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Selection of probiotic *Lactobacillus* strains with antimicrobial activity to be used as biocontrol agents in food industry

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ABSTRACT

Ninety-eight *Lactobacillus* (*Lb.*) strains were screened to select those with the best antimicrobial and probiotic properties. Firstly, a screening based on growth kinetics under gastrointestinal tract (GIT) conditions allowed to select 28 strains, which were assayed for autoaggregation, coaggregation and hydrophobicity. 7.1% were strongly autoaggregating strains after 4 h of incubation. Strains belonging to *Lb. plantarum* and *Lb. paraplantarum* species showed the highest coaggregation percentages with the pathogens tested (*Salmonella choleraesuis* and *Staphylococcus aureus*). Regarding hydrophobicity, there was correlation between the results obtained for xylene and toluene. Principal Component Analysis (PCA) of these results allowed to select 20 strains as potential probiotics, that were used for further assays of biofilms formation (ranged between 53 and 75%) and adhesion to Caco-2/TC7 cells (around 51–97%). Furthermore, 51% of the ninety-eight strains showed antimicrobial activity against four species indicators (*Salmonella choleraesuis*, *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes*). Results from all the assays performed allowed to select 12 strains attending to either their antimicrobial activity or the probiotic potential and only two of them *Lb. paracasei* Lb38 and *Lb. brevis* Lb99, stood up for both properties. Therefore, these strains could be proposed to be used as biocontrol agents in the food industry.

1. Introduction

Foodborne and food spoilage bacteria are a major concern for the food industry. Food spoilage includes physical damage and chemical changes coming from microbial growth that may cause a wide variety of infections and intoxications resulting in moderate to severe illness and death (Scallan et al., 2011; Villalobos-Delgado et al., 2019). Different methods, including thermal or high-pressure processing, food irradiation and addition of additives, are used to preserve food (Fu et al., 2016), but all of them can produce changes in chemical and organoleptic properties (Čanžek Majhenič et al., 2015).

In recent decades, the number of food additives approved for use in the food industry has drastically increased (Carocho et al., 2014; Zinöcker et al., 2018). However, consumers are currently aware of health concerns regarding use of food additives and, therefore, "natural" foods are becoming more and more attractive (Balciunas et al., 2013). In recent years, the food industry has encouragely looked for alternatives to the use of additives, as biopreservation, to ensure food safety.

Biopreservation, or biocontrol, refers to the use of epiphytic or controlled microbiota, or its metabolites, to avoid growth of spoilage and/or pathogen microorganisms, extending shelf life and enhancing the safety of foods (Gálvez et al., 2014; Oliveira et al., 2018). Microorganisms of different genera, both bacteria and fungi, have been proposed to be used as biocontrol agents (i.e. *Propionibacterium*, *Bacillus*, *Escherichia*, *Enterococcus*, *Saccharomyces*, *Aspergillus*, *Kluyveromyces*) (Ceugniez et al., 2017; Hossain et al., 2017; Ouwehand et al., 2002). The ability of lactic acid bacteria (LAB) to inhibit the growth of pathogenic bacteria is well documented (Azizi et al., 2017; Jabbari et al., 2017; Rouse & Van Sinderen, 2008; Todorov et al., 2017; Yoon et al., 2016), that is why LAB are excellent candidates to be considered as biocontrol agents. More recently some authors (Ferrari et al., 2016; Gálvez et al., 2014; Hossain et al., 2017; Muhialdin et al., 2011) have reported the use of probiotic LAB as a suitable alternative to the additives in the food and agriculture industries.

Probiotics confer health benefits to the consumers (Hill et al., 2014; Kim et al., 2019), and the mechanisms by which microorganisms exert these benefits have been widely reported from the first time they were mentioned in the literature (Lilly & Stillwell., 1965). LAB are the main

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Table 1
Strains assayed and their origins.

Species	Origin	Strains
Lb. acidophilus	Almagro eggplants	Lb30, Lb91, Lb103-104, Lb116
Lb. brevis	Almagro eggplants	Lb21, Lb23, Lb25, Lb27-28, Lb31, Lb49, Lb59-62, Lb67-68, Lb84, Lb86, Lb94, Lb99-100, Lb102, Lb105, Lb109-111, Lb115
	Wine	Lb47
	Winery air	Lb63
Lb. casei	Wine	Lb50
Lb. cellobiosus	Goat cheese	Lb2
	Wine	Lb45
Lb. curvatus	Goat cheese	Lb8-9
Lb. delbrueckii	Almagro eggplants	Lb32
Lb. fermentum	Almagro eggplants	Lb16, Lb18-20, Lb66, Lb85
Lb. hilgardii	Wine	Lb52
	Winery air	Lb64
Lb. paracasei	Goat cheese	Lb3-5, Lb24, Lb29,
	Manchego cheese	Lb38-42
	Winery air	Lb57
Lb. paraplantarum	Almagro eggplants	Lb74
Lb. pentosus	Almagro eggplants	Lb82
Lb. plantarum	Almagro eggplants	Lb11, Lb14, Lb65, Lb69-73, Lb75-79, Lb88-90, Lb93, Lb95-98, Lb101, Lb106-108, Lb112-114
	Manchego cheese	Lb33-34, Lb36-37
	Wine	Lb43-44
	Winery air	Lb53-56
Lb. rhamnosus	Goat cheese	Lb10
	Winery air	Lb58

microorganisms used as probiotics, with strains of *Lactobacillus* genus being the most abundant, followed by strains of *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Enterococcus* (Fontana et al., 2013). Traditional uses of probiotics have been focused on human and animal health (Hung et al., 2012; Kaur et al., 2015; Stella et al., 2007), but the use as biocontrol agents is currently gaining importance.

The search of useful probiotic cultures is a complex and multidisciplinary approach (Angmo et al., 2016; Ferrari et al., 2016) in which firstly it is necessary to obtain an abundant collection of strains in which the probiotic and antimicrobial properties will be determined. At this respect, although many of the published studies report have analysed samples taken in internal cavities from human beings and animals, nowadays there is an increasing interest in the study of strains isolated from traditional fermented foods such as vegetables (Argyri et al., 2013), cured meat products (De Vuyst et al., 2008), natural sourdough starters (Manini et al., 2016) or traditional alcoholic drinks (Giles-Gómez et al., 2016).

Proceedings reported by FAO-WHO (2002) to determine probiotic potential in bacteria include between others the assay of survival in the GIT and the capacity to adhere to the epithelial cells. On this later matter, both the determination of cell surface hydrophobicity and the adherence to Caco-2/TC7 cells are carried out. Caco2/TC7 cells possess functional and morphological properties like those of the human intestine cells (De Angelis et al., 2011) and are considered appropriate for this measurement (Serrano-Niño et al., 2016). Besides, it is also advisable to know the ability of the strains to aggregate themselves (autoaggregation) and to grow forming protective structures as the biofilms, because it seems essential (Lasa et al., 2005) to exert their action once in the intestine. Lastly, assays such as the *in vitro* growth inhibition of bacterial indicators or the ability to aggregate with other bacteria, including pathogens (coaggregation assays), are useful to assess the antagonistic activity of the strains.

