First characterization of the probiotic potential of Lactic aAcid Bbacteria isolated from Costa Rican pineapple silages

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批注 [HQ1]:

The present study described the isolation of several lactic acid bacteria (LAB) from Costa Rican pineapple silage and characterization of their probiotic potential. As a result, an LAB strain, *Lacticaseibacillus paracasei_6714*, was identified as a promising probiotic candidate. The work was well-designed and presented.

Major concerns:

1.The antimicrobial activity needs to be evaluated using more LAB strains of different species.
2.The safety of the LAB isolates needs to be tested.
3.*In vivo* experiments are suggested to evaluate the probiotic ability of the LAB.
4.Well-identified reference LAB strains need to be included as controls in all the assays.
5.The novelty and innovation of the study should be highlighted.
6.The manuscript should be revised by native English speakers.

Minor points:

1.The layout of the tables and figures should be optimized.
2.The references should be a consistent style.
3.Line 1, lactic acid bacteria
4.Line 33, species
5.Lines 144-145, A 1.5- kb fragment…..the primer pair 27F/1492R
6.Line 273, a 1-kb
7.Lines 278 and 295, HeLa
8.Lines 327-328, *L. paracasei*
9.Line 331, GenBank
10.Line 397, 0.3%

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Abstract

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

Methods. Twelve LAB strains were isolated from pineapple silages. The species identification was carried out considering 16S rRNA and *phe*S genes. Experiments to evaluate probiotic potential of the isolates included survival under simulated gastrointestinal environment, *in vitro* antagonistic activity (against *Salmonella* spp. and *Listeria monocytogenes*), 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 five of the isolates was 90% or higher after exposure to acidic conditions (pH: 2) and lysozyme, and the 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 strain 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 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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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 recognized as live microorganisms which, when administered in adequate amounts for a period of time, may confer health benefits on the host (Isolauri et al., 2002; Hossain et al., 2017). These microorganisms are capable of enduring gastrointestinal (GI) tract conditions, surviving host metabolic processes to 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. LAB are Gram-positive, catalase negative non-spore-forming cocci, bacilli or rods. The main energetic pathway of this group is based on the fermentation of carbohydrates, mainly glucose, to produce lactic acid alone (homofermentation) or lactic acid, CO_2 and ethanol (heterofermentation) (Ayala et al., 2019). In addition to their associated health benefits, many LAB are capable of producing 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 with a wide range of inhibitory effects or by competitive exclusion (Vieco-Saiz et al., 2019).

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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 mimic 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 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 strains 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. However, few studies have been performed in the Latin-American region in terms of screening and evaluation of new LAB strains 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 have evaluated strains obtained from environmental sources such as fruits (Veron

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et al., 2017) and the rain forest (Benavides, 2016). In terms of LAB isolation from agro-industrial by products, to the authors knowledge, just one study from Brazil has evaluated the characteristics of strains obtained from cocoa fermentation (Santos et al., 2016).

In the case of pineapple, it 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. These factors, along with characteristics common to other known probiotics, make these organisms important probiotic candidates (Ruiz-Rodríguez et al., 2019). Studies performed in fresh-cut pineapple (Russo et al., 2014; Amorim et al., 2018) confirmed the presence of LAB strains with probiotic potential. As in Costa Rica, pineapple production is one of the most important activities within the agro-industrial sector, it is possible that an important diversity of strains with biotechnological potential could be found both in the fresh fruit or the abundant waste material derived from the pineapple-derived industry that may be use for silages production.

The aim of this research was to assess the probiotic potential of autochthonous LAB isolated from Costa Rican pineapple peel silages. Selected LAB strains 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) antibiotic resistance, and d) 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 the tropics.

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Materials & Methods

Isolation of ~~b~~Bacterial ~~S~~strains

Lactic acid bacteria were isolated from pineapple peel samples that were vacuum-ensiled for 30 days. The samples were obtained from a Costa Rican company dedicated to pineapple juice

production. Twenty-five grams of each sample were 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 Faculty of Microbiology and in the Bacteriology Collection at the National Center for Food Science and Technology (CITA), University of Costa Rica.

