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Probiotic Properties of Lactobacilli and Their Ability to Inhibit the Adhesion of Enteropathogenic Bacteria to Caco-2 and HT-29 Cells

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Abstract
We evaluated the probiotic properties of lactic acid bacteria using resistance, safety, and functional assays. A preliminary subtractive screening of nineteen strains was performed based on their survival in simulated gastric and intestinal juice, and cell surface characteristics (hydrophobicity and auto-aggregation). Five strains were selected for further characterization, which included the assessment of their co-aggregation to pathogens, phenol tolerance, antimicrobial activity, and safety. Moreover, their adhesion to Caco-2 and HT-29 cells and the ability to inhibit pathogenic bacteria adhesion were evaluated. All strains had high (≥ 80.0%) survival rates in gastric and intestinal juices. Among them, *Lactobacillus brevis* CCMA 1284, *L. plantarum* CCMA 0743, and *L. plantarum* CCMA 0359 exhibited higher hydrophobicity (95.33, 96.06, and 80.02%, respectively), while *L. paracasei* CCMA 0504 and *L. paracasei* CCMA 0505 had the highest auto-aggregation values (45.36 and 52.66%, respectively). However, these last two strains were positive for the DNAse test, which is a safety concern. The CCMA 0359 and CCMA 1284 strains did not show antimicrobial activity, while the CCMA 0505 strain had a higher percentage of adhesion (4.75%) to Caco-2 cells. In the simulated competition and exclusion assays, the CCMA 0743 strain was able to reduce *Salmonella* adhesion to both cells (Caco-2 and HT-29), but only the CCMA 0743 and CCMA 0505 strains inhibited *Escherichia coli* adhesion to HT-29 cells in the competition assay. According to the results of these evaluated attributes, this strain showed to be an excellent candidate for probiotic use.

Keywords Antagonistic effects · Brazilian foods · Enteropathogenic *Escherichia coli* · Epithelial cells · Gastrointestinal tract · *Salmonella enteritidis*

Introduction

Probiotic microorganisms are recognized for their many health benefits. Among the known probiotic microorganisms, various species of lactic acid bacteria (LAB), especially those of the *Lactobacillus* genus, are widely used as probiotic cultures, as well as for the development of probiotic fermented products [1, 2]. Although there are several strains with proven probiotic properties on the market, the search for novel strains with functional and technological characteristics remains an attractive goal to satisfy increasingly demanding consumers [1, 2], contributing to improved health and reducing the risk of disease [3].

LAB can be found in a variety of food matrices such as dairy products [4], meats [5], indigenous fermented beverages [6], and cocoa [7]. Thus, these food sources are potential reservoirs of novel probiotic strains. The study and selection of new probiotic strains require a systematic approach consisting of sequential evaluations to reduce the number of candidate strains. Probiotic characteristics are reported as strain-specific [8]; therefore, the evaluation of both wild and novel strains is essential, since isolates belonging to the same species may display different properties and probiotic mechanisms.

The methods and criteria used to characterize probiotic strains include assessing their ability to tolerate stress conditions exerted by the human body, ability to interact with host epithelial cells, safety attributes (such as β-hemolysis, gelatinase, and DNAse enzyme activities), and sensitivity to

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antibiotics [9], antimicrobial activity, and competition with pathogens [10]. Moreover, cell surface properties (hydrophobicity, auto-aggregation, and co-aggregation with pathogens) and the interaction of candidate strains with human epithelial cell lines and pathogenic bacteria represent different mechanisms that can be considered in the evaluation of probiotic efficacy [11, 12]. In vitro models employing the HT-29 and Caco-2 cell lines isolated from colonic adenocarcinomas have been widely used to investigate probiotic adhesion capacity [13].

Enteropathogenic *Escherichia coli* (EPEC) and *Salmonella enterica* are important human pathogens whose virulence traits depend on their ability to adhere to epithelial intestinal cells [14, 15]. The indiscriminate use of antibiotics to combat these microorganisms has contributed to the development of resistance mechanisms [16]. In this sense, probiotics have emerged as an alternative in the treatment of bacterial infections, mainly due to the protection conferred to the host cells [17]. Some studies have indicated that the LAB may prevent or reduce the attachment of pathogens to host cells [17, 18]. Here, we evaluated the in vitro probiotic properties of wild LAB strains isolated from different fermented food products. The antagonistic effects of selected LAB strain on the adhesion of pathogens to Caco-2 and HT-29 cells were evaluated by exclusion and competition assays.

Materials and Methods

Screening of LAB Strains

Nineteen LAB strains belonging to the Culture Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras and isolated from different substrates were initially employed in this study (Table 1). Sequentially, the preselection of LAB cultures was based on their ability to survive simulated gastric and intestinal juices and cell surface characteristics (hydrophobicity and auto-aggregation). Five strains (three with higher hydrophobicity and two with higher auto-aggregation) were selected for further characterization.

