## Important declarations

Please remove this info from manuscript text if it is also present there.

### Associated Data

**New DNA/RNA/peptide etc. sequences were reported.**
**Sequences supplied by author here:**
All accessions are kept (with the same name indicated on this research) in the Bacteriology Collection at the Faculty of Microbiology and in the Bacteriology Collection at the National Center for Food Science and Technology (CITA), University of Costa Rica. Lactobacillus casei strain Lc-P6709 16S ribosomal RNA gene, partial sequence GenBank: MH753098.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753098.1?report=fasta Lactobacillus paracasei strain Lp-P6710 16S ribosomal RNA gene, partial sequence GenBank: MH753094.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753094.1?report=fasta Lactobacillus paracasei strain Lp-P6711 16S ribosomal RNA gene, partial sequence GenBank: MH753095.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753095.1?report=fasta Lactobacillus paracasei strain Lp-P6712 16S ribosomal RNA gene, partial sequence GenBank: MH753096.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753096.1?report=fasta Lactobacillus casei strain Lc-P6713 16S ribosomal RNA gene, partial sequence GenBank: MH753099.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753099.1?report=fasta Lactobacillus paracasei strain Lp-P6714 16S ribosomal RNA gene, partial sequence GenBank: MH753097.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753097.1?report=fasta Lactobacillus casei strain Lc-P6715 16S ribosomal RNA gene, partial sequence GenBank: MH753100.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753100.1?report=fasta Lactobacillus fermentum strain Lf-P6702 16S ribosomal RNA gene, partial sequence GenBank: MH753090.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753090.1?report=fasta Lactobacillus fermentum strain Lf-P6704 16S ribosomal RNA gene, partial sequence GenBank: MH753091.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753091.1?report=fasta Lactobacillus parafarraginis strain Lp-P6717 16S ribosomal RNA gene, partial sequence GenBank: MH753092.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753092.1?report=fasta Lactobacillus parafarraginis strain Lp-P6719 16S ribosomal RNA gene, partial sequence GenBank: MH753093.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753093.1?report=fasta Weissella ghanensis strain Wg-P6706 16S ribosomal RNA gene, partial sequence GenBank: MH753101.1
https://www.ncbi.nlm.nih.gov/nuccore/MH753101.1?report=fasta Lactobacillus casei strain P6709 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752084.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752084.1?report=fasta Lactobacillus paracasei strain P6710 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752080.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752080.1?report=fasta Lactobacillus paracasei strain P6711 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752081.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752081.1?report=fasta Lactobacillus paracasei strain P6712 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752082.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752082.1?report=fasta Lactobacillus casei strain P6713 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752085.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752085.1?report=fasta Lactobacillus paracasei strain P6714 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752083.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752083.1?report=fasta Lactobacillus casei strain P6715 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752086.1
https://www.ncbi.nlm.nih.gov/nuccore/MH752086.1?report=fasta Lactobacillus fermentum strain P6702

phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752076.1 https://www.ncbi.nlm.nih.gov/nuccore/MH752076.1?report=fasta Lactobacillus fermentum strain P6704 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752077.1 https://www.ncbi.nlm.nih.gov/nuccore/MH752077.1?report=fasta Lactobacillus parafarraginis strain P6717 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752078.1 https://www.ncbi.nlm.nih.gov/nuccore/MH752078.1?report=fasta Lactobacillus parafarraginis strain P6719 phenylalanine-tRNA ligase subunit alpha (pheS) gene, partial cds GenBank: MH752079.1 https://www.ncbi.nlm.nih.gov/nuccore/MH752079.1?report=fasta

**Data supplied by the author:**
Raw data of sequences (16S, 16Sa, pheS, pheSa) Assays of Resistance to the Gastrointestinal Tract (ph2, bile, lisozyme) Cell culture, antibiotics, antagonist and antimicrobial activity Plasmid DNA isolation (agarose gel)

## Required Statements

**Competing Interest statement:**
The authors declare that they have no competing interests.

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# First characterization of the probiotic potential of lactic acid bacteria isolated from Costa Rican pineapple silages

**Jannette Wen Fang Wu Wu** [1], **Mauricio Redondo-Solano** [2], **Lidieth Uribe** [3], **Rodolfo WingChing-Jones** [4], **Jessie Usaga** [5], **Natalia Barboza** [Corresp. 6]

[1] Food Technology Department, Universidad de Costa Rica, San Pedro, San José, Costa Rica

[2] Research Center for Tropical Diseases (CIET) and Food Microbiology Research and Training Laboratory (LIMA), College of Microbiology, University of Costa Rica (UCR), Universidad de Costa Rica, San Pedro, San José, Costa Rica

[3] Agronomic Research Center (CIA), Universidad de Costa Rica, San Pedro, San José, Costa Rica

[4] Animal Science Department, Animal Nutrition Research Center (CINA), Universidad de Costa Rica, San Pedro, San José, Costa Rica

[5] National Center for Food Science and Technology (CITA), Universidad de Costa Rica, San Pedro, San José, Costa Rica

[6] Food Technology Department, National Center for Food Science and Technology (CITA), Center for Research in Cellular and Molecular Biology (CIBCM)., Universidad de Costa Rica, San Pedro, San José, Costa Rica

Corresponding Author: Natalia Barboza
Email address: natalia.barboza@ucr.ac.cr

**Background.** Agro-industrial waste from tropical environments could be an important source of lactic acid bacteria (LAB) with probiotic potential.

**Methods.** Twelve LAB isolates were isolated from pineapple silages. The species identification was carried out considering 16S rRNA and *phe*S genes. Experiments to evaluate the probiotic potential of the isolates included survival under simulated gastrointestinal environment, *in vitro* antagonistic activity (against *Salmonella* spp. and *Listeria monocytogenes*), auto-aggregation assays, antibiotic susceptibility, presence of plasmids, adhesiveness to epithelial cells, and antagonistic activity against *Salmonella* in HeLa cells.

**Results.** *Lacticaseibacillus paracasei*, *Lentilactobacillus parafarraginis*, *Limosilactobacillus fermentum*, and *Weissella ghanensis* were identified. Survival of one of the isolates was 90% or higher after exposure to acidic conditions (pH: 2), six isolates showed at least 61% survival after exposure to bile salts. The three most promising isolates, based on survivability tests, showed a strong antagonistic effect against *Salmonella*. However, only *L. paracasei*_6714 showed a strong *Listeria* inhibition pattern; this isolate showed a good auto-aggregation ability, was resistant to some of the tested antibiotics but was not found to harbor plasmids; it also showed a high capacity for adhesion to epithelial cells and prevented the invasion of *Salmonella* in HeLa cells. After further *in vivo* evaluations, *L. paracasei*_6714 may be considered a probiotic candidate for food industry applications and may have promising performance in acidic products due to its origin.

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[1] Food Technology Department, University of Costa Rica, San Jose, Costa Rica

[2] Research Center for Tropical Diseases (CIET) and Food Microbiology Research and Training Laboratory (LIMA), College of Microbiology, University of Costa Rica (UCR), San Jose, Costa Rica

[3] Agronomic Research Center (CIA), University of Costa Rica (UCR), San Jose, Costa Rica

[4]Animal Science Department, Animal Nutrition Research Center (CINA), University of Costa Rica (UCR), San Jose, Costa Rica

[5] National Center for Food Science and Technology (CITA), University of Costa Rica (UCR), San Jose, Costa Rica

[6] Center for Research in Cellular and Molecular Biology (CIBCM), University of Costa Rica (UCR), San Jose, Costa Rica

Corresponding Author:

Natalia Barboza[1, 5, 6]

San Jose, 11501-2060, Costa Rica

Email address: natalia.barboza@ucr.ac.cr

## Abstract

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## Introduction

Currently, the development and intake of functional foods containing probiotic microorganisms have grown considerably due to their known health benefits and ability to prevent certain diseases (*Nami et al., 2018*). Probiotics are defined by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) as "microorganisms which when administered in adequate amounts confer a health benefit on the host" (*FAO/WHO, 2002*). Probiotics are capable of enduring gastrointestinal (GI) tract conditions, to temporarily colonize the intestinal environment and supply health effects through modulation of GI microbiota and immunogenic responses, or by producing certain beneficial metabolites of interest (*Meybodi & Mortazavian, 2017; Nami et al., 2018*). Delivery of health-promoting microorganisms is commonly done through the consumption of fermented products, most frequently dairy (Nascimento et al., 2019). However, with the increased incidence of lactose intolerance, vegetarianism, and other consumer demands, interest in the development of non-dairy probiotic foods has grown. Nevertheless, changes in matrix properties may imply variations in the probiotic physiological dynamics (*Dey, 2018*).

