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MICROBIOLOGY

In vitro selection and characterization of probiotic properties in eight lactobacillus strains isolated from cocoa fermentation

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Abstract: Traditionally, probiotic microorganisms are isolated from human and animal intestinal microbiota. However, the demand for diversification of biofunctional products has driven the search for new sources of probiotic candidates, such as fermented foods and vegetables. The present study found that strains isolated from the fermentation of fine cocoa from southern Bahia have biotechnological potential for use as a probiotic, since they showed capacity for self-aggregation and co-aggregation, antimicrobial activity against intestinal pathogens and resistance to gastrointestinal transits. Scores of importance for each property were established in order to more accurately assess the probiotic potential of the strains. The tests carried out contemplate the criteria previously established for the selection of probiotic candidates.

Key words: cocoa, lactic acid bacteria, technological properties, lactobacillus, probiotics.

INTRODUCTION

Probiotics are living microorganisms that, when administered in adequate amounts, promote beneficial effects on the host's health (FAO/WHO 2002). Among these benefits are the restoration of the intestinal microbiota (in dysbiosis situations), relief of symptoms of inflammatory and allergic diseases and modulation of the immune system (Pereira et al. 2018). Besides that, recent work has studied the influence that probiotic microorganisms can have on the development of diseases such as anxiety and depression (Huang et al. 2016). Lactic acid bacteria (LAB) and yeast are two groups of microorganisms traditionally used as probiotics in commercial products. As the market for biofunctional products constantly needs diversification in terms of products, scientific studies have increasingly focused on screening and selection of new strains and properties. These new microorganisms can be isolated from the human and animal intestinal microbiota and other types of sources, like fermented vegetables, fruits, and dairy products (Pereira et al. 2018). Due to the range of biological properties that these strains can present, the selection of microorganisms with probiotic potential occurs through a process consisting of several stages. These steps include assessing the adherence capacity, antimicrobial activity, the resistance to stressful host conditions, and safety assessment (FAO/WHO 2002, Pereira et al.2018).

In this study, properties of probiotic interest were investigated for the biotechnological application of eight strains of lactobacilli previously isolated from fine cocoa fermentation in southern Bahia (Santos et al. 2011). A series of tests was carried out and a score was established to point out the most promising strain for

future use in *in vitro* assays as a bacterium with probiotic potential.

MATERIALS AND METHODS

Growth conditions and maintenance of microorganisms

Strains of *Lactiplantibacillus plantarum* 1.1, *Lactiplantibacillus plantarum* 2.1, *Lactiplantibacillus plantarum* 2.2, *Lactiplantibacillus plantarum* A1, *Lactiplantibacillus plantarum* A2, *Limosilactobacillus fermentum* A2, *Limosilactobacillus fermentum* A5, and *Limosilactobacillus fermentum* 3.2 were previously isolated and identified by our research group (Santos et al. 2011). They were grown in MRS (Man, Rogosa e Sharpe) broth for 18 h in conditions of microaerophilia at 37 °C and stored in MRS with 30% glycerol at -80 ºC. (Zheng et al. 2020). The pathogenic bacteria used for the co-aggregation test was provided by the National Institute for Quality Control in Health - Fiocruz. Salmonella Enteritidis PT4 (IOC) and *Escherichia coli* EHEC INCQS 00171 grew up in TSA (Trypticase Soy Agar) for 18-24 h at 37 °C and were stocked in TSB (Tryptic Soy Broth) containing 30% of glycerol at -80 ºC.

Self-aggregation and Co-aggregation

The self-aggregation test was performed to assess the ability of *lactobacillus* strains to associate with each other. For this essay, the lactobacilli were washed twice with 0.9% (w/v) saline after growth and were resuspended in the same solution until A_{600nm} = 0.3. Then, a 1 mL aliquot of the solution was incubated at 37 ºC for 5 h. The system absorbance was monitored every hour and the percentage of self-aggregation was calculated in relation to the initial absorbance, using the following formula: %self-aggregation = $(OD_{inicial} - OD_{final}) / OD_{inicial} \times 100$. In order to assess the ability of lactobacilli to associate with other bacteria, the potential for co-aggregation has been determined. For this, lactobacilli strains and pathogenic strains were washed twice with 0.9% saline solution and resuspended in the same solution until A_{600nm} = 0.3. After that, each strain of *Lactobacillus* was matched with *Escherichia coli* INCQS 00170 and Salmonella Enteritidis PT4 in the proportion of 1:1 at 37 ºC for 5 h. The absorbance was also measured every hour and the percentage of co-aggregation was given by the following formula: % = [((Ax + Ay)/2) –A(x+y)] ÷ [(Ax + Ay)/2], where x and y indicate the absorbance of each lactobacilli strain (controls) and (x+y) indicates the absorbance of lactobacilli plus the pathogenic strain (Pessoa et al. 2017).

Hydrophobicity

The evaluation of microbial adhesion to solvents was carried out in order to analyze the degree of hydrophobicity of the *lactobacillus* membrane. The *lactobacillus* strains were washed twice after growth and diluted in 0.9% saline solution to an OD (optical density) corresponding to A_{600nm} = 0.7. Then, the bacterial suspension was mixed with xylene at a 1:1 ratio. The mixture was vortexed for 2 min before incubation. Thereafter, the tubes were incubated at 37 ºC for 2 h. After that time, the absorbance value of the aqueous phase was measured. The percentage of hydrophobicity was calculated according to the following formula: % hydrophobicity = $((A_0 - A_2)/A_0) \times 100$, where A_0 indicate absorbance before incubation and A_2 indicate absorbance after 2 hours of incubation (Pessoa et al. 2017).