Survival to Simulated Gastric and Intestinal Juices

The survival of LAB in simulated gastric and intestinal juices was assessed as previously described [26] with modifications. Cultures were grown for 16 h at 37 °C in sterile MRS both, then 1 mL of each culture was mixed in 9 mL sterile saline solution (NaCl 0.85% w/v) with pH adjusted to 2.0 using 1 M hydrochloric acid (HCl) containing 0.5% pepsin (Fisher Scientific, UK) (w/v). After mixing, the initial bacteria counts were determined by plating. Subsequently, samples were incubated for 90 min at 37 °C and cell viability determined by plating. Then, the simulated intestinal juice was prepared by adding oxgall (Himedia, Mumbai, India) and pancreatin (Dinâmica, Brazil) solutions to obtain final concentrations of 0.3% and 0.1% (w/v), respectively, and the pH was then adjusted to 7.0 by adding 1 M sodium hydroxide (NaOH). After mixing, samples were incubated at 37 °C for 150 min, and then viable cell counts were determined. All sample counts were determined by plating on MRS (Man Rogosa and Shape, Kasvi, Italy) agar. The experiments were repeated three times and performed in triplicate. Results were expressed as mean log colony-forming units per mL (CFU/mL). The survival rate was calculated as follows:

$$\text{Survival}\ (\%) = [\text{final}(\text{Log CFU/mL})/\text{initial}(\text{Log CFU/mL})] \times 100$$

Table 1 LAB strains obtained from CCMA

Specie	Code	Source	Reference
Lactobacillus paracasei	CCMA 0504	Apple juice kefir	[19]
Lactobacillus paracasei	CCMA 0505	Apple juice kefir	[19]
Lactobacillus paracasei	CCMA 0506	Apple juice kefir	[19]
Lactobacillus paracasei	CCMA 0354	Goat milk	[4, 20]
Lactobacillus brevis	CCMA 0355	Goat milk	[4, 20]
Lactobacillus brevis	CCMA 0351	Goat milk	[4, 20]
Lactobacillus brevis	CCMA 1284	Cauim	[21]
Lactobacillus plantarum	CCMA 0743	Cauim	[22]
Lactobacillus plantarum	CCMA 0361	Cocoa	[8]
Lactobacillus plantarum	CCMA 0359	Cocoa	[8]
Enterococcus faecium	CCMA 0416	Rice chicha	[23]
Enterococcus faecium	CCMA 0418	Rice chicha	[23]
Lactobacillus casei	CCMA 0412	Rice chicha	[23]
Lactobacillus casei	CCMA 0411	Rice chicha	[23]
Lactobacillus casei	CCMA 0784	Corn silage	[24]
Lactobacillus rhamnosus	CCMA 1431	Corn silage	[24]
Lactobacillus acidophilus	CCMA 0779	Corn silage	[24]
Lactobacillus fermentum	CCMA 0201	Yakupa	[25]
Lactobacillus fermentum	CCMA 0203	Yakupa	[25]

Determination of Cell Surface Characteristics

The hydrophobicity, auto-aggregation, and co-aggregation assays were performed according to Kaktcham et al. [27] with slight modifications.

Hydrophobicity

The cell surface hydrophobicity of each strain was assessed by measuring microbial affinity to xylene. Briefly, cells collected from a 16-h old culture were centrifuged (10,000 rpm for10

min). The resulting pellet was washed twice with sterile phosphate-buffered saline (PBS) (pH = 7.2) and re-suspended in the same buffer. The optical density at the 600 nm wavelength (OD_{600}) of the suspension was measured (A0) using a spectrophotometer (Spectrum, SP-2000UV). Thereafter, 1 mL of xylene was added to 3 mL of cell suspension and mixed by vortexing for 2 min. Then, the water and xylene phases were separated by incubation for 1 h at 37 °C. The aqueous phase was removed and the new OD_{600} was measured (A1). The percentage of the cell surface hydrophobicity was calculated using the formula:

$$\text{Hydrophobicity}\ (\%) = (1-\text{A1}/\text{A0}) \times 100$$

The strains were then classified into microorganisms of low (0–34%), moderate (35–69%), and high hydrophobicity (70–100%) [28].

Auto-Aggregation

LAB cells were harvested from a 16-h old culture in MRS broth, washed twice with PBS (pH 7.2), re-suspended in the same buffer and diluted to an OD_{600} of 0.6 ± 0.1 (approximately 7–8 Log CFU/mL). Bacterial cell suspensions were vortexed for 10 s and subsequently incubated at 37 °C for 5 h. The auto-aggregation percentage was determined using the equation:

$$\text{Auto-aggregation}(\%) = (1-\text{At}/\text{A0}) \times 100$$

where At represents the absorbance at time $t = 5$ h and A0 represents the absorbance at time $t = 0$ h.

Co-Aggregation

For co-aggregation, the LAB strains were grown in MRS broth for 16 h at 37 °C, while enteropathogenic *Escherichia coli* (EPEC) CDC 055 and *Salmonella enterica* serovar Enteritidis ATCC 564 were grown in BHI (Brain-Heart Infusion) broth for 24 h at 37 °C. Bacterial suspensions were prepared as described in the auto-aggregation test above. Equal volumes (2 mL) of LAB and human pathogen suspensions were mixed by vortexing (10 s) and incubated at room temperature without agitation for 4 h. Control tubes contained 2 mL of the suspension of each bacterial strain. The absorbances (OD_{600}) of the mixtures and controls were measured after incubation. The percentage of co-aggregation was calculated using the following formula:

$$\text{Co-aggregation}(\%) = [(\text{Alab} + \text{Apat}) - 2\text{Amix}/(\text{Alab} + \text{Apat})] \times 100$$

where A_{lab} and A_{pat} refer to the OD_{600} of the LAB cell suspension and pathogen cell suspension, respectively, in control tubes and A_{mix} represents the absorbance of the mixed bacterial suspension tested after 4 h.