The majority of probiotic bacteria belong to the lactic acid bacteria (LAB) group that are capable to produce antimicrobial compounds such as lactic acid and bacteriocins (*Soccol et al., 2010*), which makes them suitable as probiotics and bio-control organisms due to their ability to inhibit other microorganisms through the production of different metabolites or by competitive exclusion (*Vieco-Saiz et al., 2019*).

The genera *Lactobacillus* and *Bifidobacterium* are commonly used probiotics. However, *Lactococcus, Streptococcus, Enterococcus,* and selected yeasts can potentially be used as probiotics as well (*de Vrese & Offick, 2010; Ayala et al., 2019*). The selection and characterization of novel microorganisms as potential probiotics must take into account certain properties such as tolerance to low pH and high bile salt concentrations, as these conditions are

present in the GI tract environment during digestion processes (*García-Ruiz et al., 2014; Byakika et al., 2019*). Recent studies have also suggested the importance of evaluating other features such as adhesiveness to the intestinal mucosa, prolonged and stable persistence in the GI tract, and antimicrobial properties (*García-Ruiz et al., 2014*).

In the last years, probiotics have been obtained mostly from fermented dairy products or the human GI tract (*Kook et al., 2019*). Nonetheless, with the increasing demand for novel probiotics with improved health and processing properties, the search for organisms from non-traditional sources has been intensified (*Kumar et al., 2015*). Some of the unconventional sources that have recently been screened for potential probiotics include traditional fermented foods and beverages, vegetables, and vegetable wastes (*Sornplang & Piyadeatsoontorn, 2016; Ruiz-Rodríguez et al., 2019*). Different intrinsic characteristics of these matrices are considered significant factors leading to the diversity of species or isolates that can be found (*Sornplang & Piyadeatsoontorn, 2016*). In fact, LAB isolated from non-traditional foods can show better performance and high competitiveness as food additives (*Somashekaraiah et al., 2019*).

Multiple sources to isolate LAB with probiotic potential can be found in tropical and subtropical environments. In the Latin-American region, different research have been carried out in terms of screening and evaluation of new LAB isolates with health-promoting properties. Most of the studies have focused on the isolation of strains from local foods (*Maldonado et al., 2011; Melgar-Lananne et al., 2013; Ramos, 2013; Agostini et al., 2018*), food animals (*Iñiguez-Palomares et al., 2007*), and traditional beverages (*Romero-Luna et al., 2017*). A minor portion of the studies has evaluated strains obtained from environmental sources such as fruits (*Veron et al., 2017*), rain forest (*Benavides, 2016*), and agro-industrial products (*Schwan et al., 1998; Santos et al., 2016*). However, the characterization of LAB with probiotic potential has not been performed in Costa Rica yet.

The aim of this research was to assess the probiotic potential of autochthonous LAB isolated from Costa Rican pineapple peel silages. Selected LAB isolates were identified using molecular

markers and subjected to a series of *in vitro* analyses to evaluate a) resistance to GI tract conditions; b) antimicrobial properties, c) auto-aggregation ability, d) safety properties, and e) adhesion to epithelial cells. These evaluations were done as a preliminary screening for strains with potential application in fermented food applications. This is the first report of the evaluation of LAB with promissory probiotic traits from silages of pineapple residuals from Costa Rica.

## Materials & Methods

### Isolation of Bacterial Isolates

Lactic acid bacteria were isolated from twenty pineapple peel samples that were vacuum-ensiled for 30 days. The samples were obtained from a Costa Rican company dedicated to pineapple juice production (*WingChing-Jones et al., 2021*). Twenty-five grams of each sample was homogenized with 0.1 % w/v peptone water (PW) (Oxoid, Basingstoke, UK) and serially diluted in tubes containing 9 mL of deionized water. Each dilution was used to streak De Man, Rogosa, and Sharpe agar plates (MRS) (Difco, Le Pont de Claix, France) that were incubated at 35 ± 2 °C overnight in anaerobic conditions. Selected colonies were subjected to Gram staining and a posterior morphological identification. The cultures were stored as glycerol stocks (20 % v/v) at -80 °C until analyzed. All accessions are kept (with the same name indicated on this research) in the Bacteriology Collection at the Faculty of Microbiology and in the Bacteriology Collection at the National Center for Food Science and Technology (CITA), University of Costa Rica. The strain *L. casei* ATCC 393 was used as a control given that it is currently commercialized as probiotic (Sidira *et al.,* 2010; Haddaji *et al.,* 2015).

### DNA Extraction and PCR Amplification

Total nucleic acids were extracted from each isolate using a miniprep protocol (*Birnboim & Doly, 1979*). A 1.5 kb fragment of the 16S rRNA gene was amplified using the primer pair 27F/1492R

(Edwards et al., 1989). The PCR was done considering the conditions of an initial denaturation step at 94 °C for 1 min, 30 cycles of 94 °C for 40 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The master mix contained a final volume of 25 µl and included 1X reaction buffer, 0.2 mM dNTPs, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Bio-Rad, Hercules, CA, USA), and 50 ng of DNA. In addition, a ~490 bp fragment of the phenylalanyl-tRNA synthase (*phe*S) gene was amplified by PCR using the primer pair combination pheS-21-F/pheS-22-R (*Naser et al., 2005*). The reaction was performed using iProof High-Fidelity DNA polymerase (Bio-Rad) and 50 ng of DNA. The following cycling conditions were used: 98 °C for 30 s, 35 cycles of 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. PCR products were visualized by electrophoresis in a 1 % agarose gel and stained with GelRed (10.000 X) (Biotium, Fremont, CA, USA). The amplified gene fragments were sequenced in both orientations by Macrogen® (Seoul, South Korea).

**Sequencing Analysis**

The Staden package was used to assemble the obtained sequences. Sequences were aligned using the MUSCLE algorithm (MEGA 7) (*Kumar et al., 2016*). Sequences were compared with those available in the databases with the BlastN tool (*Altschul et al., 1990*). Costa Rican sequences were deposited in the GenBank (Table S1). A total of 25 LAB sequences (12 isolates from this study and 13 obtained from GenBank) were used for phylogenetic comparison. A region of 1299 nucleotides (nt) corresponding to 16S rRNA gene and a fragment of 420 nt for the *phe*S gene, were selected. A phylogenetic tree was constructed using Bayesian phylogenetic analysis. Ten million generations, eight chains, and a mixed model with sampling every 1.000 generations was considered (*Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003*). As an external group, the sequences of *L. delbrueckeii* subsp. *lactis* KTCT

159 3034 was considered for phylogenetic analysis of both genes. Sequences obtained on this
160 research are shown in bold font.
161
162 **Assays of Resistance to the Gastrointestinal Tract**
163 *Tolerance to pH 2.0.* All isolates and a control strain (*L. casei* ATCC 393) were exposed to pH
164 2.0 (Ramos et al., 2013), in order to evaluate tolerance to acidic conditions. Each isolate was
165 cultivated in MRS broth (Difco) at 35 ± 2 °C for 24 h and pH 7.0. Cells were centrifuged at 5000
166 rpm for 5 min at 24 °C, washed two times in PW (Oxoid), and resuspended in PW (Oxoid) to a
167 concentration of about $10^8$ CFU/mL. A 1 mL aliquot of the final bacterial suspension was used to
168 inoculate 50 mL of MRS broth (Difco) adjusted to pH 2.0 using 1 N HCl (Thermo Fisher
169 Scientific, Waltham, Massachusetts, USA) and cultures were incubated at 35 ± 2 °C for 3 h.
170 After 3 hours of incubation, the effect of acidity was neutralized with 1N NaOH (Thermo Fisher
171 Scientific, Waltham, Massachusetts, USA). To quantify the final bacterial population, 1 mL
172 aliquots obtained at time 0 and after 3 h incubation were serially diluted in PW (Oxoid), plated
173 on MRS agar (Difco), and incubated in anaerobic jars for 72 h at 35 ± 2 °C. The assay was
174 conducted in triplicate.
175
176 *Lysozyme resistance***.** Lysozyme resistance was evaluated using a modified version of the
177 method described by Zago et al. (2011). One milliliter of LAB cells and a control strain (*L. casei*
178 ATCC 393) was cultured in MRS broth (Difco) at 30 ± 2 °C for 24 h. After incubation, an aliquot
179 of the culture was centrifuged at 5000 rpm for 5 min at 24 °C and washed twice in phosphate
180 buffer (0.1 M, water pH 7.0). The bottom was resuspended in 2 mL of Ringer solution (8.5 g/L
181 NaCl, 0.4 g/L KCl, 0.34 g/L hydrated $CaCl_2$) (Sigma Aldrich, St. Louis, MO, USA). A sterile
182 electrolyte solution (SES) (0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$)
183 containing 100 mg/L of lysozyme (Sigma Aldrich) was used to resuspend each LAB ($10^8$
184 CFU/mL). Bacterial suspensions in SES without lysozyme were used as negative controls. Each