Preparation of Lactobacillus supernatant and antimicrobial activity

For the preparation of supernatants, *lactobacillus* strains were grown in MRS broth for 48 hin a 37 ºC oven. Then, the cultures were centrifuged at 5000

rpm for 15 min and the pellet was discarded. The recovered supernatants had their pH evaluated, and soon after that, they were filtered through membranes of 0.22 µm. The antimicrobial activity of the supernatants was analyzed using the agar diffusion technique. For this, *Escherichia coli* INCQS00171 and Salmonella Enteritidis PT4 were grown in BHI broth for 24 h at 37 ºC. After incubation, the concentration was adjusted to 1×10^8 CFU/mL using the spectrophotometer and an aliquot of 100 µL of this suspension was inoculated into Mueller-Hinton agar. Small wells were made on the agar and, then, 100 µL of the supernatant from each *lactobacillus* strain was added to the wells. After 24 h of incubation at 37 ºC, the presence or absence of inhibition halos was observed, and their diameters were measured.

Susceptibility to antimicrobials

The susceptibility of *Lactobacillus* strains to antimicrobials was evaluated using the agar diffusion method (CLSI 2015, Charteris et al. 1998). After growth in MRS for 18 h, cultures were centrifuged, washed with 0.9% saline solution and resuspended in the same solution until A_{600nm} = 0.135. A 100 µL aliquot was inoculated onto MRS plates and antibiotic discs were placed on the plates immediately afterwards. The plates were incubated in an oven at 37 °C for 18-24 h. After that time, the diameter of the inhibition halos was measured and the strains classified as sensitive (S), moderately sensitive (MS), and resistant ® (Charteris et al.1998). The tested antimicrobials were: amoxicillin (10 µg), ampicillin (10 µg), cephalothin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), chloramphenicol (30 µg), erythromycin (10 µg), gentamicin (10 µg), norfloxacin (10 µg), penicillin G (10 µg), tetracycline (30 µg), and vancomycin (30 µg).

Gastrointestinal simulation

First, the lactobacilli grew in 10 mL of MRS broth for 18-24 h at 37 ºC and then their concentration was adjusted in a spectrophotometer to 1×10^8 CFU/mL. Then, the suspension was centrifuged and the pellet was resuspended in the same volume of simulated gastric solution, with a pH=2.5 and with pepsin (3 g/L). This solution was incubated at 37 ºC for 1 h and 30 min. After that time, another solution was prepared containing 0.25% bile and 1 mg/mL pancreatin, at pH = 8. And that last suspension was incubated at 37 °C for 45 min. Bacterial counts were performed at the beginning of the test and after incubation in order to determine the cell viability of lactobacilli before and after gastrointestinal simulation.

Importance score for parameterization of *lactobacillus* strains

Importance coefficients were established for each property studied in order to assess in more detail the potentially probiotic strains (Vineetha et al. 2016). The values obtained in the tests were multiplied by the coefficients of importance previously established and the values of each property were added to obtain a single final score. The properties and their established coefficients are shown below.

Statistical analysis

All experiments were carried out in triplicates. The values presented represents the mean plus the standard deviation and were analyzed using GraphadPrism 5.0 software. The statistical differences between the values were determined using ANOVA and post Tukey test with $p < 141$ 0.05.

RESULTS

Hydrophobicity

The strains showed varying degrees of affinity to xylene and were classified as having: low (0-35%), moderate (36-70%), and high (71-100%) hydrophobicity (Piwat et al. 2015). *Lactiplantibacillus plantarum* A2 strain showed high hydrophobicity (82.8±0.21%). Four strains showed moderate hydrophobicity: *Limosilactobacillus fermentum* A2 (67.1±1.55%), *Lactiplantibacillus plantarum* 2.1 (60.6±1.41%), *Lactiplantibacillus plantarum* A1 (58.6±3.32%), and *Lactiplantibacillus plantarum* 2.2 (48.5±2.96%). *Lactiplantibacillus plantarum* 1.1, *Limosilactobacillus fermentum* A5, and *Limosilactobacillus fermentum* 3.2 presented low hydrophobicity: 19.8±3.95%; 25.7±1.06% and 30.3±1.62%, respectively. The profile of adhesion to the nonpolar xylene solvent of the studied *lactobacillus* strains is shown in Figure 1.

Self-aggregation and co-aggregation capabilities

All strains tested showed capacity to self-aggregate and the percentage obtained varied according to the strain, the highest self-aggregation value observed was for *L. plantarum* 2.1 (24.2±1.34), followed by *L. plantarum* A1 (23.6±0.49), *L. plantarum* 2.2 (22.7±0.63), *L. fermentum* A2 (22.5±1.90), and *L. plantarum* A2 (22.2±0.63). Meanwhile, *L. plantarum* 1.1, *L. fermentum* A5 and *L. fermentum* 3.2 demonstrated self-aggregation values below 20% (Figure 2). In general, *Lactiplantibacillus plantarum* isolates self-aggregated better than those of *Limosilactobacillus fermentum*, except for *Limosilactobacillus fermentum* A2. In addition, the data obtained demonstrated that the capacity for self-aggregation increased with the incubation time (Supplementary Material - Figure S1). Through the analysis of the degree of correlation between hydrophobicity and self-aggregation it was possible to establish a positive correlation ($p < 0.05$; $r = 0.77$) between these two properties. The ability to co-aggregate with pathogenic bacteria varied between the strains of lactobacilli studied and varied according to the genus and species of the pathogenic bacteria tested. After 5 h of incubation, only one strain (*Lactiplantibacillus plantarum* A1) was unable to co-aggregate with *Escherichia coli*. Among the strains that were able to co-aggregate, *Lactiplantibacillus plantarum* 2.2 (21.2±1.45) and *Limosilactobacillus fermentum* 3.2 (15.3±1.78) had the best values for co- aggregation. *L. plantarum* 1.1, *L. plantarum* 2.1, *L. plantarum* A2, *L. fermentum* A2, and *L. fermentum* A5 presented a co-aggregation below 15% (Figure 3). Five strains of lactobacilli co-aggregated with Salmonella Enteritidis. Among these strains, the best percentages of co-aggregation were for *Lactiplantibacillus plantarum* 2.1 (15.6±1.77) and *Lactiplantibacillus plantarum* 2.2 (8.9±1.0). *Lactiplantibacillus plantarum* A1, *Limosilactobacillus fermentum* A2, and *Limosilactobacillus fermentum* A5 did not co-aggregate with Salmonella Enteritidis (Figure 3). After 24 h of incubation there was a significant decrease in the ability to co-aggregate. The only strain that showed a brief increase in co-aggregation was *Lactiplantibacillus plantarum* A1, but still this increase was not significant (Figure 4). When analyzing the degree of correlation between self-aggregation and co-aggregation with Salmonella Enteritidis and *Escherichia coli*, it was possible to observe that these properties did not correlate ($r=-0.16$ and $r=0.14$, respectively).

