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Characterization of homofermentative lactobacilli isolated from kefir grains: potential use as probiotic

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Considering that several health promoting properties are associated with kefir consumption and a reliable probiotic product requires a complete identification of the bacterial species, the present work evaluates several proved markers of probiotic potential of eleven isolates of homofermentative lactobacilli isolated from kefir grains and molecular identification and genotypic diversity. Using restriction analysis of amplified ribosomal DNA (ARDRA) and analysis of the 16S–23S rRNA internal spacer region we confirmed that all homofermentative lactobacilli belong to the species *Lactobacillus plantarum*. RAPD-PCR analysis allowed the discrimination of lactobacilli in five clusters. All isolates exhibited high resistance to bile salt. High survival after one hour of exposure to pH 2·5 was observed in *Lb. plantarum* CIDCA 8313, 83210, 8327 and 8338. All isolates were hydrophilic and non autoaggregative. Isolate CIDCA 8337 showed the highest percentage of adhesion among strains. All tested lactobacilli had strong inhibitory power against *Salmonella typhimurium* and *Escherichia coli*. Seven out of eleven isolates showed inhibition against *Sal. enterica* and five isolates were effective against *Sal. gallinarum*. Only CIDCA 8323 and CIDCA 8327 were able to inhibit *Sal. sonnei*. We did not find any correlation between the five clusters based on RAPD-PCR and the probiotic properties, suggesting that these isolates have unique characteristics.

Keywords: kefir, *Lactobacillus plantarum*, probiotic properties.

Indigenous lactobacilli as well as lactobacillus strains involved in homemade or industrial fermented food have shown beneficial effects on gut health (Fernández et al. 2003; Parvez et al. 2006). High viable counts and survival rates during passage through the stomach are necessary to allow probiotics to take part in the biological role in the human intestine. Survival against the acid conditions of the stomach and bile salt are, therefore, of prime importance. In addition, adherence of probiotics to intestinal epithelium and temporary colonization of the gut are probably of crucial importance for their beneficial health effect (Ouwehand et al. 1999; Servin & Coconnier, 2003). Association of probiotic action with surface bacterial properties can be considered in almost all mentioned mechanisms (Pérez et al. 1998; Cesena et al. 2001). Characteristics ascribed to a probiotic are in general strain specific and individual strains have to be tested for each property.

A reliable probiotic product requires a complete identification of the bacterial species. In this context, molecular techniques have emerged in recent years as a complement to traditional phenotypic tests for comparing strains or species of probiotic bacteria. Amongst these methodologies, analysis of the 16S rRNA gene (Kullen et al. 2000) and 16S–23S rRNA internal spacer region (Leblond-Bourget et al. 1996; Tannok et al. 1999) have proven to be useful tools for identification. For typing purpose, RAPD technique is reported to be simple and rapid to perform, and to provide good levels of discrimination; they are therefore considered the most suitable when a large number of strains must be analysed (Seseña et al. 2004).

Kefir is fermented milk obtained by the use of kefir grains, which contain a cluster of microorganisms held together by a polysaccharide-protein matrix. A vast variety of different species of organisms forming the kefir grains, comprising yeast and bacteria, have been isolated and

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identified (Garrote et al. 2001; Witthuhn et al. 2004). Several health promoting properties are associated with kefir consumption (Farnworth, 2005; Lopitz-Otsoa et al. 2006). The beneficial action of this fermented milk can be partially attributed to the inhibition of pathogenic microorganisms by metabolic products such as organic acids produced by kefir microflora (Garrote et al. 2000). Recent studies also demonstrated stimulation of immune system by this fermented milk (Thoreux & Schmucker, 2001; Vinderola et al. 2005).

The aim of this study was to apply established in vitro assay tests to evaluate the probiotic potential of homofermentative lactobacilli isolated from kefir grains. Also, in the present work, molecular identification and genotypic diversity were performed.