DNA Extraction and PCR Amplification

Total nucleic acids were extracted from each isolate using a miniprep protocol (Birnboim & Doly, 1979). A ~1,500 kbbp fragment of the 16S rRNA gene was amplified using the primer pair 27F and 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).

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批注 [HQ2]: LAB are facultative anaerobes, if the conditions were optimal for bacterial isolation?

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Sequencing ~~a~~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 generation, eight chain, 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 3034 was considered for phylogenetic analysis of both genes. Sequences obtained on this research are shown in bold font.

Assays of Resistance to the Gastrointestinal Tract

Tolerance to pH 2.0. All isolates were exposed to pH 2.0 (Ramos et al., 2013), in order to evaluate tolerance to acidic conditions. Each strain was cultivated in MRS broth (Difco) at 35 ± 2 °C for 24 h and pH 7.0. The c~~C~~ells were centrifuged at 5,000 rpm for 5 min at 24 °C, washed two times in PW (Oxoid) and resuspended in PW (Oxoid) to a concentration of about 10^8 CFU/mL. A 1 mL aliquot of the final bacterial suspension was used to inoculate 50 mL of MRS broth (Difco) adjusted to pH 2.0 using 1 N HCl (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cultures were incubated at 35 ± 2 °C for 3 h. After 3 hours of incubation, the effect of acidity was neutralized with 1N NaOH (Thermo Fisher Scientific, Waltham, Massachusetts, USA). To quantify the final bacterial population, 1 mL aliquots obtained at time 0 and after 3 h incubation were serially diluted in PW (Oxoid), plated on MRS

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agar (Difco), and incubated in anaerobic jars for 72 h at 35 ± 2 °C. The assay was conducted in triplicate.

*Lysozyme resistance***.** Lysozyme resistance was evaluated using a modified version of the method described by Zago et al. (2011). One milliliter of LAB cells was cultured in MRS broth (Difco) at 30 ± 2 °C for 24 h. After incubation, an aliquot of the culture was centrifuged at 5,000 rpm for 5 min at 24 °C and washed twice in phosphate buffer (0.1 M, water pH 7.0). The bottom was resuspended in 2 mL of Ringer solution (8.5 g/L NaCl, 0.4 g/L KCl, 0.34 g/L hydrated $CaCl_2$) (Sigma Aldrich, St. Louis, MO, USA). A sterile electrolyte solution (SES) (0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$) containing 100 mg/L of lysozyme (Sigma Aldrich) was used to resuspend each LAB (10^8 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 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 ~~B~~bile s~~S~~alts***.** LAB tolerance to bile salts was evaluated with minor modifications (García-Ruiz et al., 2014). The isolates that showed a survival greater than 90% after exposure to pH 2 and lysozyme were selected. The strains 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 were 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.

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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 ~~a~~Assays

Antagonistic activity against pathogens. The antagonistic activity of all isolated LAB strains 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 ~~*Salmonella* 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 ~~T~~tryptic ~~S~~soy ~~B~~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 ~~B~~brain h~~H~~eart ~~i~~infusion a~~A~~gar (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 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 strain *L. paracasei*_6714, which showed inhibition zones with a diameter larger than 6 mm for both pathogens, was cultured in MRS

批注 [HQ3]: More strains of different species need to be evaluated.

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broth (Oxoid) at 35 ± 0.5 °C for 24 ± 2 h. The LAB cultures were centrifuged at 1,500 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 µL of sterile TSB (Oxoid), 50 µL of the indicator pathogen, and 50 µL of sterile MRS (Difco). Negative controls did not contain the pathogen, and the volume was adjusted with 50 µL of sterile PW (Oxoid). Microplates were incubated aerobically at 35.0 ± 0.5°C for 24 ± 2 h in high humidity conditions and the absorbance at 620 nm was measured in an Ultra Microplate Reader (Biotek instruments, Winooski, VT, USA). The results were adjusted by subtracting the absorbance value obtained for the negative control. All determinations were performed in triplicate. To analyze the inhibitory effect of the supernatant solutions on the two pathogens, two-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test were performed using JMP version 11 (SAS Institute Inc., USA). Differences were considered significant at a *P*-value of < 0.05.