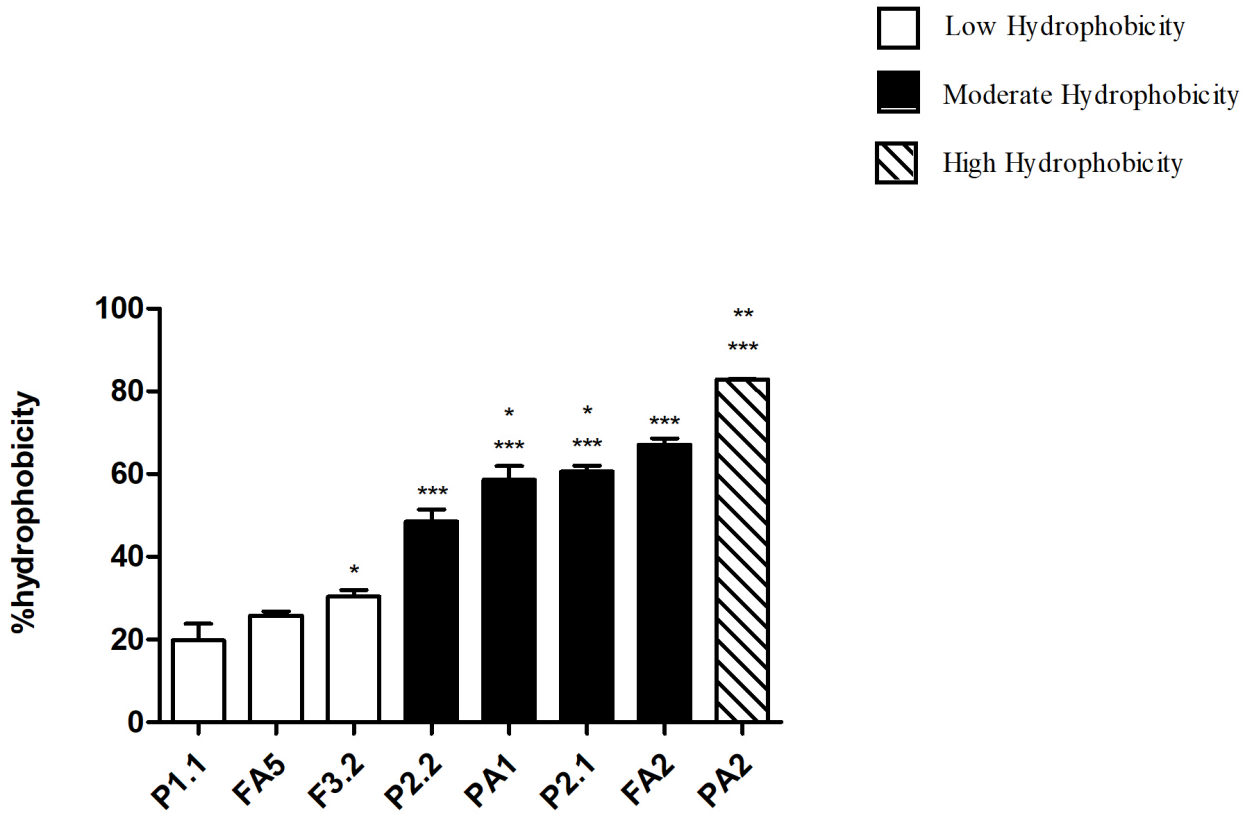

Figure 1. Classification of strains according to the degree of hydrophobicity. The hydrophobicity was evaluated from the incubation of the lactobacilli suspension with xylene and the degree of hydrophobicity was calculated from the absorbance values obtained before and after 2 hours of incubation. (*) represents statistical difference of $p<0.05$, (**) represents statistical difference of $p<0.01$ and (***) represents statistical difference of $p<0.001$. PA2 is statistically different from FA2 (**) and all other six strains studied (***); FA2 is statistically different from P2.2 (***) and all strains classified as low hydrophobicity (***); P2.1 and PA1 are statistically different from P2.2 (*) and all strains classified as low hydrophobicity (***); P2.2 is statistically different from all strains classified as low hydrophobicity (***) and F3.2 is statistically different from P1.1 (*).