Materials and Methods

Microorganisms and growth conditions

Eleven homofermentative lactobacilli (CIDCA 8312, 8313, 8323, 8327, 8331, 8334, 8336, 8337, 8338, 83114, 83210) were isolated from kefir grains and identified previously as *Lactobacillus plantarum* (Garrote et al. 2001; Bosch et al. 2006). Reference strains employed were *Lb. plantarum* DSM 20174, *Lb. rhamnosus* ATCC 7469, *Lb. alimentarius* ATCC 29643 and the heterofermentative *Lb. kefir* JCM 5818, for out-of-group comparison. Lactobacilli were grown in MRS broth (Biokar Diagnostics, Beauvais, France) during 24 h at 30 °C. *Escherichia coli, Salmonella enterica* serovar Enteritidis, *Sal. enterica* serovar Tiphymurium, *Sal. gallinarum* and *Shigella sonnei* were grown in nutritive broth (Biokar Diagnostics) during 18 h at 37 °C.

Molecular identification and genotypic diversity

DNA extraction, amplified ribosomal DNA and restriction analysis (ARDRA), 16S–23S rRNA internal spacer region analysis (ISR), random amplified polymorphic DNA (RAPD) and typing by RAPD-PCR patterns were performed according to Delfederico et al. (2006).

Hydrophobicity and Autoaggregation assay

Two millilitres of lactobacilli suspension in phosphate buffer saline (PBS) at $OD_{550\ nm}=1{\cdot}0$ (OD_0) were mixed with 0·5 ml n-hexadecane for 2 min and after phase separation, the optical density at 550 nm (OD_1) was measured. The hydrophobicity was calculated as $\%H=(OD_1-OD_0)/OD_1\times 100$.

Aggregation coefficient (AC) was calculated at t = 30 min according to Kos et al. (2003) as: $AC_t=[1-(OD_t/OD_0)]\times 100$, where OD_0 is the initial optical density of the microbial suspension at 550 nm and OD_t is the optical density after 30 min.

Resistance of lactobacilli to simulated gastrointestinal conditions

Lactobacilli were resuspended in HCl pH 2·5 to a concentration of 10^8 CFU/ml and incubated at 37 °C. Aliquots were taken immediately (0 h) and after 1 and 3 h. Serial dilutions using 0·1% tryptone were prepared and plated on MRS agar in order to determine the number of survivals. Measurement of bile resistance was performed by modified ecometric method according to Kociubinski et al. (1999).

Adhesion to Caco-2 cells

Caco-2 cells were routinely grown following the procedure described by Minnaard et al (2007). For adhesion assay, Caco-2 monolayers were incubated with 0·25 ml *Lactobacillus* suspension (2×10^8 CFU/ml) and 0·25 ml DMEM (GIBCO BRL Life Technologies Rockville, USA) for 1 h at 37 °C in a 5% CO_2 – 95% air atmosphere. Then, monolayer was washed three times with PBS and lysed by adding sterile distilled water. To determine the number of viable cells associated with Caco-2 cells, appropriate dilution in 0·1% tryptone were plated on MRS and colony counts were performed. Experiments were performed per triplicate in three consecutive cell passages.

Antimicrobial activity assay

The *Esch. coli, Sal. enterica* serovar Enteritidis, *Sal. enterica* serovar Tiphymurium, *Sal. gallinarum* and *Sh. sonnei* inhibition was screened by using spot test assay. *Lactobacillus* cultures at pH 3·9–4·2, were centrifuged for 15 min at 10·000 ***g*** and filtrated through 0·22 µm membrane filter (Millipore Corporation, Milford, USA) to obtain spent culture supernatants (SCS). A suspension of 10^8 CFU/ml of target bacteria was swabbed over nutrient agar (3 g meat extract/l, 5 g peptone/l, 15 g agar/l, pH 6·7) and 10 µl lactobacilli SCS were spotted onto the agar surface. The plates where incubated at 37 °C and the growth free inhibition zone around the spotted area was recorded. Inhibition of *Esch. coli* was also assessed by determining the growth kinetic in nutrient broth in presence of lactobacilli SCS. Artificially acidified MRS was prepared adding 120 mM-DL-lactic acid to MRS and adjusting to pH 4·0. Assays were performed in duplicate in three independent experiments.

Determination of organic acids concentration

Lactic acid concentration of SCS was measured by HPLC as described by Garrote et al. (2000).

Statistical analysis

Results were expressed as means ± standard deviation (SD) of at least three separate duplicate experiments. For

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statistical comparisons, Student's t test was performed at P value of $<0{\cdot}05$.