Phenol Tolerance

Phenol tolerance was determined according to the method described by Shehata et al. [29]. Overnight cultures of LAB strains were inoculated (1%) into MRS broth with 0.2 and 0.5% (v/v) of phenol, or without phenol. Bacterial cells in the culture broth were quantified by reading the OD_{600} after 24 h of incubation at 37 °C. The experiments were performed in duplicate.

Antimicrobial Activity

The antimicrobial activity of LAB cultures was evaluated by the agar spot test according to Arena et al. [30] with modifications. An aliquot of 5 μL of each LAB isolate previously grown in MRS broth was separately spotted on MRS agar and plates were incubated at 37 °C for 48 h to allow the expression and secretion of antimicrobial compounds produced by cultures. The indicator microorganisms were *Salmonella enterica* serovar Enteritidis ATCC 564, enteropathogenic *Escherichia coli* (EPEC) CDC 055, *Listeria monocytogenes* ATCC 19117, *Staphylococcus aureus* ATCC 5674, and *Bacillus cereus* ATCC 14579. Overnight cultures of indicator microorganisms were mixed 1:100 with BHI soft agar (0.7% w/v) and overlaid on developed colonies (8 mm diameter) of LAB isolates. After incubation at 37 °C for 24 h, plates were checked for zones of inhibition surrounding the producer colonies. The experiment was repeated three times.

Antibiotic Susceptibility

The antibiotic susceptibility of the strains was determined by the disk diffusion assay. Overnight cultures (100 μL) were spread onto MRS agar media, and antibiotic discs containing ampicillin (10 μg), vancomycin (30 μg), gentamicin (10 μg), streptomycin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), azithromycin (15 μg), penicillin (10 μg), novobiocin (30 μg), oxacillin (1 μg), and lincomycin (2 μg) were placed on the surface of the inoculated plates using sterile forceps. Inhibition zone diameters were measured after incubation at 37 °C for 24 h. The susceptibility of the isolates was categorized as resistant (R), moderately susceptible (MS), or susceptible (S) according to interpretative values [31]. The experiment was repeated three times.

In Vitro Assessment of Safety Attributes

The safety of the isolates was investigated by assessing hemolysis, DNAse activity, and gelatin hydrolysis, as described by Singh et al. [32] with modifications. Hemolytic activity was

determined by inoculating the strains on blood agar plates containing 5% sheep blood after 48 h incubation at 37 °C. The absence of an effect on blood plaques (γ-hemolysis) was considered non-hemolytic. Green-hued zones around the colonies (α-hemolysis) were considered as partial hemolytic activity, and strains showing clear areas of hydrolysis resulting from blood cell lysis around the colonies were classified as hemolytic strains (β-hemolysis). Gelatinase production by strains was analyzed using tryptone-neopeptone-dextrose (TND) agar (17.0 g tryptone, 3.0 g neopeptone, 2.5 g dextrose, 5.0 g NaCl, 2.5 g K_2HPO_4, 15 g agar, and 1 L distilled water) containing 0.4% gelatin. The LAB cultures were spot-inoculated onto plates containing the medium and incubated at 37 °C for 48 h. Enzyme production was visualized by the formation of a halo around the colony after addition to a saturated ammonium sulfate solution to confirm gelatin hydrolysis. For the DNAse test, strains were streaked on the DNAse test agar medium (Difco, USA) and the plates were incubated at 37 °C for 48 h. After this time, a 1 M HCl solution was added to the plate. A clear zone around the colonies after incubation was considered positive for DNAse production. For all tests, the *Staphylococcus aureus* ATCC 25923 strain was used as the positive control. The experiment was repeated three times.

Adhesion of LAB Strains to Caco-2 and HT-29 Cell Lines

Growth and Maintenance of Caco-2 and HT-29 Cells

The Caco-2 and HT-29 cells provided by the Cell Bank of Rio de Janeiro (BCRJ, Rio de Janeiro) were grown in modified Eagle's minimal essential medium (MEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum), 1× non-essential amino acids, and 0.1 mg/mL gentamicin. All solutions were obtained from Invitrogen, Gibco (Naerum, Denmark). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed regularly and when the cells reached sub-confluence (80–90%), they were sub-passaged.

Adhesion Assay on Caco-2 and HT-29 Cells

The adhesion capacity test for the five selected strains (based on previous assays) to the human colon adenocarcinoma cell lines (Caco-2 and HT-29) was performed according to Ramos et al. [8] with slight modifications. The Caco-2 and HT-29 cells were sub-cultured (2×10^5 cells/mL) in 24-well tissue culture plates (Sarstedt, Germany) and grown at 37 °C in a humidified atmosphere of 5% CO_2 for 21 days to promote differentiation in cell media. The culture medium was changed on alternate days.