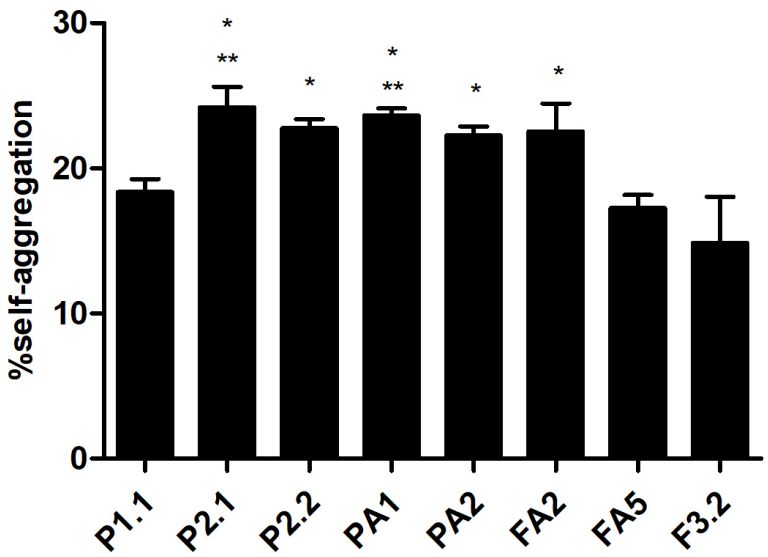

Figure 2. Self-aggregation after 5 hours of incubation. The ability of lactobacilli to aggregate among themselves was evaluated by obtaining the absorbance value before and after 5 hours of incubation of bacterial suspensions. (*) represents statistical difference of $p<0.05$ and (**) represents statistical difference of $p<0.01$. P2.1 and PA1 are statistically different from FA5 (*) and F3.2 (**); P2.2, FA2 and PA2 are statistically different from F3.2 (*).

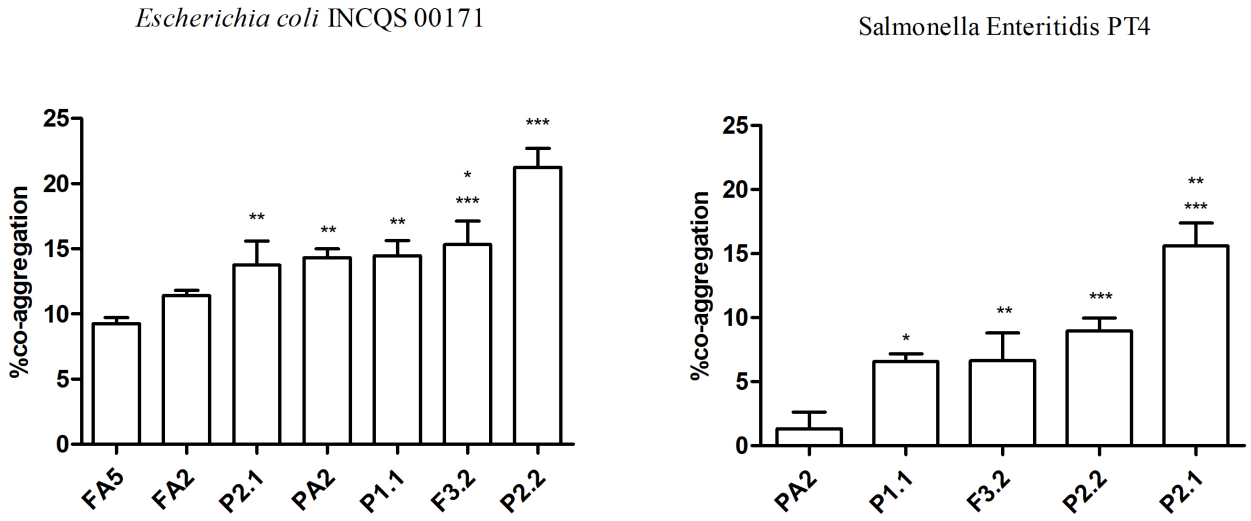

Figure 3. Co-aggregation after 5 hours of incubation. The percentage of co-aggregation evaluated the ability of lactobacilli to aggregate with pathogenic bacteria and was obtained from the measurement of absorbance before and after a 5 hours incubation. (*) represents statistical difference of p<0.05, (**) represents statistical difference of p<0.01 and (***) represents statistical difference of p<0.001. With *E.coli*, P2.2 was statistically different from all other strains studied (***); F3.2 was statistically different from FA2 (*) and FA5 (***); P1.1, PA2 and P2.1 were statistically different from FA5 (**). With *S.Enteritidis*, P2.1 was statistically different from P2.2 (**) and all other strains studied (***); P2.2, F3.2 and P1.1 were statistically different from PA2 (***) (**) (*), respectively.

