significant differences (LSD) mean comparison test ($P < 0.05$). *Lactobacillus rhamnosus* GG was used as an adherent reference strain.