For the adhesion assay, bacteria were cultured in MRS broth for 16 h at 37 °C and after washing twice with the phosphate-buffered solution (PBS), the cultures were re-suspended in the media (described above) at a concentration of approximately 10^8 CFU/mL. One milliliter of each bacteria suspension was added to cells in each well and incubated for 90 min at 37 °C in a 5% CO_2 atmosphere. Subsequently, the cells were washed three times with 1 mL of PBS to remove non-adherent bacteria cells and then lysed with 1 mL of Triton-X solution (0.1% v/v in PBS). After 10 min of incubation at 37 °C, the solution with released bacteria cells was serially diluted and plated on MRS agar. The plates were incubated at 37 °C for 48 h. Adhesion ability was expressed as the percentage ratio between the initial counts of bacteria seeded and the counts after the washing steps (CFU/mL). Experiments were performed in duplicate and repeated three times. The probiotic strain *L. paracasei* LBC-81 (Danisco A/S, Copenhagen, Denmark) was employed as a reference strain.

Inhibition of Pathogenic Bacteria Adhesion to Caco-2 and HT-29 Cells

Cell cultures were maintained as previously described. For the pathogen adhesion inhibition test, two different types of experiments were performed using modifications to previously described procedures [33]. In the competition assay, lactobacilli suspensions (10^8 CFU/mL) and *Salmonella* or *E. coli* (10^8 CFU/mL) were mixed and co-cultured simultaneously for 90 min with Caco-2 and HT-29 monolayers. In the exclusion assay, Caco-2 and HT-29 cells were first preincubated with lactobacilli suspensions (10^8 CFU/mL) for 30 min and then a *Salmonella* or *E. coli* suspension (10^8 CFU/mL) was added to each well. Cell cultures in the presence of bacteria were incubated for an additional 90 min at 37 °C in a 5% CO_2 atmosphere. Afterward, the cells were washed three times with 1 mL of PBS to remove non-adherent bacteria cells and lysed with 1 mL of Triton-X solution (0.1% v/v in PBS). After 10 min of incubation at 37 °C, the solution of released bacteria cells was spread on BHI agar and the plates were incubated at 37 °C for 16 h. After this time, enumerations of pathogen colonies were performed. Experiments were performed in duplicate and repeated three times.

Statistical Analysis

Data were analyzed by one-way analysis of variance, followed by post hoc Tukey's and Dunnett's tests for multiple comparisons. Differences were considered statistically significant when $p < 0.05$. All statistical analysis was carried out using Statistica software version 10.0 (Statsoft, USA).

Table 2 Number of LAB strains able to survive in in vitro GIT conditions and hydrophobicity and auto-aggregation capacity of the strains distributed according to percentage values

The survival rate, hydrophobicity or auto-aggregation (%)	No. of isolates ($n = 19$)		
	GIT survival rate	Hydrophobicity	Auto-aggregation
81–100	18	2	
61–80	1	1	
41–60		2	2
21–40		9	15
0–20		5	2

Results and Discussion

Survival in Simulated Gastric and Intestinal Juices and Cell Surface Properties

Eighteen isolates had survival rates ≥ 81% after exposure to simulated gastric and intestinal juices, except *L. casei* CCMA 0411 that showed a survival rate of 79.42% (Table 2). The LAB strains evaluated in the present study exhibited variable hydrophobicity values ranging from 6.67 to 96.06% (Table 2). On the other hand, approximately 68% (13) of the strains had low hydrophobicity (< 35.0%). According to Kaktcham et al. [27], the composition of the bacterial membrane influences the hydrophobicity of the cell surface and, therefore, hydrophobicity evaluation is important in estimating the ability of strains to adhere to host epithelial cells. Regarding auto-aggregation capacity, the strains had values ranging from 16.50 to 52.66% after 5 h of incubation. The highest values were found for *L. paracasei* strains (CCMA 0504 and CCMA 0505) which exhibited auto-aggregation in the range of 41.00–60.00% (Table 2). This is important because auto-aggregation allows the formation of a barrier that prevents the colonization of pathogens on surfaces of the mucosa [34].

Based on the results obtained for survival in simulated gastric and intestinal juices as well as assessments of hydrophobicity and auto-aggregation, five LAB strains were selected (Tables 3 and 4). The selected strains showed high percentages (90.06–96.50%) of survival in simulated gastric and intestinal juices, indicating that they were able to tolerate stressful conditions imposed by GIT (Table 3).

Researchers have suggested a correlation between hydrophobicity and aggregation capacity [35, 36]. All of the five selected strains, which exhibited hydrophobicity, also displayed auto-aggregation capacities after 5 h of incubation (Table 4). All selected strains were able to co-aggregate with EPEC and *S. enteritidis*, except *L. paracasei* CCMA 0504, which did not co-aggregate with *S. enteritidis* (Table 4). The co-aggregation abilities of *Lactobacillus* strains can prevent intestinal colonization by pathogenic bacteria and represent an important host defense mechanism [37, 38].