As mentioned before, the search of novel probiotics able to inhibit growth of pathogenic and spoilage microorganisms has opened new and interesting opportunities for the food industry in order to avoid use of additives. The objective of this research was the characterization of a collection of 98 lactobacilli, isolated from different fermented food and drinks, to select those with the best properties to be used both as probiotics and biocontrol agents. In order to achieve a more complete characterization of the strains, all of them were initially assayed for both the antimicrobial and the probiotic potential and finally those having the best results from both assays were selected. To find strains with probiotic potential, a preliminary screening based on kinetic of passing through the gastrointestinal tract was carried out and only those with the best properties were further analysed for autoaggregation, coaggregation and hydrophobicity. Finally, the most promising strains were tested for biofilm formation and adherence to Caco-2/TC7 cells. It is noted that each decision was made based on multifactorial statistical assay results.

2. Materials and methods

2.1. LAB strains

A total of 98 *Lactobacillus (Lb.)* strains belonging to thirteen species were assessed in this study (Table 1). They were isolated and identified in previous works from different fermented foods and drinks: Almagro eggplants, goat and sheep cheeses and wine (Nieto-Arribas et al., 2011; Pérez-Martín et al., 2014; Ruiz et al., 2010; Sánchez et al., 2005; Seseña et al., 2004). Strains were routinely grown in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain) and incubated aerobically at 30 °C. Cultures were maintained frozen at −80 °C supplemented with 20% (v/v) glycerol as a cryoprotectant.

2.2. Antimicrobial properties

The inhibitory effect of the *Lactobacillus* strains on growth of some potentially pathogenic bacteria was tested according to the "spot test" method described by Fleming et al. (1975), with slight modifications. The pathogenic bacteria (indicator) assayed were *Listeria monocytogenes* CECT 4031, *Staphylococcus aureus* CECT 86, *Salmonella choleraesuis* CECT 443 and *Bacillus cereus* CECT 148. All of them were grown in Tryptic Soy Broth (TSB), except *Listeria monocytogenes* in Brain Heart Infusion (BHI). Incubation temperature was 37 °C except for *Salmonella choleraesuis* which was incubated at 30 °C.

Surface of MRS agar plates was spotted with a drop (5 μL) of overnight cultures of the *Lactobacillus* strains. Plates were incubated for 24 h at 30 °C. After incubation, they were overlaid with 7 mL of a soft TSA or BHI agar (0.7% w/v agar) inoculated with a volume of an overnight culture of the indicator bacteria to reach a concentration of around 8 log CFU/mL. The susceptibility of the pathogens to the *Lactobacillus* strains was assessed by measuring the diameter (mm) of the zone of inhibition of bacterial growth around the spots after incubation for 24 h in optimal conditions for each indicator. Values of between 1 (diameter of the

inhibition zone = 10 mm) and 4 (diameter of the inhibition zone ≥ 30 mm) were assigned, following the procedure of Serrano-Niño et al. (2016), slightly modified. The sumatory of the four values for each strain was used to select those with the highest antimicrobial capacity. The assay was performed in triplicate.

2.3. Probiotic properties

2.3.1. Growth kinetic under gastrointestinal tract conditions

The protocol set up by Arévalo-Villena et al. (2018) was adapted for LAB. *Lactobacillus* strains were cultured for 48 h in MRS broth at 30 °C and after incubation, aliquots of 100 μL were dispensed in a 96-well plate and the absorbance at 630 nm (A_{630}) was measured using the reader Synergy HT (Biotek, EEUU). Cells were harvested by centrifugation (18500×*g* for 5 min at 4 °C), washed twice with sterile phosphate buffer saline (PBS) pH 7.2 and re-suspended in 1 mL of sterile PBS adjusted to pH 2.0 containing 3 mg/mL pepsin. These cell suspensions were incubated for 3 h at 37 °C, simulating the time that food remains in the stomach. Absorbance was monitored every hour during incubation.

After incubation, cell suspensions were again centrifuged (18500×*g*, 5 min, 4 °C) and re-suspended in 90 μL of MRS broth, prior to evaluate resistance to intestinal conditions. 10 μL of these suspensions were inoculated into 190 μL of MRS broth pH 8.0, supplemented with 1 mg/mL pancreatin and 0.5% (w/v) bile salts in a well-plate, and incubated during 22 h at 37 °C. A_{630} was read every 30 min, previous gentle stirring, using the above mentioned reader. Strain Lb15, characterized in a previous assay, was used as a positive control and non-inoculated MRS broth pH 8.0 was the negative control. All assays were carried out in triplicate.

Absorbance data were plotted against time and the following parameters were calculated: A_{max}-A_{ini}, where A_{max} is the maximum absorbance reached during incubation under intestinal conditions and A_{ini} is the initial absorbance; $m_{0.5\text{-}2.5}$ is the slope of the growth kinetic, calculated from absorbance values between 0.5 and 2.5 h of incubation under intestinal conditions, and λ is the lag phase time.

2.3.2. Autoaggregation assay

The autoaggregation assay was performed as described by Ferrari et al. (2016), with slight modifications. Briefly, *Lactobacillus* strains were grown overnight at 30 °C in MRS broth and cells were harvested by centrifugation (18500×*g* for 10 min at 4 °C), washed twice using sterile PBS, and re-suspended in the same buffer to reach the nº 0.5 McFarland turbidity standard (equivalent to 8 log CFU/mL). Suspensions were incubated at room temperature in static and absorbance at 600 nm was measured at 0, 2 and 4 h of incubation. The autoaggregation percentage was calculated using formula (1):

$$Autoaggregation(\%) = \left(1 - \frac{A_t}{A_0}\right) x100 \quad (1)$$

where A_t represents the absorbance at 2 h or 4 h incubation and A_0 the absorbance at t = 0. The assay was performed in triplicate.

2.3.3. Coaggregation assay

The ability of the *Lactobacillus* strains to coaggregate with one strain of two Potentially Pathogenic Bacteria (PAT), *Salmonella* (*S.*) *choleraesuis* CECT 443 and *Staphylococcus (St.) aureus* CECT 86, was evaluated following the procedure reported by Ferrari et al. (2016). Overnight cultures of the *Lactobacillus* strain in MRS broth and of *S. choleraesuis* CECT 443 or *St. aureus* CECT 86, in TSB (Pronadisa, Madrid, Spain), were used to prepare cell suspensions following the procedure described for the autoaggregation assay. Equal volumes (2 mL) of the *Lactobacillus* suspension and of *S. choleraesuis* CECT 443 or *St. aureus* CECT 86, were mixed and vortexed for 10 s. Control tubes, containing 4 mL of separated bacterial suspension, *Lactobacillus* strain and *S. choleraesuis* or *St. aureus* strains, were set up at the same time. The absorbance at 600 nm of the mixed and of the control suspensions was monitored at 0 time and after 5 h of incubation at room temperature in static. The percentage of coaggregation was calculated using the following equation (2):

$$Coaggregation(\%) = \frac{\left(\frac{A_{LAB}+A_{PAT}}{2}\right) - A_{MIX}}{A_{LAB}+A_{PAT}} x100 \quad (2)$$

where A_{LAB} and A_{PAT} represent the absorbance of the individual bacterial suspensions, and A_{MIX} represents the absorbance of the mixed (LAB + PAT) bacterial suspension. The assay was performed in triplicate.