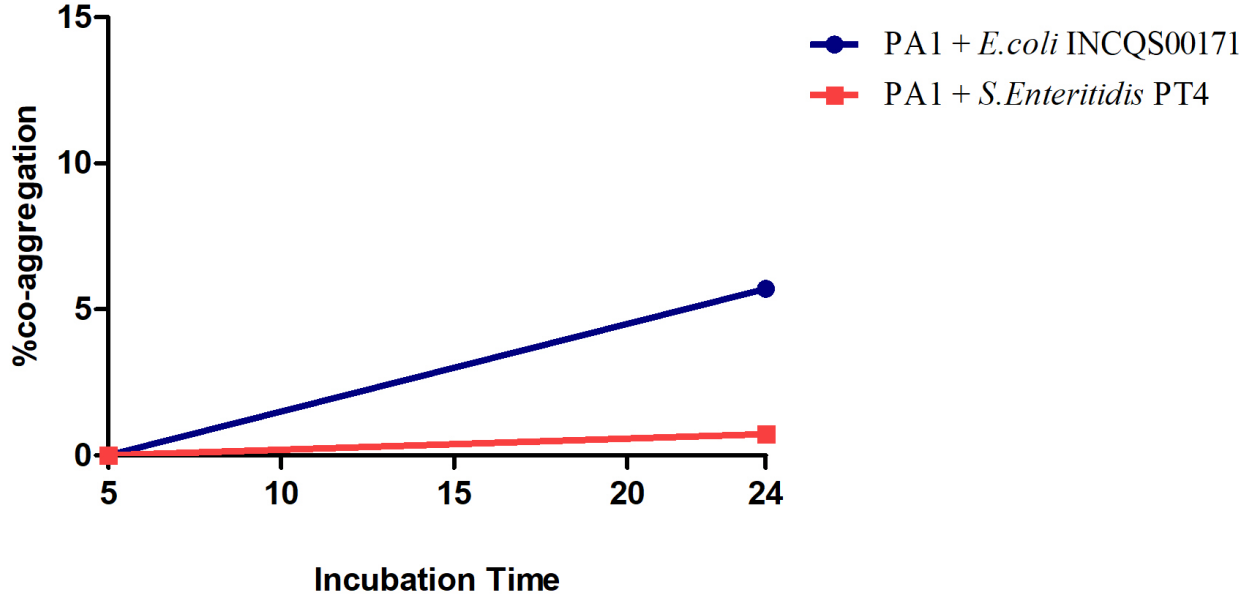

Figure 4. Co-aggregation of *Lactiplantibacillus plantarum* A1 after 24 hours of incubation. The percentage of co-aggregation of PA1 increased to 5.7% with *E.coli* and to 0.7% with S. Enteridis.

Antimicrobial activity of the supernatant

Regarding the ability of *lactobacillus* strains to interfere with the growth of intestinal pathogens, it was possible to observe through the agar diffusion technique that the supernatants of *Lactiplantibacillus plantarum* A1 (pH= 4.08), *Lactiplantibacillus plantarum* A2 (pH= 4.03), and *Lactiplantibacillus plantarum* 2.1 (pH= 4.05) inhibited the growth of *E. coli* and S. enteritidis; the sizes of the inhibition halos for *E. coli* were 6,

8, and 8 mm and for S. Enteritidis the sizes were 8, 6, and 8 mm, respectively. *Limosilactobacillus fermentum* A5 n(pH= 4.68) only inhibited the growth of S. Enteritidis, presenting a halo of inhibition of 6 mm. *Lactiplantibacillus plantarum* 1.1 (pH= 4.52), *Lactiplantibacillus plantarum* 2.2 (pH= 4.64), *Limosilactobacillus fermentum* A2 (pH= 4.63), *Limosilactobacillus fermentum* 3.2 (pH= 4.66), and the control (medium without microbial growth; pH= 6.56) did not inhibit the growth of any of the pathogenic strains used (Figure S2).

Susceptibility of *Lactobacillus* strains to antimicrobials

The susceptibility/resistance to antimicrobials of *lactobacillus* strains was evaluated using the agar disc-diffusion method and after analysis of the inhibition halos, strains were classified as resistant (R), sensitive (S), and moderately sensitive (MS). All *lactobacillus* strains studied showed resistance to vancomycin, gentamicin, streptomycin, and to inhibitors of nucleic acid synthesis (ciprofloxacin and norfloxacin). All strains were sensitive to penicillins (amoxicillin, ampicillin, and penicillin G); except for *Lactiplantibacillus plantarum* 2.2, which demonstrated a moderate sensitivity to penicillin G (Table II). All strains studied were sensitive to tetracycline, chloramphenicol, erythromycin, and clindamycin (inhibitors of protein synthesis).

Table I. Importance coefficients established for each property tested.

SELF - AGGREGATION	0.10
CO - AGGREGATION	0.10
HYDROPHOBICITY	0.20
ANTIMICROBIAL ACTIVITY	0.25
pH	0.20
BILE	0.15

Except for *Limosilactobacillus fermentum* A2, which was moderately sensitive to tetracycline; *Lactiplantibacillus plantarum* A1, which demonstrated resistance to chloramphenicol and *Lactiplantibacillus plantarum* 2.1, which was resistant to clindamycin (Table II).

Gastrointestinal simulation

Differences in cell viability were significant after incubation in gastric solution and after incubation in solution containing bile and pancreatin. Gastric simulation significantly reduced the cell viability of *Lactiplantibacillus plantarum* A2 (3.51×10^{10} para 2×10^{10} CFU/mL) and *Lactiplantibacillus plantarum* 2.1 (6.7×10^{9} para 3×10^{9} CFU/mL). After incubation in bile and pancreatin, the cell viability values obtained for each strain were: *Lactiplantibacillus plantarum* A2 (1.68×10^{7} CFU/mL) and *Lactiplantibacillus plantarum* 2.1 (4.9×10^{7} CFU/mL) (Figure 5).

Scores of importance for parameterization of *lactobacillus* strains

Parameterization started with the selection of strains that presented moderate and high hydrophobicity. From there, the scores for the properties of self-aggregation, co-aggregation and antimicrobial activity were calculated and added to obtain a partial score for each strain (Table III). The strains with the two highest scores were selected for gastrointestinal simulation (Table IV).

DISCUSSION

Screening for the establishment of microorganisms with probiotic potential is complex, involving multiple criteria and stages (Pereira et al. 2018). In addition to the properties related to the viability stability, as resistance to acidic pH, enzymes, and bile salts, currently several studies seek the selection of

Table II. Susceptibility profile of 8 strains of Lactobacillus isolated from cocoa fermentation.

Group	Antimicrobial					Susceptibility				
	Name	Disk Conc. (µg)	L.P 1.1	L.P 2.1	L.P 2.2	L.P A1	L.P A2	L.F A2	L.F A5	L.F 3.2
Cell wall synthesis inhibitors										
Penicillins	Amoxicillin	10	S	S	S	S	S	S	S	S
	Ampicillin	10	S	S	S	S	S	S	S	S
	Penicillin G	10	S	S	MS	S	S	S	S	S
Cephalosporins	Cephalothin	30	S	S	S	S	R	S	S	S
Glycopeptides	Vancomycin	30	R	R	R	R	R	R	R	R
Protein synthesis inhibitors										
Aminoglycosides	Gentamicin	10	R	R	R	R	R	R	R	R
	Streptomycin	10	R	R	R	R	R	R	R	R
Tetracyclines	Tetracycline	30	S	S	S	S	S	MS	S	S
Aminoglycosides	Gentamicin	30	S	S	S	R	S	S	S	S
Macrolides	Erythromycin	15	S	S	S	S	S	S	S	S
Lincosamides	Clindamycin	2	S	R	S	S	S	S	S	S
Nucleic acid synthesis inhibitors										
Quinolones	Ciprofloxacin	5	R	R	R	R	R	R	R	R
	Norfloxacin	10	R	R	R	R	R	R	R	R