Table 3 Survival of selected LAB strains under simulated gastric and intestinal juices conditions at 37 °C

Strains	Survival to gastric and intestinal juices			
	Initial mean count[a]	Survival after 90 min[a]	Survival after 240 min[a]	Surviving percentage (%)*
L. brevis CCMA 1284	8.64 ± 0.44	8.62 ± 0.06	8.19 ± 0.01	95.04
L. plantarum CCMA 0743	8.37 ± 0.12	7.97 ± 0.01	8.08 ± 0.06	96.50
L. plantarum CCMA 0359	8.36 ± 0.35	7.73 ± 0.08	8.02 ± 0.01	96.15
L. paracasei CCMA 0504	7.05 ± 0.05	6.84 ± 0.36	6.36 ± 0.84	90.06
L. paracasei CCMA 0505	7.17 ± 0.75	6.82 ± 0.15	6.54 ± 0.66	90.94

*No significant difference ($p > 0.05$), according Tukey test

[a] The values are reported as Log CFU/mL (mean of 3 experiments, each was carried out in triplicate ± standard error)

Table 4 Percentage of hydrophobicity, auto-aggregation, and co-aggregation of selected LAB strains

Strains	Hydrophobicity	Auto-aggregation	Co-aggregation	
			EPEC	*S. enteritidis*
CCMA 1284	95.33 ± 3.59[a]	22.09 ± 4.23[b]	2.39 ± 3.49[c]	8.08 ± 3.03bc
CCMA 0743	96.06 ± 0.26[a]	38.62 ± 2.56[ab]	13.01 ± 6.99[c]	24.04 ± 0.96a
CCMA 0359	80.02 ± 1.98[a]	20.10 ± 4.03[b]	4.57 ± 1.20[c]	13.29 ± 2.18ab
CCMA 0504	46.49 ± 4.53[b]	45.36 ± 6.30[ab]	65.15 ± 1.52[a]	NCc
CCMA 0505	35.29 ± 3.11[b]	52.66 ± 4.98[a]	38.10 ± 4.76[b]	9.76 ± 2.44bc

Mean of 3 experiments, each was carried out in triplicate ± standard error. Different letters for values at the same column mean significant different values according to the Tukey test at a 95% confidence level. NC means that no co-aggregation ability was found.

Phenol Tolerance

The effect of the two different phenol concentrations (0.2% and 0.5%) [39, 40] evaluated on the growth of the five selected LAB is shown in Fig. 1. There were differences in the sensitivities of the strains for the different evaluated phenol concentrations. As expected, the strains were more tolerant to 0.2% phenol than 0.5%. In 0.2% phenol, *L. paracasei* CCMA 0504 was the most tolerant (58.93%), followed by *L. plantarum* CCMA 0743 (53.11%). In 0.5% phenol, all the evaluated strains had less than 5% relative growth. Divisekera et al. [41] evaluated three *Lactobacillus* spp. and reported that they were not able to tolerate 0.5% phenol.

Phenols are compounds formed after bacterial degradation of aromatic amino acids and have been shown to exert toxic effects. Their presence can be affected by many factors such as diet, endogenous proteins, and gut microbiota composition [42, 43]. Phenols inhibit various species of bacteria and may, therefore, affect the diversity and metabolic activity of the intestinal microbiota, mainly by the formation of more potent inhibitory compounds, such as phenolic acids, resulting from microbial transformations of flavonols, flavan-3-ols, flavones, and anthocyanins obtained from the diet [44]. Furthermore, most polyphenols follow through the colon, where they maybe converted by many intestinal bacteria in short-chain fatty acids that can modulate intestinal microbiota composition, increasing *Lactobacillus* genus and other beneficial bacteria populations [45]. Taken together, these results suggest that phenolic compounds resulting from deamination of aromatic amino acids by the intestinal microbiota have bacteriostatic effects against some probiotic strains [9]. Thus, phenol tolerance is an interesting issue for probiotic strains characterization.

Antimicrobial Activity

Regarding antimicrobial activity, *L. plantarum* CCMA 0743 and *L. paracasei* (CCMA 0504 and CCMA 0505) demonstrated inhibitory activity against all pathogens evaluated (Table 5). The two *L. paracasei* strains and *L. plantarum* CCMA 0743 showed the highest activity against *S. aureus* and *B. cereus*, respectively. Conversely, *L. brevis* CCMA 1284 and *L. plantarum* CCMA 0359 showed no inhibitory activity for the evaluated pathogenic microorganisms.