Results and Discussion

Molecular identification and genotypic diversity of lactobacilli kefir grains

Today, microbiologists agree that a reliable classification can only be achieved by the exploration of each group of isolates by a set of techniques, generally known as the polyphasic approach. This approach implies that two sources of information must be investigated: genomic and phenotype data (Rosselló-Mora & Amann, 2001). In this work, a large region of 16S rDNA was amplified from lactobacilli and fragments of approximately 1450 bp were obtained. ARDRA patterns generated with enzymes *Nco* I, *Hinf* I and *Hae* III of all isolates and the reference *Lb. plantarum* strain share the same profile for each enzyme (data not shown). On the other hand, *Lb. rhamnosus* and *Lb. kefir* showed a differential pattern for each enzyme (data not shown).

PCR amplification of a gene region including 16S–23S ISR was made from lactobacilli isolates and from reference strains. Four amplification fragments, with sizes of approximately 1400, 750, 700 and 500 bp, were obtained in all cases, showing absence of 16S–23S ISR size polymorphism among isolates and reference strains (data not shown). Minor fragment of four isolates were cloned and sequenced and ISR flanking regions were subtracted.

The absence of 16S–23S ISR length polymorphisms among lactobacilli isolates and reference strains agree with previously published data for other lactobacilli (Fortina et al. 2003). 16S–23S ISR sizes and absence of tRNA genes in minor length 16S–23S spacer, found in this work, are in good correlation with those reported by Nour (1998) for members of the *Lactobacillus* genus.

A multiple sequence alignment of 16S–23S ISR sequences, using CLUSTAL X Program, was made. This analysis showed a similitude value higher than 98·4% among homofermentative lactobacilli and the reference *Lb. plantarum* strain (data not shown). That similitude value was higher than the cut-off value of 97·5% purpose by Tannok et al. (1999) as a criterion to identify species. On the other hand, 16S–23S ISR sequences obtained from GenBank database, showed a similitude value lower than 98%, even the closely related *Lb. paraplantarum* (Fig. 1). According to molecular data obtained, we may point out that all homofermentative lactobacilli studied belong to the species *Lb. plantarum*, in good agreement with previous phenotypic identification (Garrote et al. 2001, Bosch et al. 2006).

RAPD-PCR analysis was performed and patterns obtained are shown in Fig. 2 (right). Differences in discriminatory power of these primers became apparent after the analysis of several lactobacilli isolates. RAPD-PCR analysis showed primer Coc, specifically designed for lactic acid bacteria discrimination (Cocconcelli et al. 1997), as the best for differentiation of lactobacilli isolates belonging to *Lb. plantarum* species. The UPGMA dendrogram was obtained by combination of Coc, ERIC-2, 1254, and M13 profiles (Fig. 2, left). The similitude value between the homofermentative isolates and the reference *Lb. plantarum* strain was higher than 70%. Based on an arbitrary similitude value of 82% to define clusters, this unrooted tree has five clusters. Reference *Lb. plantarum* strain, CIDCA 8327 and 83114 belong to cluster one. Cluster two contains the isolates CIDCA 8312 and 8331. In cluster three are grouped CIDCA 8337, 83210, 8313 and 8323. In cluster four, CIDCA 8336 and 8338 and finally cluster five only contains the isolate CIDCA 8334. The reference strains *Lb. alimentarius* and *Lb. rhamnosus* are in different and most distant branches of the tree.

In this study, RAPD-PCR analysis was used as a molecular typing method to group lactobacilli isolates from heterogeneities exhibited by their profiles. This approach has often been used with a similar propose (De Angelis et al. 2001; Vásquez et al. 2002). RAPD analysis is also in good agreement with ARDRA and ISR results. In regard to species identification, five clusters obtained by RAPD-PCR analysis could be correlated with at least five different strains of species *Lb. plantarum*.

Probiotic properties of lactobacilli isolated from kefir grains

All lactobacilli studied showed a high resistance to bile and acid conditions (Table 1). Isolates CIDCA 83114, 83210 and 8336 showed highest resistance to bile. The highest resistance to acid conditions was observed for isolates CIDCA 8313, 83210 and 8338. The remaining isolates showed lower percentage of survival but none were lower than 40% after 3 h. It is important to point out that survival experiments were performed under the most unfavourable conditions. It is expected that in physiological conditions the complex food matrix could exert an additional protective action against acid damage.