strains with specific functional characteristics (Halloran & Underwood 2019). The focus of this study was to evaluate the properties related to the stability and functions of eight strains of lactobacilli isolated from cocoa fermentation. The evaluation started with the analysis of the degree of hydrophobicity of these lactobacilli and through this it was possible to observe that there were differences in the degree of hydrophobicity between the two species studied, with strains of *Lactiplantibacillus plantarum* showing predominantly moderate hydrophobicity; while the strains of *Limosilactobacillus fermentum* showed, in their majority, a low degree of hydrophobicity. In a potentially probiotic strain screening study, Reuben et al. (2019) found predominantly moderate hydrophobicity among lactic acid bacteria. According to Tang et al. (2017), the differences in the degree of hydrophobicity between strains of different species, and even the same species, can be attributed to variations in the expression levels of molecules responsible for the hydrophobic character of the microbial surface. The hydrophobic surface character is considered the initial step for colonization and binding to the host mucosa, establishing a nonspecific type of interaction with epithelial cells (Rosenberg 2006, Valeriano et al. 2014). Teichoic acid is one of the main responsible factors for the hydrophobic character of the surface of lactic acid bacteria, by anchoring to the cell membrane and acting as a mucus and receptor ligand on epithelial cells (Klopper et al. 2017). A higher degree of hydrophobicity is also associated with the presence of glycoprotein material on the surface, which is known to favor specific interactions (binding molecules, like adhesins) necessary for the mucosal adhesion process (Valeriano et al. 2014, Piwat

Table III. Partial score based on the values presented for each property mentioned by strain and on the established coefficients.

Strains	Moderate andhigh hydrophobicity	Self-aggregation	Co-aggregation E. coli	Antimicrobial activity	Co-aggregation S. Enteritidis	Antimicrobial activity	Partial score
***L. plantarum* 2.1**	12.12	2.42	1.37	2	1.56	2	21.47
***L. plantarum* 2.2**	9.70	2.27	2.12	0	0.89	0	14.98
***L. plantarum* A1**	11.72	2.36	0	1.5	0	2	17.58
***L. plantarum* A2**	16.56	2.22	1.43	2	0.13	1.5	23.84
***L. fermentum* A2**	13.42	2.25	1.14	0	0	0	16.81

et al. 2015). The ability to auto-aggregate, phenomenon that allows strains of the same species to form groups among themselves, is also related to the bacterial ability to adhere to epithelial cells (Lukic et al. 2014, Reuben et al. 2019). In general, the studied *Lactiplantibacillus plantarum* isolates self-aggregated better than those of *Limosilactobacillus fermentum*, except for *Limosilactobacillus fermentum* A2. Moreover, the obtained data demonstrated that the capacity of self-aggregation increased with the incubation time (Figure S1), as has been observed in other investigations of the probiotic properties of lactobacilli (Valeriano et al. 2014, Klopper et al. 2017). An analysis of the aggregation capacity of two strains of lactobacilli isolated from fermented[vegetables revealed values between 20% and 30%, confirming the findings of this study (Grigoryan et al. 2017). Commercially used lactobacilli showed self-aggregation percentages similar to those found in this study. There were variations, but in general the values remained between 15% and 30% (Tareb et al. 2013, Campana et al. 2017, Klopper et al. 2017, Sharma et al. 2017, Xing et al. 2017).

As with hydrophobicity, the ability to self-aggregate is generally related to the ability to adhere to cell surfaces and, indirectly, to stimulate the immune system (Nwoko & Okeke 2021). Self-aggregation has been linked to the ability to form biofilms, preventing pathogens from attaching and favoring their displacement (Alameri et al. 2022). In addition, self-aggregation is associated with the ability of probiotic microorganisms to persist in sufficient numbers in the gastrointestinal tract, to resist adverse conditions and to limit pathogens access to the mucosa (Campana et al. 2017, Gupta & Bajaj 2017, Ferreira et al. 2011). This association has already been demonstrated through the recovery of *Lactobacillus crispatus* M247 in the feces and intestinal mucosa of mice, whereas a MU5 mutant strain unable to aggregate could not be recovered (Voltan et al. 2007). Co-aggregation is a mechanism that facilitates the elimination of pathogens from the gastrointestinal tract, in addition to contributing to the formation of a barrier to the colonization of pathogens (Todorov et al. 2008, Ferreira et al. 2011, Tulumoglu et al. 2013, Campana et al. 2017, Reuben et al. 2019). In this study, the co-aggregation percentages were shown to be low to moderate and, in general, strains of *Lactobacillus* co-aggregated better with *Escherichia coli*. These findings are in accordance with other co-aggregation studies, in which low to moderate percentages were observed, including with *Lactobacillus* strains commercially used (Tuo et al. 2013,

Table IV. Final scores per strain, based on the percentage values of survival after TGI and on the established coefficients.