sample was incubated in a water bath at 37 °C for 0, 30, and 120 min. After incubation, serial dilutions were made in PW (Oxoid) and samples were plated in duplicate on MRS and incubated for 72 h at 35 °C under anaerobic conditions. Cell counts were done, and survival was determined according to the population described as the percentage of CFU/mL after 30 and 120 min relative to the bacterial population in CFU/mL at time zero. Assays were carried out in triplicate.

*Resistance to bile salts***.** LAB tolerance to bile salts was evaluated following the protocol described by García-Ruiz et al., (2014) with minor modifications. The isolates that showed a survival greater than 20% after exposure to pH 2 and lysozyme were selected. The isolates were grown overnight in MRS (Difco) and independently inoculated (2 % v/v) in fresh MRS broth (Difco) supplemented with 0.3 % bile salt (w/v) (Sigma-Aldrich). The LAB was incubated in tilted tubes at 35 ± 2 °C for 24 h and shacked at 250 rpm in a rotary benchtop incubated shaker (Lab Companion model SI-600R, Jeio Tech Company, South Korea). Counts were performed following the procedure previously described. A sample without bile salts was used as a control. Every experimental trial was performed in triplicate and the growth percentage of each culture was compared to the control.

**Antimicrobial Assays**

*Antagonistic activity against pathogens.* The antagonistic activity of all isolated LAB isolates and *L. casei* ATCC 393 against *Listeria monocytogenes* and *Salmonella enterica* was evaluated using a modified version of the overlay protocols (*Booth et al., 1977; Hütt et al., 2006; Soleimani et al., 2010*). Five *L. monocytogenes* strains were used, including four isolates from processed meat products and one reference strain (ATCC 19116). The five *Salmonella* isolates used in the study included one *Salmonella* serovar Typhimurium, one *S.* Typhi, and three isolates of undefined serotype. Before the experiments, each LAB and pathogen strain was individually

grown at 35.0 ± 0.5 °C for 24 ± 2 h in MRS (Difco) or Tryptic Soy Broth (TSB) (Oxoid), respectively. After incubation, each LAB was inoculated on MRS agar plates in a thick straight line approximately 7 cm in length and 0.5 cm from the edge; streaked plates were incubated under capnophilic conditions at 35.0 ± 0.5 °C for 24 ± 2 h. The MRS plates were then overlaid with approximately 5 ml of Brain Heart Infusion agar (BHI) (Oxoid). After solidification, plates were swabbed with a cocktail suspension prepared with the overnight cultures of each pathogen. Petri dishes were incubated at 35.0 ± 0.5 °C for 24 ± 2 h under aerobic conditions. The plates were then examined for a clear inhibition zone around the line of each LAB. Clear zones were measured, and inhibitory activity was determined (*Pan et al., 2009*). Inhibition zones with a diameter larger than 6 mm were considered a confirmation of strong antagonistic activity.

*Antimicrobial activity of the supernatants.* The antimicrobial activity of the cell-free supernatants was determined against the same pathogenic strains by using a previously described protocol with modifications (*Lourenço & Pinto, 2011*). The isolate *L. paracasei*_6714, which showed inhibition zones with a diameter larger than 6 mm for both pathogens, was cultured in MRS broth (Oxoid) at 35 ± 0.5 °C for 24 ± 2 h. The LAB cultures were centrifuged at 1500 rpm for 15 min and the supernatant was decanted and filtered (0.2 µm) into sterile test tubes. To avoid an inhibitory effect due to acid lactic exposure, the pH of the supernatant was adjusted to 7.00 with a solution of 0.1 M NaOH (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the supernatant was used immediately. An isolated colony of each pathogenic strain grown overnight on Tryptic Soy Agar (TSA) (Oxoid) was suspended in PW (Oxoid) to obtain a McFarland standard of 0.5; equal volumes of each strain suspension were mixed to obtain the cocktail solutions used in the experiments. The wells of a 96-well microplate were filled with a 50 µL of sterile TSB (Oxoid), 50 µL of the indicator pathogen solution, and variable volumes (50, 45, 40, 35, 30, 25, 20, and 15 µL) of filtered supernatant adjusted to 50 µL with sterile MRS (Difco). Positive and negative controls were included. The positive control was prepared with 50

237 µL of sterile TSB (Oxoid), 50 µL of the indicator pathogen, and 50 µL of sterile MRS (Difco).
238 Negative controls did not contain the pathogen, and the volume was adjusted with 50 µL of
239 sterile PW (Oxoid). Microplates were incubated aerobically at 35.0 ± 0.5 °C for 24 ± 2 h in high
240 humidity conditions and the absorbance at 620 nm was measured in an Ultra Microplate Reader
241 (Biotek Instruments, Winooski, VT, USA). Results were adjusted by subtracting the absorbance
242 value obtained for the negative control. All determinations were performed in triplicate. To
243 analyze the inhibitory effect of the supernatant solutions on the two pathogens, two-way
244 analysis of variance (ANOVA) followed by Tukey's honest significant difference test were
245 performed using JMP version 11 (SAS Institute Inc., USA). Differences were considered
246 significant at a *P*-value of < 0.05.
247
248 **Auto-aggregation Assays**
249 The auto-aggregation assay was performed following the protocol described by *Rastogi et al.*
250 (*2020*) with some modifications. *L. paracasei*_6714, *L. fermentum*_6702 and *L. casei* ATCC 393
251 (control) were grown in MRS broth at 35 ± 2 °C for 24 h and were later harvested through
252 centrifugation (10.000 × g for 15 min, 4 °C), washed twice with phosphate buffer solution (PBS)
253 (50 mM KH2PO4/K2HPO4, pH 6.8) (Sigma-Aldrich, San Luis, Missouri, USA) and resuspended
254 in PBS to obtain an absorbance of around 0.8 at 600 nm. 3 mL of bacterial suspension was
255 vortexed and incubated at room temperature for 4 hours. Every hour, 0.1 mL of upper
256 suspension was transferred to 3.9 mL of PBS and the $OD_{600}$ was measured. PBS was used as
257 blank.
258 The auto-aggregation percentage was then calculated using the equation:

259 $$\frac{[Ao - At]}{Ao} * 100 = \%Cellular\ Auto - aggregation$$

260 Where $A_t$ is the $OD_{600}$ at time t (t=1,2,3,4) and $A_o$ is the $OD_{600}$ at t= 0.
261

**Safety Assays**

*Antibiotic resistance.* The antibiotic sensitivity of isolate *L. paracasei*_6714 was evaluated by following the swab and agar disk diffusion method (Hudzicki, 2013). A complete set of antibiotics comprising different families was used. The LAB isolate was cultured in MRS broth (Oxoid) at 35 ± 0.5 °C for 24 ± 2h and the suspension of the test isolate was swabbed on solidified Müller-Hinton agar (Oxoid) using a sterile cotton swab. Antibiotic disks impregnated with ciprofloxacin (5 µg), vancomycin (30 µg), penicillin (10 IU), amoxycillin with clavulanic acid (30 µg), erythromycin (15 µg), amikacin (30 µg), streptomycin (10 µg), tetracycline (30 µg) and chloramphenicol (30 µg) (Liofilmchem, Vie a Scozia, Italy) were placed on the agar plates. Plates were incubated at 35 ± 0.5 °C for 24 ± 2 in capnophilic conditions. After incubation, the diameter of the inhibition zones was measured and compared with the standards established by the Clinical and Laboratory Standard Institute (*Sharma et al., 2016; Wolupeck et al., 2017*). Experimental trials were performed in triplicate.