The surface properties, such as aggregation and hydrophobicity, are thought to be linked to the ability to interact with epithelial cells and/or undesirable bacteria (Gusils et al. 1999). The aggregation coefficient at 60 min was zero in all *Lb. plantarum* studied (data not shown). *Lb. plantarum* isolates have a hydrophilic surface with a hydrophobic index from 0 to 5·8% and showed different capacities to adhere to Caco-2 cells (Table 1). In agreement with other reports (Ouwehand et al. 1999; Schillinger et al. 2005), no significant correlation could be observed between cell surface hydrophobicity of *Lb. plantarum* isolates and the adhesion to epithelial cells. Thus, hydrophobicity could contribute to adhesion, but it is not a prerequisite for an adherence capacity. Among *Lb. plantarum* isolates, the highest adhesion (10·5%) was observed with the isolate CIDCA 8337.

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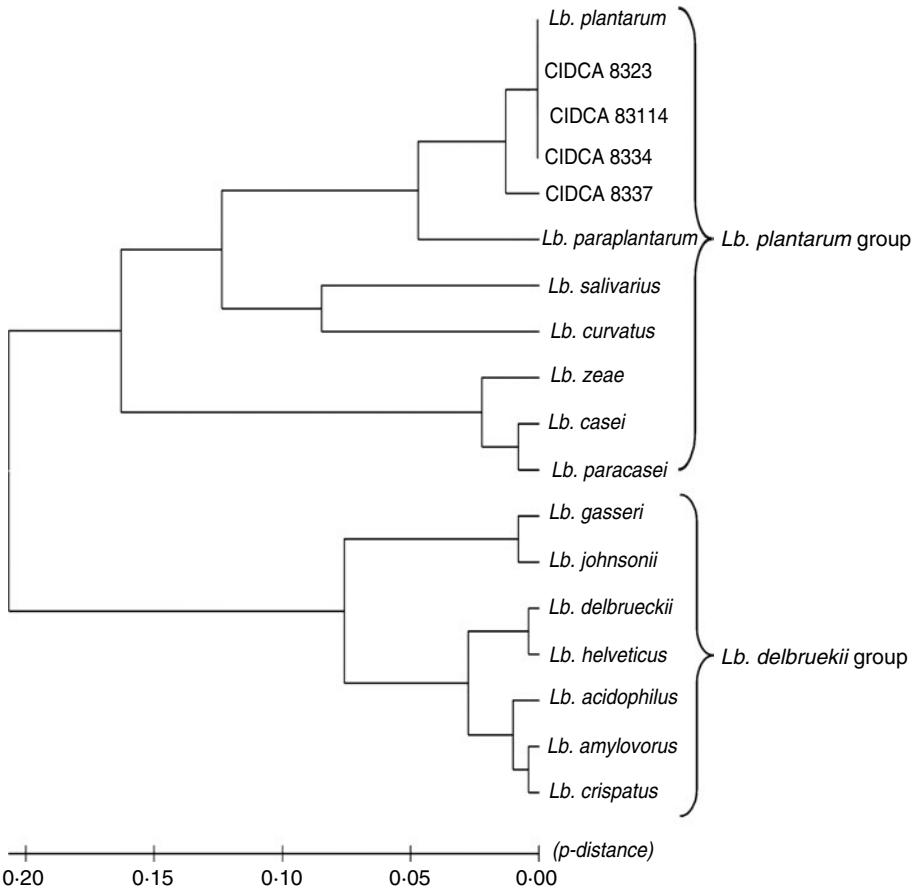

Fig. 1. Unrooted phylogenetic tree generated on the basis of 16S–23S ISR from four homofermentative isolates, and 14 reference strains obtained from GenBank data base.