2.3.4. Cell surface hydrophobicity

Adhesion to xylene and toluene of *Lactobacillus* strains was assayed as described by Serrano-Niño et al. (2016). Cell suspensions of the LAB strains were prepared as described in section 2.3.2, and after reading the absorbance at 600 nm, a volume of the hydrocarbon (xylene or toluene) of 1/5 of that of the bacterial suspension, was added. The mixture was vortexed for 2 min and incubated at room temperature for 20 min. The aqueous phase was removed and the absorbance measured at 600 nm. The hydrophobicity (%) was calculated according to the following equation (3):

$$Hydrophobicity(\%) = \left(\frac{A_0 - A}{A_0}\right) x100 \quad (3)$$

where A_0 represents the absorbance in the suspension before hydrocarbon was added and A represents the absorbance of the aqueous phase after incubation. This assay was performed in triplicate.

2.3.5. Biofilm formation

Biofilm formation was monitored as described by Speranza et al. (2011). A flask containing 50 mL of MRS broth with a glass slide (2.5 × 7.6 cm) inside was inoculated with an overnight culture of the *Lactobacillus* strain assayed to reach a concentration of 8 log CFU/mL. After incubation during 24 h at 37 °C, the slide was aseptically removed, washed with sterile PBS and introduced into another flask containing 50 mL of sterile PBS. This was submitted to sonication at a constant power of 20% for 3 min using an UP50H ultrasonic processor (Hielscher, Teltow, Germany), to suspend the biofilm adhered to the surface of the slide. Then, appropriate dilutions of the suspension in sterile saline solution were plated onto MRS agar. After incubation during 48 h at 30 °C the colonies were counted, and results expressed as CFU/mL following the method described by Corral-Lugo et al. (2012). A flask containing 50 mL of MRS broth, without a glass slide inside, which was inoculated with the same volume of the overnight culture of the *Lactobacillus* strain was used as control. Results were expressed as the percentage of cells adhered to the glass with respect to the cells population in the control. The assay was performed in triplicate.

2.3.6. Adhesion to Caco-2/TC7 cells

Adhesion assays were performed as reported by Pinto et al. (2020) using Caco-2/TC7 cells, derived from a human epithelial colorectal adenocarcinoma, which were grown in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, Lonza, BioWhittaker™, Belgium) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin–streptomycin and glutamine (2 mM), following the procedure described by Golowczyc et al. (2007). Cells in subculture passage between 23 and 30 were seeded at a concentration of 2.5×10^5 cells/well in a 24-well tissue culture plates, and incubated at 37 °C in a 5% CO_2-95% air atmosphere. Culture medium was changed every two days. Caco-2/TC7 cells were used at post confluence after 7 days of culture (differentiated cells). One hour before adhesion assay, cells were washed twice with sterile PBS and supplemented DMEM media was replaced by DMEM without FBS and antibiotics. Cells were maintained at 37 °C in a 5% CO_2-95% air atmosphere up to the adhesion assay.

Overnight cultures of the *Lactobacillus* strains were prepared in MRS

Table 2
Antimicrobial activity of Lactobacillus strains. Number of positive strains (diameter of inhibition zone ≥ 10 mm) against each of the pathogens and number of strains inhibiting different number of pathogens.

Antimicrobial activity	Number of strains (n = 98)
Pathogens	
Salmonella choleraesuis	72
Staphylococcus aureus	80
Bacillus cereus	73
Listeria monocytogenes	79
Number of pathogens inhibited	
4	50
3	18
2	20
1	9
0	1

broth to reach a cell concentration of 8 log cells/mL, and then centrifuged (18500×*g* for 10 min at 4 °C). The pellet was resuspended in 0.5 mL of DMEM without FBS or antibiotics and added onto the monolayer culture of Caco-2/TC7. Plates were incubated for 3 h at 37 °C in a 5% CO_2–95% air atmosphere. After incubation, the monolayer in each well was washed three times with sterile PBS and detached adding 0.5 mL of 1% (v/v) Triton X100 (Sigma)/well. In this suspension a count of the *Lactobacillus* population was carried out by plating the appropriate dilutions onto MRS agar plates, and incubation during 48 h at 30 °C. Results were expressed as the percentage of cells adhered to the Caco-2 cells with respect to the *Lactobacillus* population inoculated. All experiments were performed in triplicate.

2.4. Statistical analysis

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

3. Results and discussion

3.1. Antimicrobial activity

Determination of antimicrobial activity deserves a great relevance in selection of microorganisms to be used as biocontrol agents. Table 2 shows the results obtained for the 98 *Lactobacillus* strains against four potentially pathogenic bacteria.

A 51% of the strains showed antimicrobial activity against all the indicators, including all those belonging to the species *Lb. acidophilus, Lb. brevis* (except strain Lb28)*, Lb. casei, Lb. delbrueckii, Lb. hilgardii, Lb. paraplantarum, Lb. pentosus* and *Lb. rhamnosus*. Results for the remaining species were strain dependent. Values for antimicrobial activity, calculated from the summatory of values for each indicator, ranged between 16 (strains Lb101 and Lb93) and 0 (Lb71). Strains *Lb. plantarum* Lb93, Lb101, showed the highest antimicrobial activity against the four indicators, with diameter of inhibition zones ≥30 mm for all of them, followed by *Lb. plantarum* Lb56 and Lb78, *Lb. brevis* Lb99 and *Lb. paracasei* Lb38. *Lb. plantarum* Lb71 was the only strain unable to inhibit the growth of any of the indicators used. Almeida Júnior et al. (2015) and Serrano-Niño et al. (2016) reported similar results for strains of *Lactobacillus* species, *Leuconostoc mesenteroides* and *Enterococcus faecium*, suggesting that antimicrobial activity of LAB is a strain-specific property.

In reference to the capacity of LAB to inhibit growth of Gram-positive or Gram-negative indicators, discrepancies have been found between authors. Some of them (Almeida Júnior et al., 2015; Aymerich et al., 2000; Dias et al., 2013; Saraoui et al., 2016) report that Gram-positive bacteria are more sensitive, but inhibition of Gram-negative bacteria has also been displayed (Cálix-Lara et al., 2014; Iranmanesh et al., 2014; Olnood et al., 2015; Schirru et al., 2012). In this study, while 91.8% of the *Lactobacillus* strains inhibited some of the Gram-positive indicators, only 73.5% inhibited growth of *S. choleraesuis. Staphylococcus aureus* was the indicator inhibited by the largest number of strains (81.6%).

It is important to highlight that the assay of antimicrobial activity performed does not allow to know the mechanism of inhibition. Therefore, it would be convenient to go in depth in the study of the underlying mechanisms (Armin et al., 2015; De Angelis et al., 2012) with the strains displaying antagonism effect.

3.2. Probiotic potential

3.2.1. Growth kinetic under gastrointestinal tract conditions

Resistance to conditions in the GIT is an essential requirement for probiotic microorganisms because otherwise, they would not be able to reach the intestine in a viable way to carry out their functions.