*Plasmid DNA isolation. L. paracasei*_6714 was cultured in MRS broth (Oxoid) at 35 ± 0.5 °C for 24 ± 2 h. Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, Hilde, Germany). The DNA was run and visualized in a 0.8 % agarose gel stained with GelRed® (Biotium, Fremont, CA, USA). Plasmid size was estimated using a using a 100 bp MassRuler DNA ladder (Thermo Fisher Scientific).

**Cell Culture Assays**

*Preparation of cell monolayer.* The *in-vitro* adhesion of *L. paracasei*_6714 was assayed using HeLa cells (kindly supplied by the Research Center for Tropical Diseases), University of Costa Rica). Cells were cultured in a monolayer of Eagle's Minimum Essential Media (EMEM) (Thermo Fisher Scientific) supplemented with 10 % v/v fetal bovine serum, 20 µM glutamine per mL, 50 U penicillin G, and 50 µg/mL of streptomycin. Cultured cells were incubated at 35 ± 0.5

°C in a modified atmosphere of 5 % $CO_2$ and 95 % $O_2$ until used. Before experiments were conducted, the EMEM (Thermo Fisher Scientific) was discarded and cells were washed with 5 mL of 10X PBS (Sigma-Aldrich). Cells were then covered with a solution of 2.5 mL of trypsin and EDTA 0.05 (GIBCO, Thermo Fisher Scientific) with phenol red (GIBCO, Thermo Fisher Scientific) and incubated for 3 min to promote cell separation. Detached cells were resuspended in 2.5 ml of EMEM (Thermo Fisher Scientific), and a small volume was obtained for cell quantification using a Neubauer chamber. A 12-well microplate was filled with different volumes of cell suspensions and 2 mL of EMEM (Thermo Fisher Scientific) to obtain a cell concentration of $10^6$ cells/ml and then incubated for 48 h, as previously indicated.

*In-vitro cell adhesion assay.* A modified version of a previously published methodology was used (*Gopal et al., 2001; Tsai et al., 2005*). *L. paracasei*_6714, at a concentration of about $10^7$ CFU/mL in EMEM (Thermo Fisher Scientific), was placed over a monolayer of HeLa cells previously grown on a glass slide incubated inside a 12-well microplate. Microplates were then incubated for 2 h at 35 ± 0.5 °C. After incubation, cells were washed twice with PBS (Sigma-Aldrich), fixed with 10 % of paraformaldehyde for 10 min, washed twice with PBS (Sigma-Aldrich), and then stained with crystal violet for 5 min. The stained slides were washed with PBS (Sigma-Aldrich) to remove the excess dye and observed under a light microscope. LAB adhesion was evaluated by quantifying the mean number of bacterial cells attached to the HeLa cell monolayer in 5 randomly selected microscopic fields. *L. paracasei* counts were determined for an average of 26 epithelial cells. A positive control with *L. fermentum*_6702 (low adhesion capacity isolate determined in preliminary assays not included here) was included for comparison.

**Antagonistic Effect of *L. paracasei* Against *Salmonella* Invasion in HeLa Cells**

313 *Treatment assay.* A modified version of a previous published methodology was used (*Giannella*
314 *et al., 1973*). *Salmonella* serovar Typhimurium was grown on TSB (Oxoid) at 35 ± 0.5 °C for 24
315 ± 2 h and diluted in antibiotic-free EMEM to obtain a concentration of about $10^7$ CFU/mL. *L.*
316 *paracasei*_6714 was grown in MRS (Oxoid) incubated under the same conditions and then
317 diluted as described for *Salmonella*. A volume of 1 mL of each culture suspension was added to
318 each cell monolayer inside the 12-well microplate. Plates were centrifuged at 1600 rpm for 5
319 min and then incubated for 0, 3, and 24 h under the same conditions described for cell
320 maintenance. After incubation, wells were washed two times with PBS and then kept for 1 h in
321 fresh EMEM (Thermo Fisher Scientific) medium containing 100 µg/mL of gentamicin. After
322 gentamicin exposure, each well was washed twice with PBS (Sigma-Aldrich) and cells were
323 then lysed with ultrapure water for 10 min. Appropriate dilutions in PW (Oxoid) were spread onto
324 TSA (Oxoid) and xylose lysine deoxycholate agar (XLD) (Oxoid). The plates were incubated at
325 35 ± 0.5 °C overnight. Bacterial counts were used to calculate the invasion rate. A positive
326 control of *Salmonella* was included. Experiments were performed in triplicate.
327 *Protection assay.* The protocol described for the treatment assay was modified to include pre-
328 exposure of each cell monolayer to *L. paracasei*_6714 for 3 and 24 h before infection with
329 *Salmonella*.
330

## 331 Results

332 A total of twelve different LAB morphotypes were isolated from twenty pineapple silages with
333 increasing levels of urea. Considering the 16S rRNA sequence and *phe*S gen the isolates
334 correspond to *L. paracasei* (seven isolates), *Lentilactobacillus parafarraginis* (two isolates),
335 *Limosilactobacillus fermentum* (two isolates), and *W. ghanensis* (one isolate) (Table 1 and
336 Table S1). When the sequences obtained in this research and those selected from GenBank
337 (www.genbank.com) were considered, a clear cluster was established (Fig. 1). Equivalent

length portions of both genes were used to resolve the species groups obtained. The species were renamed according to the novel classification of *Zheng et al.* (*2020*). Isolates of *L. paracasei* were also previously characterized with multilocus typing sequences (MTLS). Results were reported by *Wing Ching-Jones et al.* (*2021*).

After exposure to acidic conditions (pH 2.0), all LAB isolates were viable, but just one isolate (*L. parafarraginis* 6719) showed a population that survived more than 90 %. No reduction was observed in the population of the control samples (pH 6.0) as expected (Table 2) and total reduction was observed in the case of the control isolate *L. paracasei* ATCC 393. A higher rate of survival was also observed for *L. paracasei* (isolates: 6710 and 6715) with values of 52.6 % and 42.9 %, and *L. fermentum* (isolates: 6702 and 6704) with values of 31.2 % and 22.1 %, respectively. On the other hand, eight isolates showed more than 90 % of survival after 30 min exposure to lysozyme but just six of them were able to fulfill these criteria after 120 min of exposure. Among those isolates showing higher resistance to low pH, just isolates 6704 and 6710 had a survivability of more than 90 % to lysozyme after 120 min of exposure. Interestingly, *L. parafarraginis* 6719 was very sensitive to the effect of lysozyme (13.1 % of survival after 120 min). Given that any of the LAB isolates fulfilled the selection criteria, isolates showing higher resistance to both conditions (pH and lysozyme) were selected for the bile tolerance test. Survival was lower than 10 % in all the cases, but higher resistance was observed for *L. parafarraginis* 6719 (8.8 %) and *L. fermentum* 6702 (2.1 %). Still, tolerance to bile salts was lower for the control strain (*L. casei* ATCC 393), a commercially available probiotic culture, when compared with the other isolates.

The antagonistic activity of the twelve isolates and the control (*L. casei* ATCC 393) from this study against selected pathogens is shown in Table 3 and Fig. S1. Three isolates produced strong inhibition zones against *Salmonella*. Nevertheless, when the isolates were evaluated against *L. monocytogenes*, only one isolate (*L. paracasei*_6714) produced an inhibition zone with a diameter greater than the reference criteria (6 mm). According to these results, the

antimicrobial activity of the supernatant of *L. paracasei*_6714 was evaluated and the results are shown in Table 4. Significant inhibition of *Salmonella* was observed with 20 µL of the supernatant, while up to 50 µL were required to obtain the same effect for *Listeria*.

The auto-aggregation ability of *L. paracasei*_6714*, L. fermentum*_ 6702, and *L. casei* ATCC 393 (control) was measured at four consecutive time intervals (1, 2, 3, and 4 hours). The results conveyed in Fig. 2 in which is shown a steady increase in auto-aggregation by the studied isolates. After 4 h, *L. fermentum*_6702 showed the lowest auto-aggregation percentage, while *L. casei* ATCC 393 and *L. paracasei*_6714 presented a good auto-aggregation ability, suggesting an effective cell adhesion capacity.