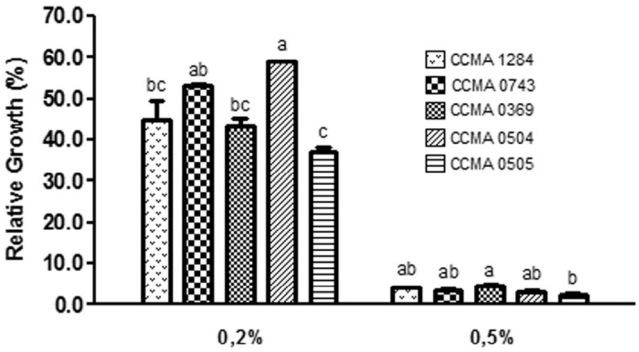

Fig. 1 Effect of phenol concentration on the growth of the five selected LAB strains. Bars indicate standard error of the mean of three independent experiments. The different superscript letters at the same phenol concentration indicate significant differences ($p < 0.05$) by the Tukey test

Table 5 Inhibitory activity of selected LAB strains

Strains	Indicator strains				
	SE	EC	BC	LM	SA
CCMA 1284	–	–	–	–	–
CCMA 0743	++	++	+++	++	++
CCMA 0359	–	–	–	–	–
CCMA 0504	+	+	++	++	+++
CCMA 0505	+	+	++	++	+++

– no inhibition, + inhibition zone 9–11 mm, ++ inhibition zone 12–14 mm, +++ inhibition zone ≥ 15 mm, SE *S. enteritidis* ATCC 564, EC *E. coli* CDC 055, BC *B. cereus* ATCC 14579, LM *L. monocytogenes* ATCC 19117, SA *S. aureus* ATCC 5674

Lactobacillus spp. have been identified with different antibacterial activities against a range of human pathogens [46].

Antibiotic Susceptibility

The evaluated strains were sensitive to at least one antibiotic from the cell wall synthesis inhibitor and protein synthesis inhibitor classes. All strains were sensitive to ampicillin and chloramphenicol, resistant to vancomycin, streptomycin, and gentamicin and showed some sensitivity to erythromycin (Table 6). Only the two *L. paracasei* strains were moderately susceptible or susceptible to lincomycin, azithromycin, and penicillin, while the *L. brevis* CCMA 1284 strain was resistant to these three antibiotics. Several *Lactobacillus* spp. have been reported to be vancomycin-resistant [26, 31], which was also observed in the present study. Antibiotic resistance may become a risk if associated with gene transfer [47]. However, in most cases, it is not a cause for concern as it maybe not of the transmissible type, nor is it a specific characteristic of the microbial genus or species [48]. Therefore, these resistance mechanisms may be intrinsic to the strain as demonstrated by Handwerger et al. [49], who reported on vancomycin-resistant *Lactobacillus* spp. These authors suggested that the antibiotic-resistant strains, which are not associated with gene transfer, are interesting candidates for concomitant therapy or after antibiotic use, thereby decreasing the adverse effects of these drugs. However, this parameter was not evaluated in the present study and should be considered for further characterization of selected candidates. Finally, all strains showed some degree of susceptibility to novobiocin (a gyrase inhibitor), while only the two *L. paracasei* strains were susceptible to 1 μg oxacillin (an inhibitor of cell wall synthesis); both antibiotics had no proposed scores.

In Vitro Assessment of Safety Attributes

The determination of safety characteristics is one of the criteria for selecting novel probiotic strains [50]. All five isolates did not show hemolytic and gelatinase activities when compared to the positive control strain, *S. aureus* ATCC 25923. Regarding DNAse activity, *L. paracasei* CCMA 0504 and CCMA 0505 strains were positive. Previous studies have reported the presence of extracellular DNAse in *L. plantarum* [51] and that the secretion of this enzyme may be found in milk-related *Lactobacillus* [52]. In the present study, the positive DNAse strains were isolated from kefir. Although the production of these enzymes has been considered as a virulence factor [53], nucleases secreted by *Lactobacillus* spp. have demonstrated activity against Gram-negative bacteria and bacteriophages and may be associated with nutritional functions [52].

Adhesion of LAB Strains to Caco-2 and HT-29 Cell Lines

The adhesion capacities of beneficial bacteria and pathogens may be affected by the in vitro cell line used for evaluation as well as the mechanisms of the strain interacting with superficial components of intestinal cells; this is mainly related to the production (or not) of mucus [13]. The present study evaluated LAB adhesion to two different cell lines, Caco-2 and HT-29. The percentage adhesion of the strains did not differ ($p > 0.05$) from the positive control strain *L. paracasei* LBC-81 on HT-29 cells. On the other hand, *L. paracasei* CCMA 0505 showed higher ($p < 0.001$) adhesion capacity (4.75%) than the positive control strain (0.85%) on Caco-2 cells (Fig. 2). Adhesion capacity is influenced by cell surface

Table 6 Antibiotic susceptibility of selected LAB strains evaluated by disc diffusion method

Strains	Diameter of inhibition zone in mm of the antibiotic tested[a]										
	VAN30	AMP10	PEN10	S10	GEN10	CLO30	E15	AZI15[b]	L2[c]	OXA1[d]	NV30[d]
CMA 1284	0 (R)	22 (S)	14 (R)	0 (R)	11 (R)	26 (S)	18 (S)	13 (R)	0 (R)	0	12
CCMA 0743	0 (R)	20 (S)	20 (MS)	0 (R)	0 (R)	24 (S)	16 (MS)	12 (R)	0 (R)	0	15
CCMA 0359	0 (R)	28 (S)	18 (R)	0 (R)	11 (R)	28 (S)	18 (S)	14 (MS)	0 (R)	0	16
CCMA 0504	0 (R)	24 (S)	28 (S)	7 (R)	0 (R)	24 (S)	22 (S)	20 (S)	10 (MS)	10	11
CCMA 0505	0 (R)	24 (S)	26 (MS)	0 (R)	0 (R)	18 (S)	26 (S)	21 (S)	10 (MS)	10	14