When the 98 *Lactobacillus* strains were incubated for 3 h in simulated gastric conditions, three growth kinetic models were observed. 84.7% of

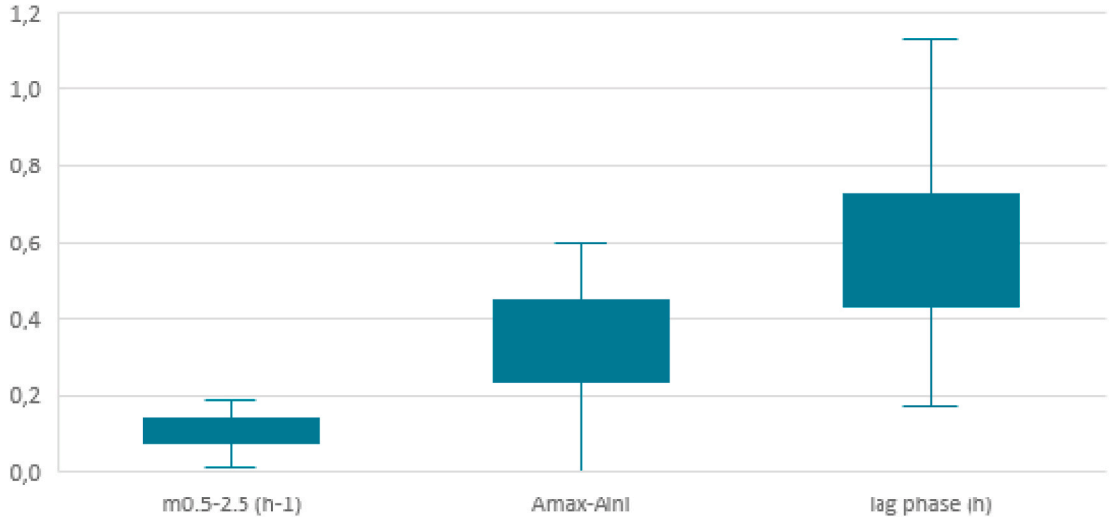

Fig. 1. Minimum value, quartile 1, quartile 2, quartile 3 and maximum value for each of the kinetic parameters under GIT conditions calculated from values for all the strains assayed. Units for each of them are shown in brackets.

Table 3
Growth kinetics under gastrointestinal conditions of the selected strains.

Species	Strain	A_{max}-A_{ini}	m (h^{-1})	λ (h)
Lb. paracasei	Lb4	$0.62 \pm 0.03^{j*}$	$0.15 \pm 0.00^{c,d,e,f,g}$	0.33 ± 0.04^{a}
Lb. rhamnosus	Lb10	$0.54 \pm 0.04^{g,h,i}$	$0.15 \pm 0.02^{e,f,g,h,i}$	$0.65 \pm 0.06^{i,j,k,l}$
Lb. paracasei	Lb24	0.63 ± 0.03^{j}	$0.18 \pm 0.01^{i,j}$	$0.63 \pm 0.04^{g,h,i,j,k}$
Lb. paracasei	Lb29	0.36 ± 0.04^{a}	$0.12 \pm 0.03^{a,b,c}$	$0.64 \pm 0.08^{h,i,j,k,l}$
Lb. acidophilus	Lb30	$0.37 \pm 0.04^{a,b}$	0.11 ± 0.01^{a}	$0.54 \pm 0.01^{e,f}$
Lb. brevis	Lb31	$0.48 \pm 0.01^{d,e,f,g}$	$0.16 \pm 0.00^{f,g,h,i,j}$	$0.68 \pm 0.02^{j,k,l,m}$
Lb. delbrueckii	Lb32	$0.52 \pm 0.05^{g,h}$	0.18 ± 0.00^{j}	$0.69 \pm 0.04^{j,k,l,m}$
Lb. plantarum	Lb34	$0.50 \pm 0.03^{d,e,f,g,h}$	$0.15 \pm 0.00^{d,e,f,g,h}$	$0.51 \pm 0.05^{d,e}$
Lb. plantarum	Lb36	$0.50 \pm 0.05^{d,e,f,g,h}$	$0.16 \pm 0.01^{e,f,g,h,i}$	$0.62 \pm 0.02^{g,h,i,j}$
Lb. plantarum	Lb37	$0.57 \pm 0.04^{h,i,j}$	$0.16 \pm 0.02^{e,f,g,h,i}$	$0.52 \pm 0.03^{d,e,f}$
Lb. paracasei	Lb38	$0.38 \pm 0.04^{a,b,c}$	$0.12 \pm 0.01^{a,b,c}$	$0.55 \pm 0.03^{e,f}$
Lb. paracasei	Lb41	$0.45 \pm 0.05^{c,d,e,f}$	$0.11 \pm 0.01^{a,b}$	$0.68 \pm 0.04^{j,k,l,m}$
Lb. plantarum	Lb71	$0.51 \pm 0.04^{d,e,f,g,h}$	$0.13 \pm 0.03^{a,b,c,d,e}$	$0.47 \pm 0.06^{c,d}$
Lb. plantarum	Lb72	$0.44 \pm 0.10^{b,c,d}$	$0.14 \pm 0.01^{c,d,e,f,g}$	$0.61 \pm 0.02^{g,h}$
Lb. plantarum	Lb73	$0.58 \pm 0.03^{h,i,j}$	$0.14 \pm 0.01^{c,d,e,f,g}$	$0.39 \pm 0.03^{a,b}$
Lb. paraplantarum	Lb74	$0.54 \pm 0.09^{g,h,i}$	$0.17 \pm 0.02^{h,i,j}$	$0.70 \pm 0.05^{l,m}$
Lb. plantarum	Lb76	$0.38 \pm 0.03^{a,b,c}$	$0.12 \pm 0.01^{a,b,c}$	0.72 ± 0.01^{m}
Lb. plantarum	Lb88	$0.52 \pm 0.04^{f,g,h}$	$0.13 \pm 0.03^{a,b,c,d,e}$	$0.66 \pm 0.03^{i,j,k,l,m}$
Lb. plantarum	Lb90	$0.61 \pm 0.03^{i,j}$	$0.12 \pm 0.01^{a,b,c,d}$	$0.54 \pm 0.05^{e,f}$
Lb. plantarum	Lb93	$0.57 \pm 0.05^{h,i,j}$	$0.12 \pm 0.01^{a,b,c}$	$0.41 \pm 0.02^{b,c}$
Lb. plantarum	Lb98	$0.53 \pm 0.03^{g,h}$	$0.14 \pm 0.02^{b,c,d,e,f}$	$0.69 \pm 0.04^{k,l,m}$
Lb. brevis	Lb99	$0.52 \pm 0.04^{e,f,g,h}$	$0.13 \pm 0.01^{a,b,c,d,e}$	$0.58 \pm 0.03^{f,g}$
Lb. acidophilus	Lb103	$0.45 \pm 0.04^{c,d,e,f}$	$0.17 \pm 0.01^{g,h,i,j}$	$0.71 \pm 0.03^{l,m}$
Lb. acidophilus	Lb104	$0.45 \pm 0.05^{c,d,e,f}$	$0.17 \pm 0.01^{h,i,j}$	$0.70 \pm 0.05^{l,m}$
Lb. plantarum	Lb107	$0.44 \pm 0.05^{c,d}$	$0.16 \pm 0.01^{e,f,g,h,i}$	$0.54 \pm 0.02^{e,f}$
Lb. brevis	Lb110	$0.44 \pm 0.03^{c,d,e}$	$0.14 \pm 0.01^{c,d,e,f}$	0.72 ± 0.07^{m}
Lb. brevis	Lb111	0.33 ± 0.03^{a}	$0.13 \pm 0.01^{a,b,c,d}$	$0.62 \pm 0.02^{g,h,i}$
Lb. brevis	Lb115	$0.52 \pm 0.02^{g,h}$	$0.13 \pm 0.02^{a,b,c,d,e}$	$0.53 \pm 0.04^{e,f}$
		A_{max}-A_{ini}	m (h^{-1})	λ (h)
Mean		0.49	0.14	0.59
Maximum		0.63	0.18	0.72
Minimum		0.33	0.11	0.33

Data shown are mean ± SD of triplicate values of independent experiments. *Different letters in the superscript indicate significant statistical differences ($p < 0.05$) between strains for each parameter according to the Student-Newman-Keuls test.

the strains showed a slight decrease in the absorbance during incubation; 10.2% maintained the initial absorbance during incubation, while for 5.1% an increase of the absorbance, ranged between 0.100 and 0.500 AU, was observed displaying that they were able to grow in the simulated gastric conditions. All the strains showing this last growth kinetic model belonged to *Lb. plantarum*, *Lb. paracasei* and *Lb. brevis* species.