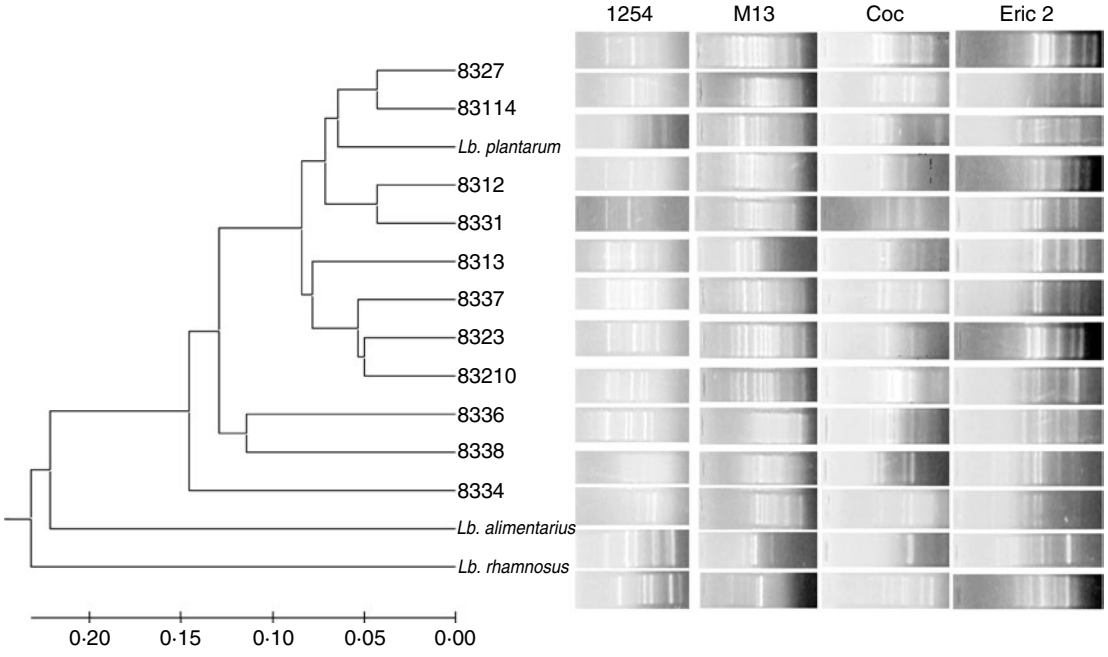

Fig. 2. RAPD-PCR obtained with Coc, ERIC-2, 1254 and M13 primers and combined dendrogram obtained from RAPD-PCR profiles.

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Table 1. Hydrophobicity, adhesion to Caco-2 cells and resistance to simulated gastrointestinal conditions of homofermentative lactobacilli isolates from kefir

Values are the mean ± SD of three independent assays

Lb. plantarum strain	Hydrophobicity (% H)	Percentage adhesion to Caco-2/TC-7 cells	Bile salt tolerance	Percentage survival at pH 2·5	
				1 h	3 h
CIDCA 8312	2·00 ± 0·30	0·97 ± 0·02	+	77·2 ± 2·3	56·3 ± 5·2
CIDCA 8313	0·30 ± 0·05	1·23 ± 0·40	++	91·2 ± 1·2	61·4 ± 1·3
CIDCA 83114	3·45 ± 0·65	3·52 ± 0·63	+++	68·0 ± 0·5	53·5 ± 2·5
CIDCA 8323	1·04 ± 0·26	1·40 ± 0·65	++	56·3 ± 1·3	41·1 ± 2·5
CIDCA 8327	3·25 ± 0·45	1·75 ± 0·52	++	85·0 ± 3·6	42·8 ± 2·1
CIDCA 83210	0·35 ± 0·10	1·25 ± 0·15	+++	100 ± 1·6	67·4 ± 2·3
CIDCA 8331	4·10 ± 0·30	1·30 ± 0·20	+	70·1 ± 2·1	67·4 ± 1·2
CIDCA 8334	2·25 ± 0·50	2·70 ± 0·05	+	40·3 ± 5·3	42·3 ± 0·8
CIDCA 8336	2·50 ± 0·20	2·73 ± 0·27	+++	77·0 ± 2·6	51·0 ± 1·8
CIDCA 8337	5·80 ± 0·85	10·50 ± 0·05	++	68·5 ± 2·0	55·5 ± 2·2
CIDCA 8338	2·30 ± 0·35	1·52 ± 0·22	++	93·7 ± 1·5	65·2 ± 1·0

Table 2. Antimicrobial activity of homofermentative lactobacilli against gram negative pathogens

Lb. plantarum strain	Inhibition of:				
	Sal. typhimurium	*Sal. enterica*	*Sal. gallinarum*	*Sh. sonnei*	*Esch. coli*
CIDCA 8312	+	–	+	–	nd
CIDCA 8313	+	–	–	–	+
CIDCA 83114	+	+	–	–	+
CIDCA 8323	+	+	–	+	+
CIDCA 8327	+	+	+	+	+
CIDCA 83210	+	–	+	–	+
CIDCA 8331	+	+	+	–	+
CIDCA 8334	+	+	+	+/–	+
CIDCA 8336	+	+	–	–	+
CIDCA 8337	+	–	–	–	+
CIDCA 8338	+	+	–	–	+