The antibiotic susceptibility of *L. paracasei*_6714 is shown in Table 5. The isolate was resistant to most of the tested compounds. The only exceptions were amoxicillin with clavulanic acid and erythromycin, where an intermediate sensitivity was observed. In addition, the *L. paracasei*_6714 isolate isolated was not found to harbor plasmids, which indicates a low probability of transferring the antibiotic resistance feature (Fig. S2).

The results for the adhesion to HeLa cells are found in Table 6. According to the cell counts, the adhesion capacity of *L. paracasei*_6714 was 200 % higher than that of *L. fermentum* (control isolate). The enological capacity of the studied isolate to prevent pathogen invasion is shown in Table 7. In the treatment assay, the adhesion of the pathogen was reduced by approximately 11 %. On the other hand, in the protection assay, pathogen reduction was between 10 % and 20 %.

## Discussion

Hostile conditions associated with environmental traits of pineapple peel silages, make the LAB isolated from this matrix, important probiotic or with biotechnological potential. Lactobacilli were the most common group found in this research. These results are similar to other reports of LAB

389 isolated from fermented products (*Sáez et al., 2018*), particularly from pineapple and pineapple
390 waste (*Mardalena & Erina, 2016; Arshad et al., 2018*). This finding is not surprising due to the
391 exceptional genetic diversity of the *Lactobacillus* genus, which has recently divided into 23 novel
392 genera (*De Bruyne et al., 2010; Dicagno et al., 2010; Zheng et al., 2020*). On the other hand,
393 many *Weissella* isolates have been obtained from fermentation processes and characterized as
394 heterofermentative bacteria. In fact, *W. ghanensis* was first isolated from cacao fermentation
395 (*De Bruyne et al., 2010*).
396 Isolates were further characterized for their probiotic potential to provide favorable effects on the
397 human gut (*Pan et al., 2009*). Probiotic evaluation of novel strains must include tolerance to the
398 GI tract, antimicrobial activity, susceptibility to antibiotics, and adhesion to mammalian cells,
399 among others (*Byakika et al., 2019*). The group of tests for GI tolerance are aimed to evaluate
400 whether the strains are able to survive exposure to acid and enzymes and eventually the transit
401 through the stomach and intestines (*Ramos et al., 2013; García-Ruiz et al., 2014; Hernández-*
402 *Alcántara, 2018*). In this study, a low tolerance to low pH was observed for most of the isolates,
403 with the exception of *L. parafarraginis*_6719 which showed the highest survival response (more
404 than 90 %). It is important to point out the need to evaluate hundreds of strains to select those
405 that can survive acidic environments (*Ramos et al., 2013*). However, resistance for all the
406 isolates was higher when compared with the control. It is hypothesized that the tolerance to
407 acidic conditions observed in this study may be related to the ensilage process, in which the
408 LAB that survive the last stages were subjected to acidic pH for a prolonged period of time
409 (*Muraro et al., 2021)*. Besides, these results indicate that some of the isolates may be able to
410 survive the normal gastric environment. It is worth noting that the average pH during human
411 digestion is around 2.0 - 3.0 with gradients from 1.8 to 4.0 during 2 to 3 h periods
412 (*Maragkoudakis et al., 2006*). Also, the high survival of LAB to lysozyme exposure in this study
413 was similar to the results previously reported (*García-Ruiz et al., 2014*) where survival greater
414 than 80 % were observed for isolates of *L. pentosaceus, L. casei,* and *L. plantarum* after

incubation for 120 min; however, survival was around 50 % for some isolates. Lysozyme resistance of LAB has been attributed to the peptidoglycan structure in the bacteria cell wall, the physiological state of cells, and the enzyme concentration in the medium (*Cunningham et al., 1991; Delfini et al., 2004*). The ability to survive in the presence of bile is another important characteristic of potential probiotic strains (*García-Ruiz et al., 2014, Hernández-Alcántara et al., 2018*). In the case of probiotics, it was established that survival limits for bile salts should be 50 % or higher after exposure to a concentration of 0.3 % (*Mathara et al., 2008*). Using these criteria, any of the isolates in this study (after pH and lysozyme tests) were classified as bile-resistant. Still, *Bifidobacterium*, other *Lactobacillus* strains, *Pediococcus pentosaceus,* and some yeasts have been reported as bile resistant according to these criteria (*Delgado et al., 2008; Jensen et al., 2012; Turchi et al., 2013; García-Ruiz et al., 2014*). To obtain accurate colonization of the host GI tract, a high bile tolerance is a desirable characteristic for bacteria aimed to be used as probiotics (*Luo et al., 2012; Byakika et al., 2019*). In this research, it was found that bile survival is strain-related instead of LAB species-related and these data are in agreement with previous reports (Delgado et al., 2008; Maldonado et al., 2012).

Inhibitory activity against foodborne pathogens is a desirable trait for bacteria with probiotic potential (*Hütt et al., 2006*). Previous reports have shown that some LAB strains are able to inhibit both Gram-positive and Gram-negative bacteria by the secretion of organic acids or other antimicrobial compounds such as bacteriocins (*Alakomi et al., 2000; Vieco-Saiz et al., 2019*). For example, a strong antimicrobial potential was reported for *L. acidophilus* NIT against *Salmonella* Typhimurium, *Escherichia coli,* and *Clostridium difficile* (*Pan et al., 2009*). Similar findings were observed from this study as *L. paracasei*_6714 was active against both *Salmonella* and *L. monocytogenes*. A previous report by *Hütt et al.* (*2006*) also found an important level of diversity in the antimicrobial activity of different LAB strains, highlighting the importance of an extensive evaluation of newly isolated strains.

The antimicrobial capacity of *L. paracasei*_6714 in solid media was further corroborated with the supernatant test. Bacterial metabolites in the medium such as lactic acid, acetic acid, diacetyl, and others may be responsible for the observed inhibitory effect (*Çon & Gökalp, 2000*). Inhibition by *L. paracasei*_6714 was still observed, even though the supernatant was previously neutralized with NaOH. This suggests that other compounds, such as extracellular proteins as bacteriocins, may be responsible for the observed effect. Several lactobacilli species can excrete antimicrobial proteins (*Mora-Villalobos et al., 2020*). This property is advantageous in terms of host colonization and competition with other bacteria as other microorganisms are inhibited by the excreted metabolites or through competitive exclusion mechanisms based on competition for binding sites and nutrients (*Vieco-Saiz et al., 2019*). *L. paracasei*_6714 is able to synthesize extracellular compounds that can inhibit both *Salmonella* and *L. monocytogenes* and it may be able to inhibit pathogens during *in vivo* applications.

According to García-Cayuela *et al.* (2014), auto-aggregation is a probiotic property that allows the organism to form cell aggregates which in turn increases the adhesion of cells to the epithelial lining of the intestine and therefore, allowing better colonization of the probiotic organism in the gut. The percentage of auto-aggregation obtained for *L. paracasei*_6714 after 4 h during this study is greater than 48 % (Rastogi *et al.*, 2020), suggesting a good adhesion capability.

Concerning susceptibility to antibiotics, an important level of resistance was observed for *L. paracasei*_6714, especially to vancomycin. This antibiotic is considered one of the last resource treatments for multidrug-resistant pathogens, and as a result, this trait is a major concern (Sharma *et al*., 2016). Previous studies have linked intrinsic resistance to glycopeptides in lactobacilli with the ability to replace the terminal d-alanine residue with d-lactate or d-serine in the muramyl pentapeptide, which prevents vancomycin binding (*Sharma et al., 2016*). Antibiotic resistance is considered an advantage for probiotic strains as it facilitates the process of host colonization and survival to eventual exposure to antibiotic treatment (*Bacha et al., 2010; Sharma et al., 2014*). Nevertheless, there may be a risk of transfer of this feature from antibiotic-

resistant strains to foodborne pathogens, since most of the resistance genes are located in gene hotspots along with mobile elements such as plasmids (*Oliveira et al., 2017*). However, as no plasmids were detected in *L. paracasei*_6714, the risk for transferring antibiotic resistance traits to other bacteria during *in vivo* applications should be low.