Next, all the strains were incubated for 22 h in simulated intestinal conditions. Representation of absorbance versus incubation time displayed that for 6% of strains absorbance remained constant or decreased slightly during this time, and were therefore discarded. For the remaining 94% an increase in absorbance, ranging between 0.115 and 0.598 AU was observed, after a lag period of between 0 and 30 min. After the exponential growth phase, values of absorbance stabilized until the end of incubation. It is important to highlight that despite the fact that 84.7% of the strains did not grow during incubation in gastric conditions, some of them did it when they were later incubated under simulated intestinal conditions.

From the values of absorbance measured during incubation, the following kinetic parameters were calculated: slope (m), lag phase duration (λ) and increase in absorbance (A_{max}-A_{ini}), which were used as input variables for the univariate statistical analysis using box plots. Fig. 1 shows the distribution of the strains for each of these parameters. From these results and with the aim of selecting the most resistant strains, it was considered that only those meeting the requirements m ≥ Q2 (0.11 h^{-1}); A_{max}- A_{ini} ≥ Q2 (0.23) and λ ≤ Q3 (0.72 h) should be used in the next assays. Twenty-eight strains were selected and their kinetic parameters are shown in Table 3. It should be noted that 42.8% of them belonged to *Lb. plantarum,* being *Lb. paracasei* and *Lb. brevis* the following most abundant species.

For each parameter significant differences between the strains were obtained. For the difference in absorbance (A_{max}-A_{ini}) ($\alpha = 0.05$; $F = 13.08$; $p = 0.00$) the strains included in the subset for which the difference of absorbance was the highest were Lb4, Lb24, Lb37, Lb73, Lb90 and Lb93, followed by strains Lb10 and Lb74 (included in the next subset). For the slope ($\alpha = 0.05$; $F = 7.13$; $p = 0.00$), the strains Lb24, Lb31, Lb32, Lb74, Lb103 and Lb104 were those showing the highest values. The lag phase duration was the parameter with the greatest differences ($\alpha = 0.05$; $F = 26.59$; $p = 0.00$) and gathered the strains Lb4 and Lb73 as the best ones in the first subset (the lowest value of lag phase duration) followed by strains Lb34, Lb37, Lb71 and Lb93 which belonged to the second, third and fourth subsets. According to these results, it was concluded that the strains (in the next order) *Lb. paracasei* Lb4, *Lb. plantarum* Lb73, *Lb. plantarum* Lb37 and *Lb. paracasei* Lb24, belonging to the subsets with the best values for the different parameters, would be the most resistant to simulated GIT conditions.

Somashekaraiah et al. (2019) also report that LAB isolates from neera, a fermented drink from India, had a high survival rate after undergoing the sequential stomach-intestine process, although during stomach conditions they showed a decrease in their viability. It should be highlighted that in the literature found, the authors evaluate tolerance to factors such as the pH or the enzymes existing in GIT, separately, instead of carrying out studies where these factors act jointly, simulating the real physiological conditions. It is the case of Missaoui et al. (2019), who found that all LAB isolated from fermented seeds, tolerated low pH, gastrointestinal enzymes and bile salts, being *Lb. plantarum* the species that stood out for its survival rate, as in the present work. Likewise, Barache et al. (2020) and Yusuf et al. (2020), evaluated LAB isolated from kefir grains and fruits, respectively, and found a considerable survival capacity at low pH and in presence of bile salts.

Resistance to gastric and intestinal conditions has been found to be both species and strain-dependent property within the genus *Lactobacillus,* as reported by other authors (Missaoui et al., 2019; Tokatli et al., 2015). It is known that lactobacilli are an important population in the human intestinal microbiota, (Nicholson et al., 2012). So, finding *Lactobacillus* strains with probiotic properties, would be the best option to repopulate the damaged microbiota or to contribute to its strengthening.

3.2.2. Autoaggregation assay

The ability to autoaggregate in strains to be used as probiotics seems to be essential in order to reach a high cell density in the GIT, ensuring adherence to intestinal epithelial cells and avoiding a potential colonization by pathogenic microorganisms (Ogunremi et al., 2015; Sánchez et al., 2011).

Results for the 28 selected strains are shown in Fig. 2. Values of autoaggregation after 2 h of incubation ranged from 0% to 79.7%, while values after 4 h of incubation ranged from 8.1% to 93.8%. According to

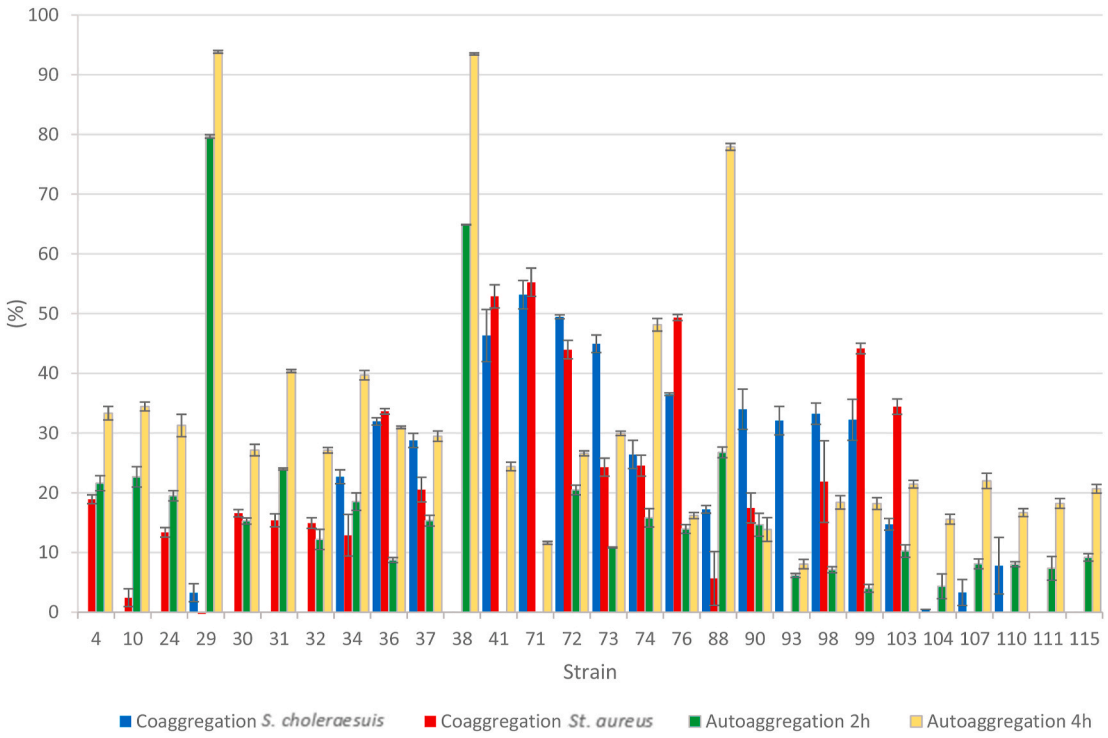

Fig. 2. Mean values of coaggregation with *S. choleraesuis* and St. aureus strains, and of autoaggregation after 2 and 4 h of incubation. Bars represent standard errors of the mean (n = 3).