Strains	pH resistance (%)	Score	Bile Resistance (%)	Score	Final score
***L. plantarum* 2.1**	45	9	4.8	0.72	31.19
***L. plantarum* A2**	57	11.4	0.7	0.105	35.34

Campana et al. 2017). Observation of these variations according to species, strain, and pathogens, allows to affirm that co-aggregation is a strain-specific property (Gueimonde et al. 2006, Reuben et al. 2019). Analyzes of the degree of correlation between the properties of hydrophobicity, self- aggregation and co-aggregation showed that hydrophobicity and self-aggregation correlate positively (*r* value close to +1), but self-aggregation and co-aggregation do not (*r* value close to 0). The positive correlation indicates that hydrophobicity and self-aggregation increase proportionally, and this can be corroborated by the fact that the strains that showed the best hydrophobicity percentages were also those strains that expressively self-aggregated. Of the eight strains studied, four showed antagonistic activity against gram-negative bacteria. *Lactiplantibacillus plantarum* 2.1, A1, and A2 formed inhibition halos for the two pathogenic strains tested, but *Limosilactobacillus fermentum* A5 only presented a halo of inhibition for Salmonella Enteritidis. The size of the halos formed did not vary much, staying in the 6 - 8 mm. Mabeku et al. (2020) obtained similar findings to those presented during the analysis of the antagonistic activity of culture supernatants of lactic acid bacteria isolated from fermented cocoa juice, with the size of the inhibition halos varying between 5 – 10 mm. The sizes of the inhibition halos shown are not in line with what was expected for *Enterobacterales* (CLSI 2015). However, this does not preclude the application of these strains as a strategy in the biocontrol of pathogens; since the size of the halos obtained for strains commercially available also is not in accordance with the recommendations. A strain of *Lactobacillus plantarum* W21 isolated from a commercial product inhibited the growth ofS. Enteritidis and *E. coli*, with halos of 10.01 mm and 10 mm respectively (Campana et al. 2017). In addition, other substances present in the supernatant, unrelated to antimicrobial action, may limit the formation of halos (Arena et al. 2016). The antimicrobial activity of lactic acid bacteria can be a consequence of several agents, such as decreased pH levels; production of substances with bactericidal or bacteriostatic action (bacteriocins or similar substances) and end products of primary metabolism (lactic acid, acetic acid, hydrogen peroxide, among others) (Tulumoglu et al. 2013). Although no analysis of the product secreted by lactobacilli has been performed, the acidity of the supernatants after cultivation indicates that the antimicrobial agent may be an organic acid derived from metabolism.

The antimicrobial susceptibility test is one of the main safety tests carried out when prospecting for potentially probiotic bacteria. The problem related to antibiotic resistance is the risk of transferring resistance to the resident microbiota. Therefore, strains that are candidates for probiotics - whether for human or animal use - should be evaluated and monitored for antibiotic resistance (Tulumoglu et al. 2014, Gupta & Bajaj 2017). In general, the susceptibility profile presented

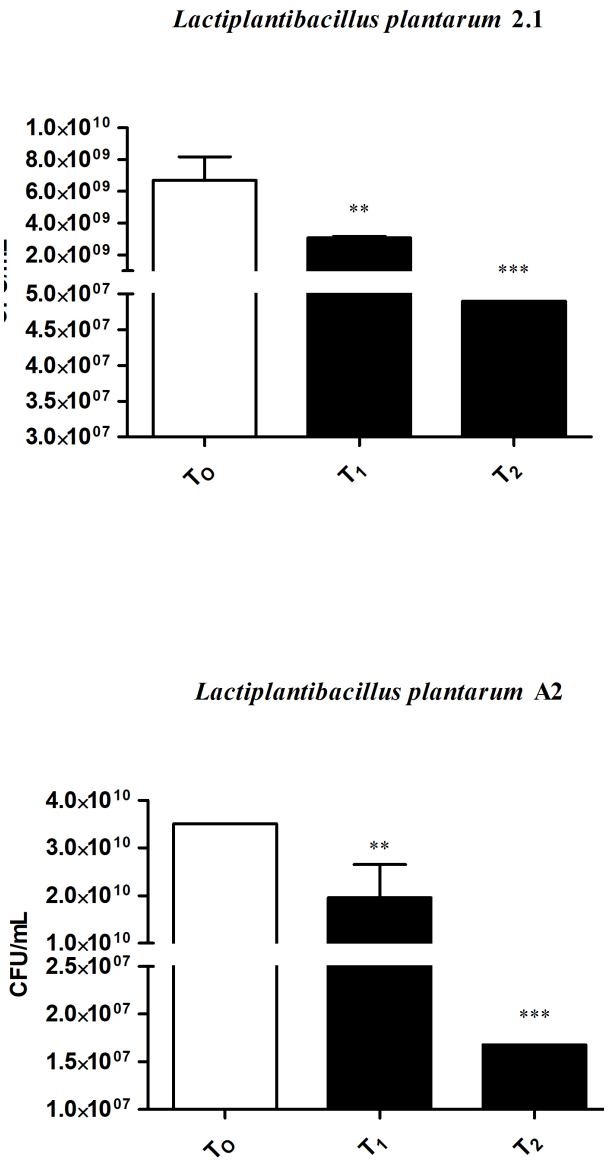

Figure 5. Cell viability in CFU/mL after simulated gastrointestinal transit. *L.plantarum* A2 e *L.plantarum* 2.1 were incubated in an acidic pepsin solution for 1h30min and, later, in a 0.25% solution of bile and pancreatin for 45min. Cell viability was calculated from the comparison between the cell counts of the initial inoculum and the counts after incubation in the solutions. (**) represents statistical difference compared to T_0 (p<0.01); (***) represents statistical difference compared to T_0 (p<0.001).

by the strains studied in this work is similar to the profile reported for other strains of lactobacilli. All strains of lactobacilli studied (100%) exhibited resistance to vancomycin, gentamicin, streptomycin, and inhibitors of nucleic acid synthesis (ciprofloxacin and norfloxacin). Similarly, most strains (five out of six – 83.3%) studied by Reuben et al. (2019) were resistant to vancomycin, ciprofloxacin, and streptomycin and 50% were resistant to gentamicin. Resistance to glycopeptides (vancomycin); aminoglycosides (gentamicin and streptomycin) and inhibitors of nucleic acid synthesis (ciprofloxacin and norfloxacin) is known to be intrinsic/chromosomal in lactobacilli. Thus, the possibility of horizontal transfer of resistance is very remote (Tulumoglu et al. 2013, 2014, Sharma et al. 2017, Shao et al. 2015, Colautti et al. 2022).