The susceptibility of the isolates was scored as resistant (R), moderately susceptible (MS), and susceptible (S) according to the cut-off values proposed by Charteris et al. (1998)

[a] Antibiotics: VAN = vancomycin (30 μg); AMP = ampicillin (10 μg); PEN = penicillin (10 μg); S = streptomycin (10 μg); GEN = gentamicin (10 μg); CLO = chloramphenicol (30 μg); E = erythromycin (15 μg); AZI = azithromycin (15 μg); L = lincomycin (2 μg); OXA = oxacilina (1 μg); NV = novobiocin (30 μg)

[b] Reference to macrolides group

[c] Reference to lincosamides group

[d] No values proposed

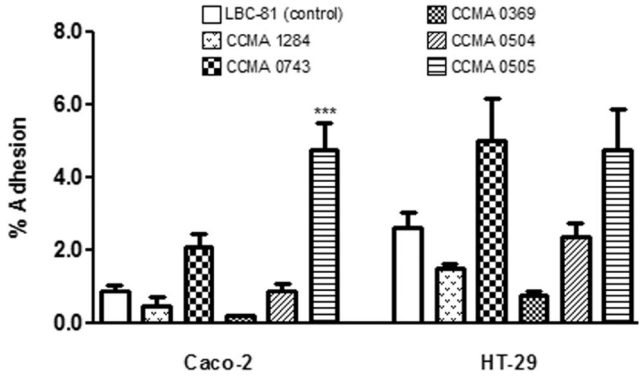

Fig. 2 Adhesion capacity of LAB strains to Caco-2 and HT-29 cells. The adhesion capacity is calculated using the ratio of the number of bacterial cells that remained attached to the total number of bacterial cells initially added to each well. Asterisks indicate statistically significant differences: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ compared to the control, using Dunnett's test. The results are expressed as the mean ± SEM of three independent assays

components and by specific adhesive proteins expressed on this surface that can confer varying degrees of adhesive properties [54].

Inhibition of Pathogenic Bacteria Adhesion to Caco-2 and HT-29 Cells

The inhibition of *Escherichia coli* (EPEC) CDC 055 and *Salmonella enterica* serovar Enteritidis ATCC 564 adhesion by the five selected LAB strains was evaluated by competition and exclusion assays. A significant reduction (0.7 to 1.7 Log CFU/mL) in *E. coli* adhesion to Caco-2 cells by the competition test was observed in the presence of all evaluated strains except CCMA 1284, while in the exclusion test, CCMA 1284 and CCMA 0743 were not able to reduce pathogen counts ($p \geq 0.05$) (Fig. 3a). On the other hand, for the assays using HT-29 cells, only CCMA 0743 and CCMA 0505 were able to significantly ($p < 0.01$) reduce *E. coli* adhesion (Fig. 3b).

The adhesion of *S. enteritidis* to Caco-2 cells was significantly ($p < 0.05$) reduced by competition with CCMA 1284, CCMA 0743, and CCMA 0359 strains, but in the exclusion assay, only the CCMA 0743 strain reduced ($p < 0.001$) pathogen counts (approximately 2.5 Log CFU/mL) (Fig. 3c). All the five evaluated LAB strains were able to inhibit ($p < 0.05$) the adhesion of *S. enteritidis* to HT-29 cells in both exclusion and competition assays (Fig. 3d).