Finally, the cell culture test was performed to evaluate the ability of *L. paracasei*_6714 to adhere to intestinal epithelial cells and mucosal surfaces. This is a prerequisite for gut colonization by probiotics (*Janković et al., 2012*). Colonization and adhesion may be determined by the aggregation of LAB cells (*Collado et al., 2007*), which is favored by the formation of a film that contributes to the exclusion of pathogens (*Gopal et al., 2001; Tsai et al., 2005*). Precisely, *L. paracasei*_6714 showed a significant level of adhesion to HeLa cells associated with a reduced level of cell infection by *Salmonella*. Likewise, it was found that LAB reduced cell infection by *E. coli* by 31 % to 52 % (*García-Ruiz et al., 2014*).

## Conclusions

Pineapple has been associated with the presence of diverse groups of LAB such as *Lactobacillus* and *Weisella*; these bacteria are adapted to the hostile conditions imposed by the nature of this matrix. As in Costa Rica, pineapple production is one of the most important activities within the agro-industrial sector, it might be possible to find an important diversity of strains with potential biotechnological applications in both, the fresh and/or in the by-products derived from the pineapple industry that are used as silage material or are regarded as a waste.

This is the first study analyzing bacteria with potential probiotic features from Costa Rican sources. The results confirm that agro-industrial by-products, specifically silages, may be an important source of promising LAB strains with a potential probiotic and biotechnological profile. At least one of the isolates (*L. paracasei*_6714) obtained could be a potential probiotic candidate

based on its *in vitro* characteristics and behavior. Additional studies, including encapsulation, could improve survival in the GI environment. This isolate showed important antagonistic activity against pathogens of public health concern, antibiotic resistance without the presence of plasmids, and a good adhesion pattern in cell cultures. Further studies to assess its potential use as a beneficial culture in the food industry are highly recommended. Additional tests may include, among others, tolerance to sodium chloride, production of bile salt hydrolase, *in vivo* tests using animal models, experiments to evaluate the behavior of the isolate in different food matrices, and production of exopolysaccharides.

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**Table legends**

**Table 1 Sequence of primers used for identification of lactic acid bacteria (LAB) from this research. Notes.** [a]Location on the genome of strain *L. paracasei* ATCC 334 (GenBank accession no. CP000423) of the primers.

**Table 2 Resistance/tolerance to pH 2.0, lysozyme and bile salts of lactic acid bacteria (LAB) isolated from pineapple silage. Notes.** *ND*, not determined. Mean values (± standard deviation, $n = 3$).

**Table 3 Inhibition halo of *Salmonella enterica* and *Listeria monocytogenes* grown on culture media pre-inoculated with different lactic acid bacteria (LAB) isolated from pineapple silage. Notes.** + Inhibition zone between 0- and 3-mm diameter (weak), ++ Inhibition zone between 3- and 6-mm diameter (good), +++ Inhibition zone larger than 6 mm diameter (strong).

**Table 4 Absorbance values obtained to evaluate the antimicrobial activity of the supernatant of *L. paracasei*_6714 against *Salmonella* and *L. monocytogenes.* Notes.** Mean values (± standard deviation, $n = 3$). Values not sharing a common letter represent significantly different values ($P < 0.05$).

**Table 5 Antibiotic resistance/susceptibility of *L. paracasei*_6714. Notes.** Mean values (± standard deviation, $n = 3$). *R,* resistant. *I*, intermediate.

**Table 6 Adhesion of *L. paracasei*_6714 to HeLa cells per microscopic field.**

**Table 7 Antagonistic effects of *L. paracasei*_6714 on *Salmonella* Typhimurium invasion of HeLa cells. Notes.** Mean values (± standard deviation, $n = 3$). Values not sharing a common letter represent significantly different values ($P < 0.05$). [a]Post-inoculation time with *Salmonella* Typhimurium.

**Figure legends**

**Figure 1: Phylogeny based on Bayesian analysis and considering the partial sequences of the 16S rRNA gene (1299 nucleotides (nt)) (A) and phenylalanyl-tRNA synthase gene**

767 **(*phe*S) (420 nt) (B) of lactic acid bacteria (LAB) isolated from ensiled pineapple peels**.
768 Probabilities are indicated at nodes. As an external group. *L. delbrueckeii* subsp. *lactis* KTCT
769 3034 was used as an external sequence for both figures. Sequences obtained on this research
770 are shown in bold font.
771 **Figure 2: Cellular auto-aggregation ability of selected lactic acid bacteria (LAB) isolated**
772 **from pineapple waste and comparison with *L. casei* ATCC 393.** Data are reported as mean ±
773 SD.
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775 **SUMPELENTAL MATERIAL**
776 **Table S1 GenBank accession numbers of 16S rRNA gene and phenylalanyl-tRNA synthase**
777 **gene (*phe*S) sequences from lactic acid bacteria (LAB) isolated from pineapple peel silage.**
778 **Figure S1: Picture of plaques and the observed inhibition halo of *L. paracasei*_6712 and *L.***
779 ***paracasei*_6714 against *L. monocytogenes* (A, B) and *Salmonella* sp. (C, D).**
780 **Fig. S2: Picture of gel red stained agarose gel (0.8 %) electrophoresis.** Gel order: 100 bp
781 MassRuler DNA ladder, miniprep of *L. paracasei*_6714, and miniprep of positive control.
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## Table 1(on next page)

Sequence of primers used for identification of lactic acid bacteria (LAB) from this research.

[a]Location on the genome of strain *L. paracasei* ATCC 334 (GenBank accession no. CP000423) of the primers.

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**Table 1 Sequence of primers used for identification of lactic acid bacteria (LAB) from this research.**

| Primer name | Forward primer (5'→ 3') | Reverse primer (5'→ 3') | Location[a] |
|---|---|---|---|
| 27F/1492R | AGA GTT TGA TCC TGG CTC AG | ACG GCT ACC TTG TTA CGA CTT | 259 513…261 026 |
| *pheS*-21-F/*pheS*-22-R | CAYCCNGCHCGYGAYATGC | CCWARVCCRAARGCAAARCC | 1 670 081…1 670 575 |

2 **Notes.**

3 [a]Location on the genome of strain *L. paracasei* ATCC 334 (GenBank accession no. CP000423) of the primers.

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## Table 2(on next page)

Resistance/tolerance to pH 2.0, lysozyme and bile salts of lactic acid bacteria (LAB) isolated from pineapple silage.

*ND*, not determined. Mean values (± standard deviation, $n = 3$).

**Table 2 Resistance/tolerance to pH 2.0, lysozyme and bile salts of lactic acid bacteria (LAB) isolated from pineapple silage.**