Del Re et al. (2000), autoaggregation percentages equal or lower than 10% correspond to strains which are unable to autoaggregate, while those autoaggregating as naturally show percentages above 10%. Percentages for strongly autoaggregating strains are equal or greater than 80%. Nevertheless, Vallejo et al. (2008) consider that strains having autoaggregation percentages higher than to 65% are strongly autoaggregating.

Following Del Re et al. (2000) criteria, 60.7% and 96.4% of the assayed strains were able to autoaggregate at 2 h and 4 h, respectively and 7.1% of them were strongly autoaggregating strains after 4 h incubation. Any strain was strongly autoaggregated after 2 h of incubation. Statistical analysis of values displayed significant differences between strains ($\alpha = 0.05$; $F = 1884.21$; $p = 0.00$). Strains showing the highest values at 4 h of incubation were *Lb. paracasei* Lb29 and Lb38, with values of 93.8% and 93.5% respectively, followed by *Lb. plantarum* Lb88 (77.9%). These values were higher than those found by Ferrari et al. (2016) and Serrano-Niño et al. (2016) who reported values around 37% at 4 h of incubation for different LAB isolates.

Comparison of the average values of autoaggregation percentages for strains of each species displayed the highest values for *Lb. paracasei* both at 2 h (37.1%) and 4 h (55.3%) incubation, while *Lb. acidophilus* had the lowest values, also at both incubation time, 10% and 21.4%, respectively.

3.2.3. Coaggregation assay

Coaggregation with pathogens is also considered an important characteristic for the probiotic strains because it helps to avoid colonization of GI tract by pathogens (Cozzolino et al., 2020) and may constitute an important defense mechanism against infection (Rickard et al., 2003).

Values of coaggregation for the 28 selected strains ranged from 0% to 53.2% with *S. choleraesuis* and from 0% to 55.3% with *St. aureus* (Fig. 2). Solieri et al. (2014) reported that values below 20% are indicative of weak coaggregation ability and according to this criterion, 53.6% and 60.7% of strains would show weak coaggregation with *S. choleraesuis* and *St. aureus,* respectively. Statistical analysis of values obtained

Table 4
Hydrophobicity values of the selected strains with the hydrocarbons xylene and toluene.

Species	Strain	Hydrophobicity (%)	
		Xylene	Toluene
Lb. paracasei	Lb4	51.71 ± 0.27^{k} *	52.58 ± 1.26^{k} *
Lb. rhamnosus	Lb10	57.14 ± 2.29^{l} *	56.60 ± 0.36^{n} *
Lb. paracasei	Lb24	48.50 ± 1.22^{j}	67.42 ± 0.15^{p}
Lb. paracasei	Lb29	35.96 ± 0.93^{g}	$64.79 \pm 1.20^{\circ}$
Lb. acidophilus	Lb30	44.97 ± 1.05^{i}	$65.15 \pm 0.25^{\circ}$
Lb. brevis	Lb31	40.41 ± 0.86^{h}	54.74 ± 1.18^{m}
Lb. delbrueckii	Lb32	39.36 ± 0.63^{h} *	$53.54 \pm 0.35^{l,m}$ *
Lb. plantarum	Lb34	37.34 ± 1.41^{g} *	38.90 ± 1.14^{g} *
Lb. plantarum	Lb36	37.06 ± 1.67^{g}	$53.73 \pm 0.87^{l,m}$
Lb. plantarum	Lb37	29.48 ± 0.71^{f}	57.40 ± 1.19^{n}
Lb. paracasei	Lb38	$1.51 \pm 0.30^{a,b,c}$	13.91 ± 0.33^{e}
Lb. paracasei	Lb41	-	-
Lb. plantarum	Lb71	-	45.24 ± 0.59^{i}
Lb. plantarum	Lb72	-	-
Lb. plantarum	Lb73	23.23 ± 0.50^{e}	26.31 ± 1.11^{f}
Lb. paraplantarum	Lb74	-	-
Lb. plantarum	Lb76	-	-
Lb. plantarum	Lb88	-	$8.58 \pm 0.55^{c,d}$
Lb. plantarum	Lb90	-	47.63 ± 0.37^{j}
Lb. plantarum	Lb93	-	43.23 ± 0.72^{h}
Lb. plantarum	Lb98	13.24 ± 1.91^{d}	7.69 ± 0.89^{c}
Lb. brevis	Lb99	$0.62 \pm 0.13^{a,b}$ *	0.53 ± 2.31^{a} *
Lb. acidophilus	Lb103	2.59 ± 1.43^{c} *	2.72 ± 0.06^{b} *
Lb. acidophilus	Lb104	-	-
Lb. plantarum	Lb107	-	-
Lb. brevis	Lb110	$1.92 \pm 0.44^{b,c}$ *	2.70 ± 0.05^{b} *
Lb. brevis	Lb111	-	9.62 ± 0.28^{d}
Lb. brevis	Lb115	-	-

Data shown are mean ± SD of triplicate values of independent experiments. Different letters in the superscript indicate significant statistical differences ($p < 0.05$) between strains for each parameter, according to the Student-Newman-Keuls test. (*) means no significant differences between results from both hydrocarbons for the same strain. (–) means no hydrophobic character.