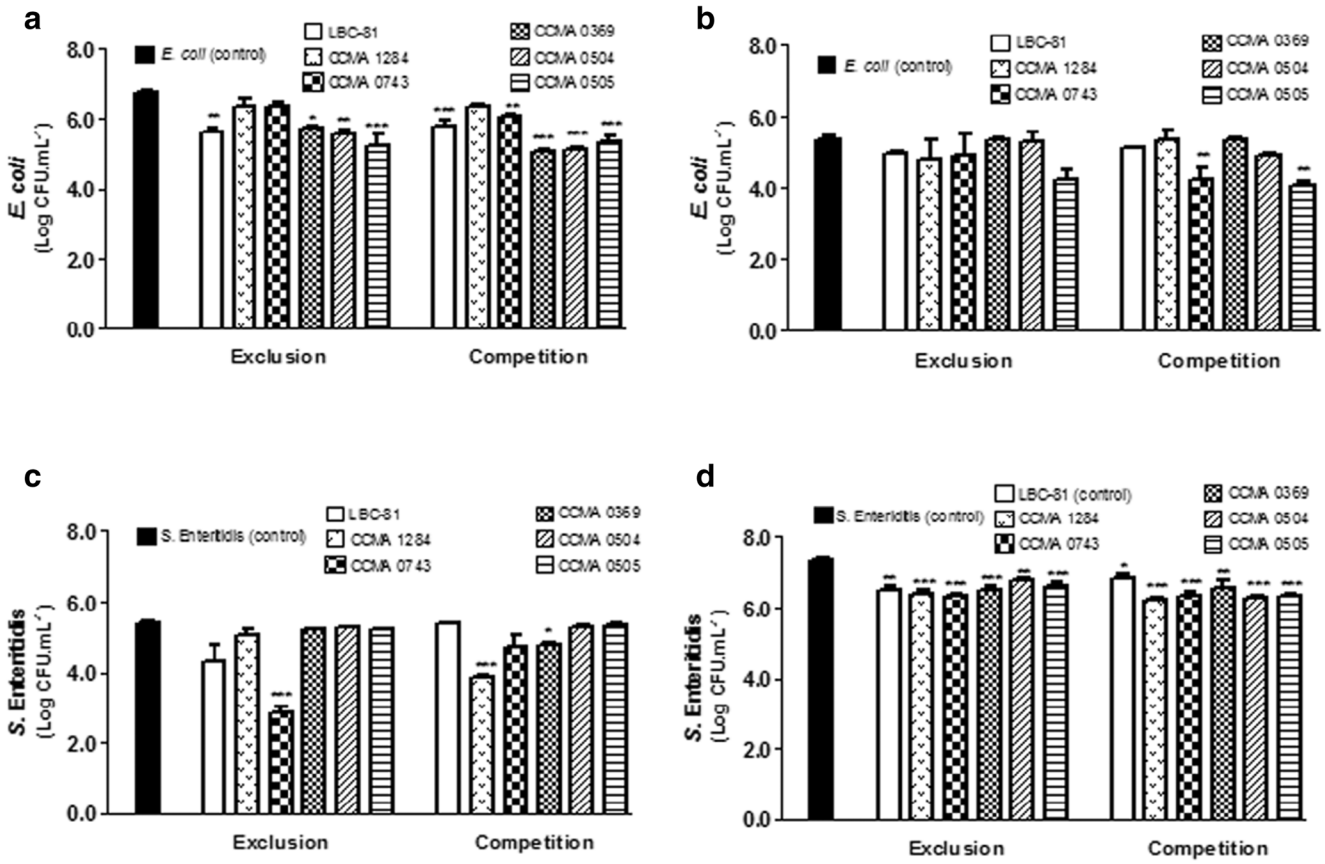

Fig. 3 Effect of *Lactobacillus* strains on the adhesion of enteropathogens to intestinal cell lines. Caco-2 (**a**, **c**) and HT-29 (**b**, **d**) cells were incubated with *E. coli* and *S. enteritidis* alone, or in the presence of *Lactobacillus* strains. Asterisks indicate significant differences: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, compared to the control, according to Dunnett's test. The results are expressed as the mean ± SEM of three independent assays

Studies have reported that *Lactobacillus* strains may inhibit pathogen adhesion by preventing their colonization through competitive exclusion, a highly specific mechanism that is strain-dependent for both probiotics and pathogens [18]. However, adhesion inhibition may be related to different mechanisms such as antimicrobial substances produced by LAB, competition for eukaryotic cell receptors and substrates, the intestinal-mucosal barrier, immunomodulation, and co-aggregation [33]. In the present study, there was a reduction of approximately 1 Log CFU/mL of *S. enteritidis* to HT-29 cells in the presence of *L. plantarum* CCMA 0743 by the exclusion assay.

According to Gagnon et al. [13], *Salmonella* has higher adhesion and invasion capacities in mucus-producing intestinal cell models (HT-29-MTX) than in non-mucus-producing cells (Caco-2). The adhesion of *S. enteritidis* to the Caco-2 and HT-29 cells observed in this study corroborate the results of these authors, as we observed adherent bacteria counts of 5.4 Log CFU/mL and 7.3 Log CFU/mL in non-mucus-producing (Caco-2) and low mucus-producing (HT-29) models, respectively (Fig. 3c and d). In contrast, EPEC adhered more strongly to Caco-2 cells (6.8 Log CFU/mL) than HT-29 cells (Fig. 3a). There is evidence that the ability of pathogenic bacteria to colonize and invade cells of different mucosal surfaces is directly related to the expression of specific proteins, pili, fimbriae, and flagella [55].

Conclusion

Of the 19 strains that were able to tolerate and survive in simulated gastric and intestinal juices, only five expressed remarkable cell surface characteristics (hydrophobicity and auto-aggregation). These strains were able to reduce the colonization and invasion of EPEC and *S. enteritidis* in human epithelial cells (Caco-2 and HT-29), with *L. plantarum* CCMA 0743, *L. paracasei* CCMA 0504, and *L. paracasei* CCMA 0505 also exhibiting antimicrobial activity towards pathogenic bacteria. However, special attention should be given to *L. paracasei* strains due to their ability to secrete DNAse, whose properties and mechanisms of action must be elucidated.

Among these strains, *L. plantarum* CCMA 0743, which is isolated from cauim, an indigenous fermented beverage for infants, exhibited interesting probiotic properties, making it the most promising candidate. However, in vivo evaluation of these probiotic effects is required to confirm these findings. Moreover, the analysis of the potential of this probiotic strain for biotechnological development is still needed before its therapeutic applications can be defined.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals Not applicable.

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