+: Clearly defined zone of inhibition, –: No inhibition zone, nd: Not determined

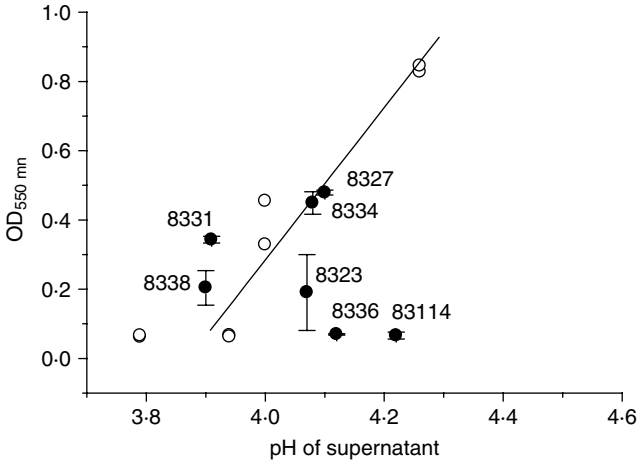

Fig. 3. Growth inhibition of *Esch. coli* in nutrient broth supplemented with acidified MRS (○) or SCS of *Lb. plantarum* isolates (●). Values are the mean ± SD of three independent assays.

There are several reports about the antagonistic activity of lactobacilli against Gram-negative pathogens (Servin, 2004; Tsai et al. 2005). Over the last few years, studies of *Lb. plantarum* as a potential probiotic have been reported (de Vriesa et al. 2006). All *Lb. plantarum* SCS studied in the present work have inhibitory power against *Sal. typhimurium* and *Esch. coli* (Table 2). Seven out of eleven isolates showed inhibition against *Sal. enterica*. Only five SCS tested were effective against *Sal. gallinarum*. A weak activity was observed against *Sh. sonnei* where only two isolates, CIDCA 8323 and 8327 were positive. Our results were in accordance with previous reports about *in vitro* assays for testing antagonist activity of *Lb. plantarum* (Çon & Gökalp, 2000; Obadina et al. 2006). No inhibitory effect of artificially acidified MRS (120 mM-lactic acid) was observed on any of the tested pathogens. This lactic acid concentration was used taking into account the average concentration of lactic acid in *Lb. plantarum* SCS which ranged from 118 to 130 mM.

Growth kinetic of *Esch. coli* in nutrient broth was recorded in the presence of SCS or artificially acidified MRS (Fig. 3). Maximal OD reached by *Esch. coli* was affected by pH. A linear correlation was observed between pH 3·8 and 4·1 with data obtained from curves with acid MRS and some SCS. However SCS of isolates 8323, 8324, 8336 and 83114 produced higher inhibition at pHs between 4·1 and 4·3. This means that the inhibitory effect should be ascribed not only to the lactic acid produced by lactobacilli. It is known that some *Lb. plantarum* produce other metabolic compounds such as bacteriocins and/or bacteriocin-like substances, that are active against certain pathogens (Šušković et al. 1997; Toksoy et al. 1999; Kostinek et al. 2005). Further work will be necessary to determine molecule(s) involved in antimicrobial effect.

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In this work, we studied the possible relationship between the five clusters based on RAPD-PCR and the probiotic properties for each isolate tested. We did not find a correlation, suggesting that these isolates have unique characteristics and in each cluster there are isolates with different probiotic properties.

All the strains tested, showed good properties as potential probiotics, but considering the ability to inhibit in vitro all pathogens tested and its tolerance to lactic acid and bile salts, we believe that *Lb. plantarum* CIDCA 8327 could be proposed as a good probiotic candidate. Although *Lb. plantarum* CIDCA 8327 has low adhesion to Caco-2 cells compared with other strains such as CIDCA 8337, it is important to consider that studies with Caco-2 cells can be useful to collect background information of probiotic strains but adhesion is not always necessary for probiotic effect of lactobacilli in the gut.

The results obtained in vitro indicates the possible utilization of certain strains isolated from kefir as probiotics. Further studies on the capacity of *Lb. plantarum* isolates to colonize the intestine and their protective action against infections using in vivo experiment are needed.

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