| LAB strain | Tolerance to pH 2.0 | | | | Resistance to lysozyme | | | | | | Resistant to bile at 0.3 % | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $t_{30}$ | | | | $t_{120}$ | | | | | |
| | Control (log CFU/ml) | Initial population (log CFU/ml) | Final population (log CFU/ml) | Survival (%) | Control (log CFU/ml) | Initial population (log CFU/ml) | Final population (log CFU/ml) | Survival (%) | Final population (log CFU/ml) | Survival (%) | Control (log CFU/ml) | Initial population (log CFU/ml) | Final population (log CFU/ml) | Survival (%) |
| *L. casei* ATCC 393 (control) | 8.50 | 8.1 ± 1.7 | 0.00 ± 0 | <90% | 8.25 | 7.39 ± 0.09 | 7.59 ± 0.18 | 100% | 8.45 ± 0.06 | 100% | 8.95 | 9.0 ± 1.2 | 3.8 ± 1.1 | <50% |
| *L. paracasei*_6709 | 6.83 | 6.56 ± 0.06 | 2.94 ± 0.02 | <90% | 8.37 | 8.26 ± 0.18 | 8.37 ± 0.10 | 100% | 8.21 ± 0.14 | 90.31 ± 10.7 | ND | ND | ND | ND |
| *L. paracasei* _6710 | 6.99 | 6.57 ± 0.09 | 6.28 ± 0.05 | <90% | 7.96 | 7.93 ± 0.17 | 7.95 ± 0.14 | 100% | 7.95 ± 0.16 | 100% | 9.61 | 9.2 ± 0.5 | 5.5 ± 0.4 | <50% |
| *L. paracasei* _6711 | 7.70 | 7.6 ± 0.7 | 6.49 ± 0.06 | <90% | 7.97 | 7.96 ± 0.16 | 8.02 ± 0.16 | 100% | 7.86 ± 0.16 | <90% | ND | ND | ND | ND |
| *L. paracasei*_6712 | 6.83 | 6.79 ± 0.01 | 5.71 ± 0.02 | <90% | 8.45 | 8.16 ± 0.05 | 8.27 ± 0.07 | 100% | 8.49 ± 0.35 | 100% | ND | ND | ND | ND |
| *L. paracasei* _6713 | 6.18 | 5.99 ± 0.004 | 5.27 ± 0.01 | <90% | 8.02 | 8.12 ± 0.15 | 8.15 ± 0.16 | 100% | 8.03 ± 0.11 | <90% | ND | ND | ND | ND |
| *L. paracasei* _6714 | 5.92 | 5.69 ± 0.05 | 4.55 ± 0.07 | <90% | 8.13 | 8.40 ± 0.25 | 8.23 ± 0.06 | <90% | 8.30 ± 0.15 | 100% | ND | ND | ND | ND |
| *L. paracasei*_6715 | 7.04 | 5.98 ± 0.07 | 5.6 ± 0.1 | <90% | 7.72 | 8.27 ± 0.28 | 8.08 ± 0.13 | <90% | 7.93 ± 0.04 | <90% | 9.76 | 9.6 ± 0.2 | 7.1 ± 0.5 | <50% |
| *L. fermentum*_6702 | 6.99 | 6.48 ± 0.02 | 5.97 ± 0.03 | <90% | 8.51 | 8.41 ± 0.06 | 8.48 ± 0.32 | 100% | 8.29 ± 0.14 | <90% | 8.30 | 8.3 ± 0.1 | 6.5 ± 0.5 | <50% |
| *L. fermentum*_6704 | 6.90 | 6.59 ± 0.02 | 5.93 ± 0.04 | <90% | 8.50 | 8.36 ± 0.16 | 8.35 ± 0.20 | 97.5 ± 10 | 8.41 ± 0.17 | 100% | 10.23 | 9.5 ± 0.6 | 7.5 ± 0.5 | <50% |
| *L. parafarraginis*_6717 | 6.79 | 6.67 ± 0.01 | 5.766 ± 0.004 | <90% | 8.16 | 8.50 ± 0.01 | 6.57 ± 0.02 | <90% | 6.44 ± 0.01 | <90% | 8.91 | ND | ND | ND |
| *L. parafarraginis*_6719 | 7.70 | 7.64 ± 0.01 | 7.62 ± 0.01 | 95.4 ± 2.3 | 8.00 | 7.82 ± 0.15 | 7.59 ± 0.16 | <90% | 6.93 ± 0.11 | <90% | 9.08 | 9.04 ± 0.04 | 8.00 ± 0.1 | <50% |
| *W. ghanensis*_6706 | 5.48 | 5.64 ± 0.06 | 4.4 ± 0.1 | <90% | 6.30 | 6.88 ± 0.18 | 6.19 ± 0.24 | <90% | 6.18 ± 0.03 | <90% | ND | ND | ND | ND |

**Notes.**

*ND*, not determined. Mean values (± standard deviation, *n* = 3).

## Table 3(on next page)

Inhibition halo of *Salmonella enterica* and *Listeria monocytogenes* grown on culture media pre-inoculated with different lactic acid bacteria (LAB) isolated from pineapple silage.

+ Inhibition zone between 0- and 3-mm diameter (weak), ++ Inhibition zone between 3- and 6-mm diameter (good), +++ Inhibition zone larger than 6 mm diameter (strong).

**Table 3** **Inhibition halo of *Salmonella enterica* and *Listeria monocytogenes* grown on culture media pre-inoculated with different LAB strains isolated from pineapple silage.**

| Strain | Halo | |
|---|---|---|
| | ***Salmonella*** | ***Listeria*** |
| *L. paracasei*_6709 | ++ | + |
| *L. paracasei*_6710 | ++ | ++ |
| *L. paracasei*_6711 | ++ | + |
| *L. paracasei*_6712 | +++ | ++ |
| *L. paracasei*_6713 | ++ | ++ |
| *L. paracasei*_6714 | +++ | +++ |
| *L. paracasei*_6715 | + | + |
| *L. fermentum*_6702 | ++ | + |
| *L. fermentum*_6704 | + | + |
| *L. parafarraginis*_6717 | ++ | ++ |
| *L. parafarraginis*_6719 | ++ | + |
| *W. ghanensis*_6706 | +++ | ++ |
| *L. paracasei* ATCC 393 | + | + |

**Notes.**

+ Inhibition zone between 0- and 3-mm diameter (weak), ++ Inhibition zone between 3- and 6-mm diameter (good), +++ Inhibition zone larger than 6-mm diameter (strong).

## Table 4(on next page)

Absorbance values obtained to evaluate the antimicrobial activity of the supernatant of *L. paracasei*_6714 against *Salmonella* and *L. monocytogenes.*

Mean values (± standard deviation, $n = 3$). Values not sharing a common letter represent significantly different values ($P < 0.05$).

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**Table 4** **Absorbance values obtained to evaluate the antimicrobial activity of the supernatant of *L. paracasei*_6714 against *Salmonella* and *L. monocytogenes*.**

| Supernatant volume (µL) | Absorbance at 620 nm | |
|---|---|---|
| | ***Salmonella*** | ***L. monocytogenes*** |
| 50 | $0.062 \pm 0.007^{cd}$ | $0.043 \pm 0.05^{bc}$ |
| 45 | $0.09 \pm 0.04^{cd}$ | $0.13 \pm 0.02^{a}$ |
| 40 | $0.055 \pm 0.008^{d}$ | $0.128 \pm 0.004^{a}$ |
| 35 | $0.08 \pm 0.03c^{d}$ | $0.14 \pm 0.01^{a}$ |
| 30 | $0.15 \pm 0.06^{bcd}$ | $0.11\pm 0.05^{ab}$ |
| 25 | $0.16 \pm 0.03^{bcd}$ | $0.113 \pm 0.004^{ab}$ |
| 20 | $0.19 \pm 0.03^{bc}$ | $0.129 \pm 0.003^{a}$ |
| 15 | $0.24 \pm 0.01^{ab}$ | $0.13 \pm 0.01^{a}$ |
| Positive control | $0.34 \pm 0.08^{a}$ | $0.151 \pm 0.007^{a}$ |

2 **Note.**

3 Mean values (± standard deviation, $n = 3$). Values not sharing a common letter represent
4 significantly different values ($P < 0.05$).

## Table 5(on next page)

Antibiotic resistance/susceptibility of *L. paracasei*_6714.

Mean values (± standard deviation, $n = 3$). *R*, resistant. *I*, intermediate.

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**Table 5 Antibiotic resistance/suceptibility of *L. paracasei*_6714.**

| Antibiotic | Halo (inhibition zone) | Interpretation |
|---|---|---|
| Ciprofloxacin | 5.3 (± 0,6) | R |
| Vancomycin | 0.0 (± 0) | R |
| Penicillin | 11.0 (± 1.0) | R |
| Amoxycilin with clavulanic acid | 15.0 (± 0,5) | I |
| Eritromycin | 15.2 (± 0,3) | I |
| Amikacin | 6.0 (± 0) | R |
| Streptomycin | 3.7 (± 0,6) | R |
| Tetracycline | 8.8 (± 1) | R |
| Chloramphenicol | 10.3 (± 0,6) | R |

2 **Notes.**

3 Mean values (± standard deviation, *n* = 3). *R,* resistant. *I*, intermediate.

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## Table 6(on next page)

Adhesion of *L. paracasei*_6714 to HeLa cells per microscopic field.

**Table 6** **Adhesion of *L. paracasei*_6714 to HeLa cells per microscopic field.**

| Strain | LAB adherence to epithelial cells |
|---|---|
| *L. paracasei*_6714 | 403 ± 18 |
| *L. fermentum*_6702 | 164 ± 16 |

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## Table 7 (on next page)

Antagonistic effects of *L. paracasei*_6714 on *Salmonella* Typhimurium invasion of HeLa cells.

Mean values (± standard deviation, $n = 3$). Values not sharing a common letter represent significantly different values ($P < 0.05$). [a]Post-inoculation time with *Salmonella* Typhimurium.