Safety Assays

Antibiotic resistance. The antibiotic sensitivity of strain *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 strain was cultured in MRS broth

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(Oxoid) at 35 ± 0.5 °C for 24 ± 2h and the suspension of the test strain 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, Alemania, Country??). 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 1 kb MassRuler DNA ladder (Thermo Fisher Scientific).

Cell Culture Assays

Preparation of ~~C~~cell ~~M~~monolayer. The *in- vitro* adhesion of *L. paracasei*_6714 was assayed using HeLa~~A~~ cells (kindly supplied by the Research Center for Tropical Diseases (CIET), 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 ~~of~~ glutamine per mL, 50 U ~~of~~ 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 ~~P~~phosphate- ~~B~~buffered ~~S~~solution (PBS) (Sigma-Aldrich, San Luis, Missouri, USA). Cells were then covered with a solution of 2.5 mL of trypsin and EDTA

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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 strain determined in preliminary assays not included here) was included for comparison.

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

Treatment assay. A modified version of a previous methodology was used (Giannella et al., 1973). *Salmonella* serovar Thypimurium was grown on TSB (Oxoid) at 35 ± 0.5 °C for 24 ± 2 h and diluted in antibiotic-free EMEM to obtain a concentration of about 10^7 CFU/mL. *L. paracasei*_6714 was grown in MRS (Oxoid) incubated under the same conditions and then diluted as described for *Salmonella*. A volume of 1 mL of each culture suspension was added to

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each cell monolayer inside the 12-well microplate. Plates were centrifuged at 1,600 rpm for 5 min and then incubated for 0, 3 and 24 h under the same conditions described for cell maintenance. After incubation, wells were washed two times with PBS and then kept for 1 h in fresh EMEM (Thermo Fisher Scientific) medium containing 100 µg/mL of gentamicin. After gentamicin exposure, each well was washed twice with PBS (Sigma-Aldrich) and cells were then lysed with ultrapure water for 10 min. Appropriate dilutions in PW (Oxoid) were spread onto TSA (Oxoid) and ~~X~~xylose ~~L~~lysin d~~D~~eoxycholate ~~A~~agar (XLD) (Oxoid). The p~~P~~lates were incubated at 35 ± 0.5 °C overnight. Bacterial counts were used to calculate the invasion rate. A positive control of *Salmonella* was included. Experiments were performed in triplicate.

Protection a~~A~~ssay. The protocol described for the treatment assay was modified to include pre-exposure of each cell monolayer to *L. paracasei*_6714 for 3 and 24 h before infection with *Salmonella*.

Results

A total of twelve different LAB morphotypes were isolated from pineapple silages. Considering the 16S rRNA sequence and *phe*S gen the isolates correspond to ~~*Lacticaseibacillus* L.~~ *paracasei* (seven strains), *Lentilactobacillus parafarraginis* (two strains), *Limosilactobacillus fermentum* (two strains), and ~~*Weissella* W.~~ *ghanensis* (one strain) (Table 1~~,~~ and Table S1). When the sequences obtained in this research and those selected from GenB~~b~~ank (www.genbank.com) were considered, a clear cluster was established. Equivalent length portions of both genes were used to resolve the species groups obtained. The species were renamed according to novel classification of Zheng et al. (2020).

After exposure to acidic conditions (pH 2.0) all LAB strains were viable, but at least seven showed a population reduction above 90 %. No reduction was observed in the population of the control samples (pH 6.0) as expected (data not shown). Higher rate of survival was observed for

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*L. parafarraginis*_6719, *L. paracasei* (isolates: 6710 and 6715), and *L. fermentum* (isolates: 6702 and 6704). On the other hand, the survival to lysozyme ranged between 77.5 and 100-%, with similar results after 120 min of exposure. Five of the LAB isolates with 90-% or higher survival after exposure to lysozyme also showed high resistance to acidic conditions, according to the established criteria. These strains were selected and further evaluated for resistance to bile salt and survival was higher than 61-% for all strains subjected to a medium containing 0.3-% bile salts. Survival data for all strains exposed to simulated GI conditions are summarized in Table 2.