In the present study, seven strains (87.5%) were sensitive to penicillins (amoxicillin, ampicillin, and penicillin G); except for *Lactiplantibacillus plantarum* 2.2, which demonstrated moderate sensitivity to penicillin G (Table II). These findings are similar to those found by Reuben et al. (2019), in which two strains (33.33%) also demonstrated moderate sensitivity to penicillin G. In contrast, when analyzing the susceptibility profile of lactic acid bacteria isolated from fermented cocoa juice, Mabeku et al. (2020) found that none of the strains studied showed resistance to penicillins and chloramphenicol. Seven of the strains (87.5%) studied were sensitive to tetracycline, chloramphenicol, and clindamycin (protein synthesis inhibitors). The exceptions were *Limosilactobacillus fermentum* A2, who was moderately sensitive to tetracycline; *Lactiplantibacillus plantarum* A1, who demonstrated resistance to chloramphenicol, and *Lactiplantibacillus plantarum* 2.1 that was resistant to clindamycin (Table II). The differences observed in the susceptibility profile of the strains can be attributed to the species and strain-dependent character of resistance to antimicrobials (Sharma et al. 2015, Klopper

et al. 2017, Sharma et al. 2017, Reuben et al. 2019). It is worth mentioning that the evaluation carried out in this study was preliminary and a reassessment of resistance by molecular methods to determine whether the resistance is intrinsic or extrinsic is necessary to complement it in the future. Regardless, with well-established functional properties, molecular gene deletion or silencing techniques can solve problems arising from the presence of transmissible resistance genes (Colautti et al. 2022).

Despite issues involving transfer of resistance genes, the total absence of antibiotic resistance can be a disadvantage. This is because the administration of probiotic strains resistant to certain antibiotics can preserve or assist the restoration of the resident microbiota during or after antibiotic therapy, in cases of disbioses (Sabir et al. 2010, Reuben et al. 2019). Either way, probiotics should not be used indiscriminately. It is important to establish the appropriate target population. Individuals with pre-established health conditions that lead to compromised immune systems should not be eligible for use of probiotics in conjunction with antibiotic therapy (Rossi et al. 2022).Resistance to adverse effects caused by gastrointestinal transit is also a criterion evaluated when choosing potentially probiotic strains, especially when the goal is to select probiotics with action on intestinal disorders. When other features and application forms are explored, as in the case of adjuvant action in the treatment of dysbiosis of the vaginal tract, this criterion is not so necessary (Chenoll et al. 2019). Tolerance to acidic pH and the presence of proteolytic enzymes creates an efficient barrier to the entry of bacteria into the intestinal tract. In the intestine, bile acts as a selective factor capable of affecting the composition of the intestinal microbiome. Thus, lactic acid bacteria also need to resist the physiological concentration of bile salts so that they can survive and colonize the intestine and be considered probiotics (Sabir et al. 2010, Horacková et al. 2017, Liu et al. 2020).

In the present study, cell viability after simulated GIT decreased significantly. Viability decreased more after incubation in bile and pancreatin than after incubation in acid pH and pepsin; result that was already expected, since the antibacterial properties of bile (mainly against gram-positive bacteria) are already known (Horacková et al. 2017). In contrast, Nemska et al. (2019) found that the studied lactic acid bacteria by their group were more

resistant to the action of bile salts than to the effects of acidic pH, during the analysis of functional characteristics of lactobacilli isolated from dairy products. In general, probiotic candidates appear to have intrinsic mechanisms to tolerate acidity and the presence of proteolytic enzymes, preventing cell damage (Gupta & Bajaj 2017). Although there is no consensus on this, Goh & Klaenhammer (2010) suggested that the survival of gastric juice was related to the aggregation and adhesion properties, since the cell viability of *Lactobacillus acidophilus* after incubation in simulated gastric juice reduced considerably when *apf* (aggregation-promoting factor) was inactivated. Like the other probiotic properties, the ability to resist gastrointestinal transit is strain-specific and cannot be extrapolated to other strains. Therefore, it is normal to have differences in the survival rate between microorganisms of the same species (Horacková et al. 2017, Nemska et al. 2019). Despite the significant reduction, at the end of the process, viability was within the intervals suggested by both researchers and regulatory agencies: the Food and Agriculture Organization of the United Nations (FAO/WHO 2002) proposed that a probiotic product should contain between 10^6 and 10^7 CFU/g; similarly, Shah (2007)

and Pereira et al. (2018) recommended that cell viability in a commercial product remains at a minimum of 10^6 CFU/g. Parameterization is useful in screening studies because it reduces probiotic candidates according to their functional properties. In addition, it can target the use of these candidates (Vineetha et al. 2016). The two strains with the highest partial scores were selected for evaluation of resistance to gastrointestinal transit (Table III). The final scores obtained for each *lactobacillus* – based on the importance coefficients established for the properties studied and partial scores (Tables I and III) - allowed to indicate the most promising strains regarding the probiotic potential. Among the lactobacilli studied, *Lactiplantibacillus plantarum* 2.1 and *Lactiplantibacillus plantarum* A2 were the two strains with the highest final scores and can be considered strains with potential biotechnological use (Table IV). However, further tests are needed in order to observe the role of these strains and their supernatants in biological models of infection *in vitro* and *in vivo* and elucidate on possible and more specific probiotic mechanisms of action. These strains have been shown to have a hydrophobic surface, self-aggregating and co-aggregation properties, the ability to resist a low pH and to inhibit the growth of pathogens. The data obtained may be useful in future studies to guide the use of these candidates and to elucidate on possible and more specific probiotic mechanisms of action.

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SUPPLEMENTARY MATERIAL

Figure S1, S2.

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