**Table 7 Antagonistic effects of *L. paracasei*_6714 on *Salmonella* Typhimurium invasion of HeLa cells.**

| Assays | Log CFU /mL *Salmonella* | Cell HeLA adhesion (%) |
|---|---|---|
| Treatment | 5,3 ± 0,1$^{B}$ | 65 ± 1$^{B}$ |
| Protection (3 h)[a] | 5,4 ± 0,2 $^{B}$ | 66 ± 2$^{B}$ |
| Protection (24 h)[a] | 4,6 ± 0,1 $^{C}$ | 56 ± 1$^{C}$ |
| Control | 6,2 ± 0,1 $^{A}$ | 76 ± 2$^{A}$ |

**Notes.**

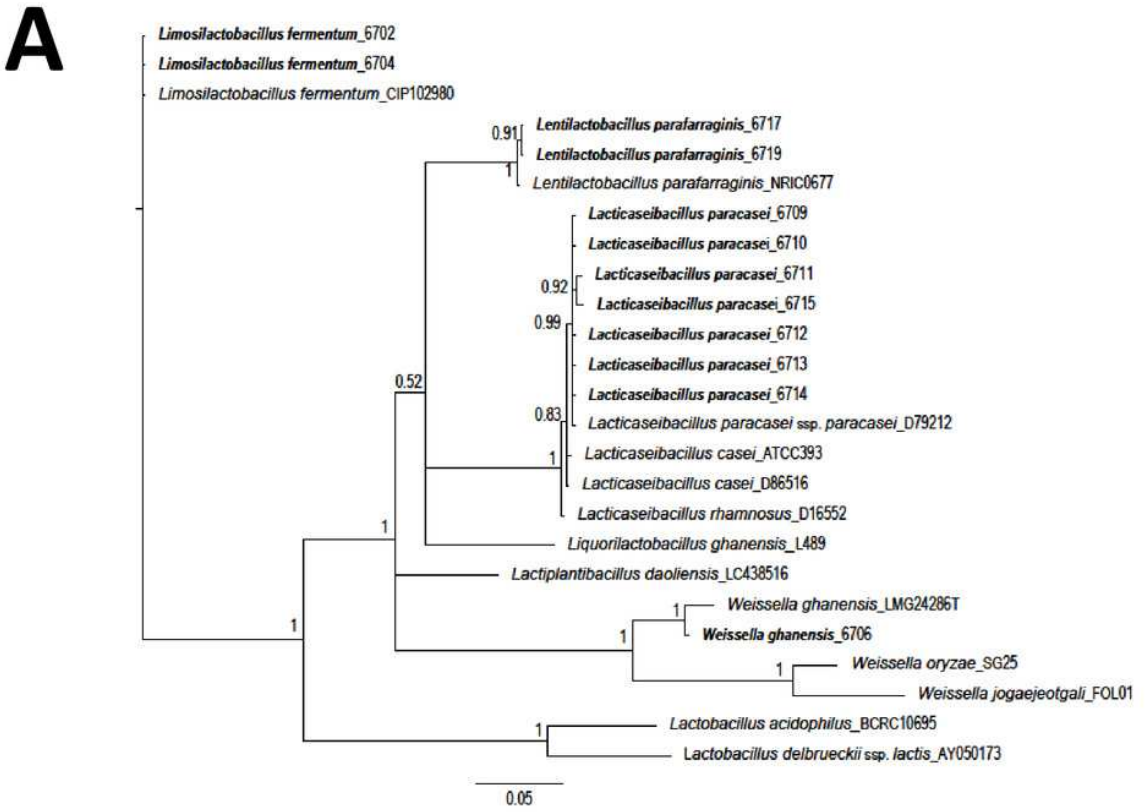
Mean values (± standard deviation, *n* = 3). Values not sharing a common letter represent significantly different values ($P < 0.05$).

[a]Post-inoculation time with *Salmonella* Typhimurium.

# Figure 1

Phylogeny based on Bayesian analysis and considering the partial sequences of the 16S rRNA gene (1299 nucleotides (nt)) (A) and phenylalanyl-tRNA synthase gene (*phe*S) (420 nt) (B) of lactic acid bacteria (LAB) isolated from ensiled pineapple peels.

Probabilities are indicated at nodes. As an external group. *L. delbrueckeii* subsp. *lactis* KTCT 3034 was used as an external sequence for both figures. Sequences obtained on this research are shown in bold font.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Lacticaseibacillus paracasei_6713
Lacticaseibacillus paracasei_6714
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Lacticaseibacillus casei_D86516
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Lactiplantibacillus daoliensis_LC438516
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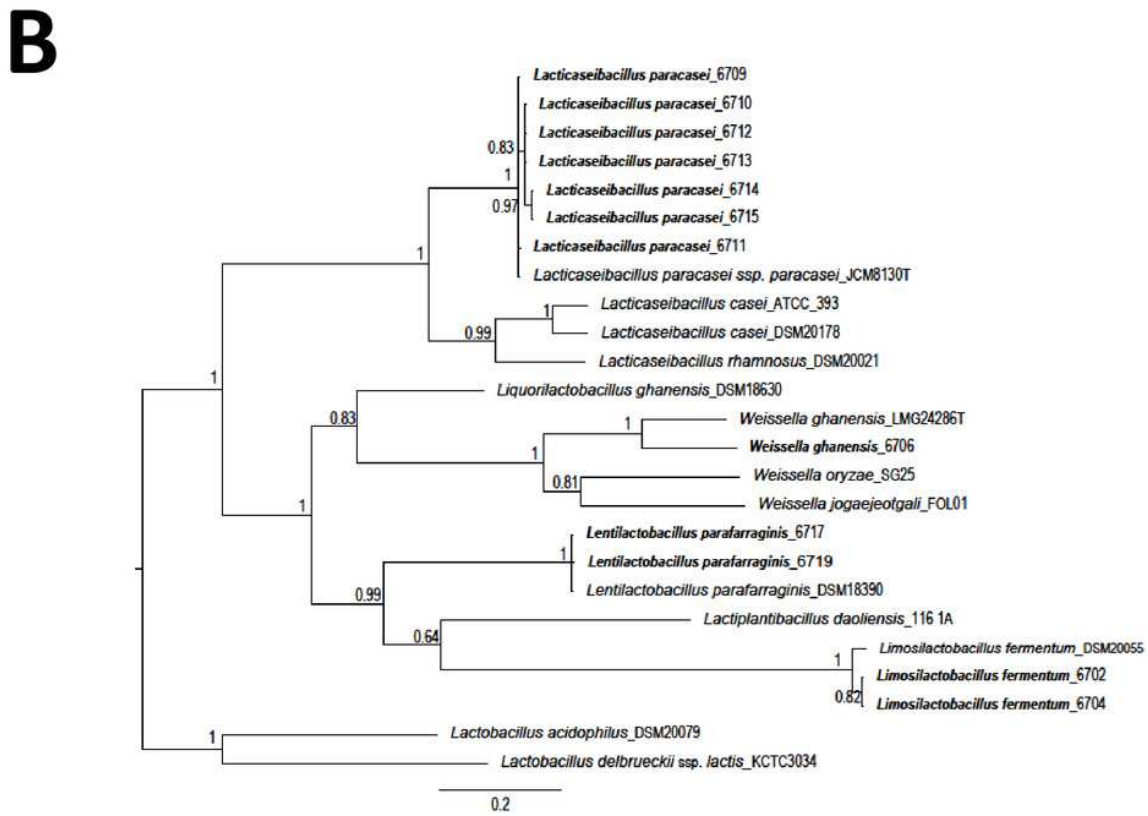
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Lacticaseibacillus paracasei_6711
Lacticaseibacillus paracasei ssp. paracasei_JCM8130T
Lacticaseibacillus casei_ATCC_393
Lacticaseibacillus casei_DSM20178
Lacticaseibacillus rhamnosus_DSM20021
Liquorilactobacillus ghanensis_DSM18630
Weissella ghanensis_LMG24286T
Weissella ghanensis_6706
Weissella oryzae_SG25
Weissella jogaejeotgali_FOL01
Lentilactobacillus parafarraginis_6717
Lentilactobacillus parafarraginis_6719
Lentilactobacillus parafarraginis_DSM18390
Lactiplantibacillus daoliensis_116 1A
Limosilactobacillus fermentum_DSM20055
Limosilactobacillus fermentum_6702
Limosilactobacillus fermentum_6704
Lactobacillus acidophilus_DSM20079
Lactobacillus delbrueckii ssp. lactis_KCTC3034
0.2

# Figure 2

Cellular auto-aggregation ability of selected lactic acid bacteria (LAB) isolated from pineapple waste and comparison with *L. casei* ATCC 393.

Data are reported as mean ± SD.