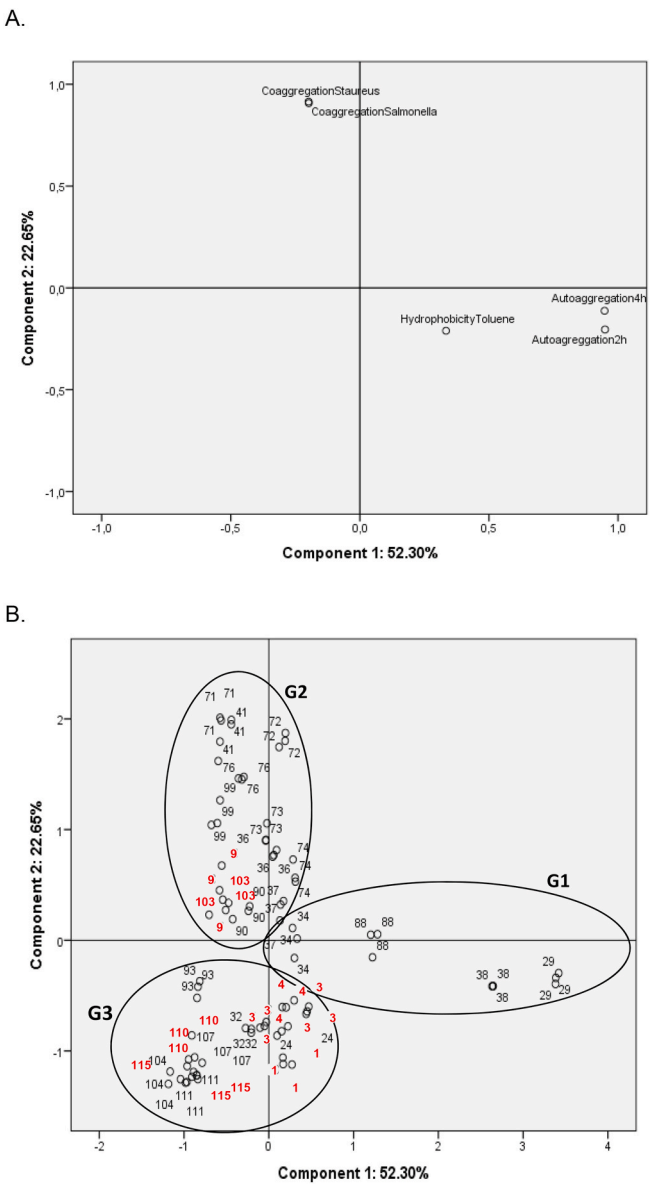

Fig. 3. Principal Component Analysis runs with coaggregation, autoaggregation and hydrophobicity parameters recovered for the strains selected for their kinetic parameters. (A) Projection of the variables; (B) Distribution of strains as a function of the variables. Group 1 (G1) includes strains with highest autoaggregation and hydrophobicity values; Group 2 (G2) includes strains with the highest coaggregation values and Group 3 (G3) includes strains with medium-to-low values for coaggregation, autoaggregation and hydrophobicity. Strains discarded are marked in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

displayed significant differences between strains for both *S. choleraesuis* ($\alpha = 0.05$; $F = 485.69$; $p = 0.00$) and *St. aureus* ($\alpha = 0.05$; $F = 387.92$ $p = 0.00$), in concordance with some authors (Collado et al., 2008; Solieri et al., 2014). The highest coaggregation percentages for *S. choleraesuis* were displayed by the strains *Lb. plantarum* Lb71 (53.2%), Lb72 (49.5%) and *Lb. paracasei* Lb41 (46.3%) while the highest values for *St. aureus* were those of the strains Lb71 (55.3%), Lb41 (52.9%) and Lb76 (49.4%), all of them belonging to *Lb. plantarum* species. These values were much higher than those reported by Ferrari et al. (2016) and Solieri et al. (2014), with values for *Salmonella* of 27.9% and 22.8% for LAB isolates from goat milk and cheeses, respectively, after 5 h of incubation. Collado et al. (2008) reported a value of coaggregation for *St. aureus* of 24.9%, using an incubation time of 4 h, highlighting that values are strain-dependent and variable with time and incubation conditions.

When the values of coaggregation for the different species were calculated, as an average of those from the different strains, it was obtained that *Lb. plantarum* and *Lb. paraplantarum* had the highest coaggregative capacity with both *S. choleraesuis* (32.3% and 26.4%, respectively), and *St. aureus* (23.7% and 24.5%, respectively).

3.2.4. Cell surface hydrophobicity

Hydrophobic interactions are the strongest long range non-covalent interactions. Hence, cell surface hydrophobicity can offer a competitive advantage in the adhesion of LAB, and it can be regarded as a critical factor for adherence to human intestinal epithelial cells (Han et al., 2017). The strains were tested for their cell surface hydrophobicity, using the hydrocarbons xylene and toluene, in order to estimate their adhesion ability.

Results are shown in Table 4, and it can be observed that, with the exception of strains, Lb71, Lb88, Lb90, Lb93 and Lb111, which only showed a decrease in absorbance when were treated with toluene, the results obtained with both hydrocarbons were quite similar. A good correlation between the results from the two solvents was observed with strains *Lb. rhamnosus* Lb10, *Lb. paracasei* Lb24, *Lb. acidophilus* Lb30, *Lb. brevis* Lb31, *Lb. delbrueckii* Lb32 and *Lb. plantarum* Lb36, which showed the highest hydrophobicity percentages. However, results from the t-Student test indicated that the behaviour of these 6 strains with the solvents was significantly different in most cases (Table 4). In general, higher hydrophobicity values were obtained when toluene was used.

Hydrophobicity percentages varied from 0.6% (*Lb. brevis* Lb99) to 57.1% (*Lb. rhamnosus* Lb10) with xylene, with 39.3% of the strains having a percentage higher than 20%. For toluene values ranged between 1.1% (*Lb. brevis* Lb99) and 67.4% (*Lb. paracasei* Lb24). Fifty percent of the strains had values higher than 20%. These values are in concordance with those reported by other authors (Das et al., 2016; Dowarah et al., 2018; Han et al., 2017) for LAB. However, it is important to highlight that values can change if conditions of incubation differ (Somashekaraiah et al., 2019). These results display that LABs are naturally hydrophilic, and that hydrophobicity is a strain-dependent property.

3.2.5. Principal Component Analysis

Results from autoaggregation, coaggregation and hydrophobicity were used as input variables to run a PCA in order to select the strains to be used in the next assays. These are closely related adhesion properties and therefore it was considered interesting to analyse them together, instead of separately.

Fig. 3 shows PCA results. The analysis accounted for 75% of the total variability and the contribution of Principal Component 1 and 2 was 52.3% and 22.7%, respectively. Autoaggregation (after 2 and 4 h incubation) and hydrophobicity (with toluene) variables were positively related to the Principal Component 1, whereas co-aggregation variable was related to the Principal Component 2. The factorial distribution of LAB pinpointed the existence of 3 groups. Group 1 (G1) characterized by high autoaggregation and hydrophobicity values; group 2 (G2) gathered the strains with the highest values in coaggregation, and group 3 (G3) included the LAB with medium-to-low values of coaggregation, autoaggregation and hydrophobicity.

From the analysis of these results, only 8 strains were eliminated. The remaining 20 strains were representatives of the three groups, but showed different characteristics. So, we avoided discarding any strain with good properties. The selected strains were: *Lb. paracasei* Lb29, Lb38, Lb88 and *Lb. plantarum* Lb34 and Lb37 included in G1; *Lb. plantarum* Lb36, Lb71, Lb72, Lb73, Lb74, Lb76, Lb90, *Lb. paracasei* Lb41 and *Lb. brevis* Lb99, included in G2; and *Lb. paracasei* Lb24, *Lb. delbrueckii* Lb32, *Lb. plantarum* Lb93 and Lb107, *Lb. acidophilus* Lb104 and *Lb. brevis* Lb111, included in G3. They were used for the following two assays.

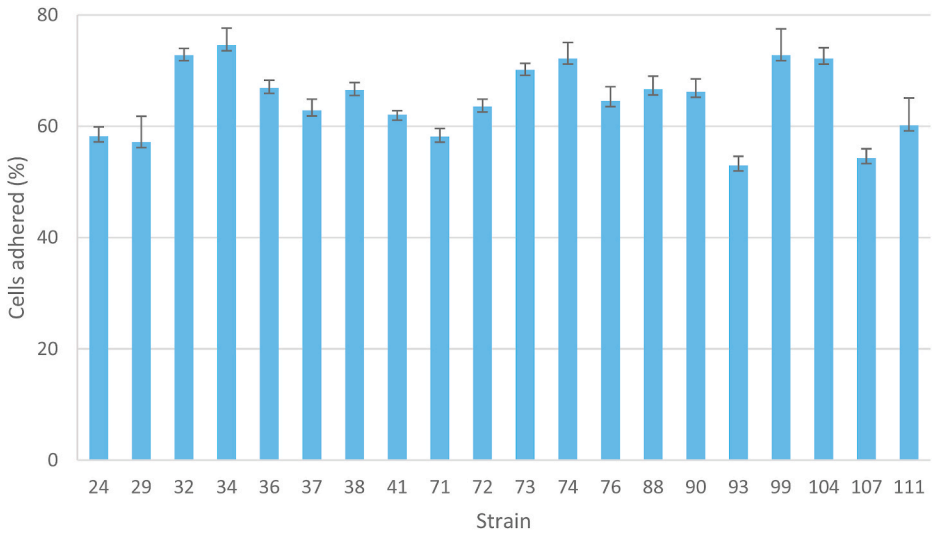

Fig. 4. Percentage of cells adhered to the glass with respect to the cells population in the control. Bars represent standard errors of the mean (n = 3).

Table 5
Minimum, maximum and medium values of adhesion (%) and strains included in each of the grades.

	Adhesion (%)														
	Low			Moderate			Moderate-high			High			Very high		
	Min	Max	Medium	Min	Max	Medium	Min	Max	Medium	Min	Max	Medium	Min	Max	Medium
	51	55	53	61	70	65	71	78	75	82	85	83	94	97	95
Strains	72, 99			73, 76, 71, 37, 34, 90, 93, 24, 36			41, 32, 74, 111, 104			88, 107			29, 38		

Min: minimum value; Max: maximum value; Medium: medium value.

3.2.6. Biofilm formation

Biofilm-forming capacity is considered an interesting property for probiotic strains, since it can promote the colonization and longer permanence of LAB in the mucosa of the host, avoiding settlement by pathogenic bacteria (Terraf et al., 2012).

This property was evaluated by comparison of counts of cells adhered to the glass with respect to the population in the control. Values between 53% and 75% were obtained. As shown in Fig. 4, percentage for strain *Lb. plantarum* Lb34 was significantly higher than for the remaining strains (values in Duncan's test, $\alpha = 0.05$; $F = 40.12$; $p = 0.00$), although there was not much intra-group difference (rms = 6.06). *Lb. delbrueckii* Lb32, *Lb. paraplantarum* Lb74, *Lb. brevis* Lb99 and *Lb. acidophilus* Lb104, showed also high biofilm-forming capacity with values around 72–73%, without significant differences between them.

Few studies have extensively investigated probiotic functions associated with biofilms. Jones and Versalovic (2009) demonstrated that *Lb. reuteri* strains, isolated from niches of the human body, form biofilms and can produce an antimicrobial agent and pro- or anti-inflammatory factors. Bujňáková and Kmeť (2012) found 4 strains (*Lb. fermentum* 202, *Lb. gallinarum* 7001, *Lb. rhamnosus* 183, and *Lb. plantarum* L2-1) which manifested an outstanding potential to inhibit selected intestinal pathogens, and simultaneously demonstrated strong biofilm-forming capacity. Aoudia et al. (2016) also found other lactobacilli (*Lb. plantarum* and *Lb. fermentum*) able to grow as biofilm. These studies corroborate our data, and confirm the ability of *Lactobacillus* species to form biofilm.

3.2.7. Adhesion to Caco-2/TC7 cells

The ability of adhesion to intestinal cells can provide information about the capability of strains to colonize the gastrointestinal tract epithelium. An *in vitro* assay using the Caco-2/TC7 intestine-derived cell line was carried out and the results were expressed as percentage of lactobacilli cells adhered to the Caco-2 cells, with respect to the population inoculated. The percentages ranged from 51% to 97%, with significant differences between strains ($\alpha = 0.05$; $F = 38.47$; $p = 0.00$). Duncan's test managed to differentiate 10 homogeneous subsets, which were classified in five grades, designed as low, moderate, moderate-high, high or very high adhesion capacity, for an easier discussion of the results (Table 5). In each grade were included strains without significant differences between them.

Only two strains were included in each of the low and very high adhesion grades, while the remaining 16 were included in the moderate, moderate-high and high adhesion grades. Strains *Lb. paracasei* Lb29 and Lb38 had the highest percentages of adhesion, 97 and 94% respectively, being significantly different to those of the remaining strains. It showed that adhesion capacity is a strain-dependent property, in concordance with other authors (Duary et al., 2011; Pinto et al., 2020).

Percentages reported by authors for different LABs are variable and while Duary et al. (2011) obtained 10% adherence for *Lb. plantarum*, Haghshenas et al. (2015) found a 70.5% adhesion for the same species, in consonance with our results (67%). The percentages of adhesion reported for other LAB are variable and values of 66% for *Lb. rhamnosus* (Haghshenas et al., 2015), 64–74% for *Enterococcus durans* (Nami et al., 2019) or 34% for *Pediococcus pentosaceus* strains (Damodharan et al., 2015) have been described.

Taking into account subsets generated from Duncan's test for both tests (biofilm formation and *adhesion to Caco-2/TC7 cells*), the strains that stood out for presenting the best probiotic properties were *Lb. delbrueckii* Lb32, *Lb. paraplantarum* Lb74, *Lb. acidophilus* Lb104, *Lb. paracasei* Lb38 and *Lb. plantarum* Lb88 showed percentages of adhesion moderate-high or very high and a high biofilm-forming capacity. Therefore, it could be concluded that these 5 strains showed the most promising probiotic potential of between the 98 initially analysed. However, others as *Lb. brevis* Lb99 showing both high coaggregation value and high biofilm-forming capacity, could also be used for this purpose.

4. Conclusions

The results of the present work has allowed to select 12 strains among the 98 *Lactobacillus* strains assayed, attending to either their antimicrobial activity (*Lb. plantarum* Lb56, Lb78, Lb93 and Lb101, *Lb. brevis* Lb99 and *Lb. paracasei* Lb38) or the probiotic potential (*Lb. delbrueckii* Lb32, *Lb. paracasei* Lb38, *Lb. paraplantarum* Lb74, *Lb. plantarum* Lb88, *Lb. acidophilus* Lb104 and *Lb. brevis* Lb99).

Two of them, the strains *Lb. paracasei* Lb38 and *Lb. brevis* Lb99, stood out for both properties, a high antimicrobial activity, against both Gram-positive and Gram-negative potentially pathogenic strains, as well as for having interesting probiotic properties, the highest autoaggregation and hydrophobicity values and a high coaggregation value and high biofilm-forming capacity, respectively. Therefore, these two strains could be used as biocontrol agents in order to protect against pathogenic microorganisms and to improve healthcare and food safety, avoiding the use of additives.

CRediT authorship contribution statement

S. Rodríguez-Sánchez: Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing. **P. Fernández-Pacheco:** Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing. **S. Seseña:** Writing – review & editing. **C. Pintado:** Data curation, Resources, Writing – original draft. **M. Ll Palop:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

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