The antagonistic activity of the twelve isolates from this study against selected pathogens is shown in Table 3 and Figure S1. Three strains produced strong inhibition zones against *Salmonella*. Nevertheless, when the strains were evaluated against *L. monocytogenes*, only one strain (*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 antibiotic susceptibility of *L. paracasei*_6714 is shown in Table 5. The strain 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 strain isolated was not found to harbor plasmids, which indicates a low probability of transferring the antibiotic resistance feature (data not shown).

~~Results~~ 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 strain to prevent pathogen invasion is shown in Table 7. In the treatment assay, adhesion of the pathogen was reduced by

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approximately 11-%. On the other hand, in the protection assay, pathogen reduction was between 10-% and 20-%.

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Discussion

Lactobacilli were the most common group found in this research. Thise results is are similar to other reports of LAB isolated from fermented products (Sáez et al., 2018), particularly from pineapple and pineapple waste (Mardalena & Erina, 2016; Arshad et al., 2018). This finding is not surprising due to the exceptional genetic diversity of the *Lactobacillus* genus, which has recently divided in 23 novel genera (De Bruyne et al., 2010; Dicagno et al., 2010; Zheng et al., 2020). On the other hand, many *Weissella* isolates have been obtained from fermentation processes and characterized as heterofermentative bacteria. In fact, *W. ghanensis* was first isolated from cacao fermentation (De Bruyne et al., 2010).

Isolates were further characterized for their probiotic potential to provide favorable effects on the human gut (Pan et al., 2009). Probiotic evaluation of novel strains must include tolerance to the GI tract, antimicrobial activity, susceptibility to antibiotics and adhesion to mammalian cells (Byakika et al., 2019). The group of tests for GI tolerance are aimed to evaluate whether the strains are able to survive exposure to acid and enzymes and eventually the transit through the stomach and intestines (Ramos et al., 2013; García-Ruiz et al., 2014; Hernández-Alcántara, 2018). In this study, a high tolerance to low pH was observed for all the strains (Tripathi and & Giri, 2014), with the exception of *L. paracasei*_6709 which showed the lowest survival response. It is important to point out the need to evaluate hundreds of strains to select those that can survive acidic environments (Ramos et al., 2013). It is hypothesized that high tolerance to acidic conditions observed in this study may be related with the ensilage process, in which the LAB that survive the last stages were subjected to acidic pH for a prolonged period of time (Muraro et al., 2021). Besides, these results indicate that some of the isolates may be able to survive the

normal gastric environment. It is worth noting that the average pH during human digestion is around 2.0 - 3.0 with gradients from 1.8 to 4.0 during 2 to 3 h periods (Maragkoudakis et al., 2006). Also, the high survival of LAB to lysozyme exposure in this study was similar to the results previously reported (García-Ruiz et al., 2014) where survival greater than 80-% were observed for strains of *L. pentosaceus, L. casei* and *L. plantarum* after incubation for 120 min; however, survival was around 50-% for some strains. 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, five strains selected in this study (after pH and lysozyme tests) were classified as bile-resistant. Similar results have been reported for *Bifidobacterium*, other *Lactobacillus* strains, *Pediococcus pentosaceus* and some yeasts (Delgado et al., 2008; Jensen et al., 2012; Turchi et al., 2013; García-Ruiz et al., 2014). To obtain an 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

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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 are able to 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 are able to inhibit both *Salmonella* and *L. monocytogenes* and it may be able to inhibit pathogens during *in vivo* applications.

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 an 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 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 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).

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Conclusions

To our knowledge, this is the first study analyzing bacteria with potential probiotic features from Costa Rican sources and one of few studies obtaining LAB with biotechnological potential from agro-industrial waste in Latin America. The r~~R~~esults from this study confirm that agro-industrial byproducts, specifically silages, may be an important source of promising LAB strains with potential probiotic 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. Although this strain survived simulated GI conditions, additional studies, including encapsulation, could improve survival in the GI environment. This strain 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 strain in different food matrices and production of exopolysaccharides.

Acknowledgements

This work was supported by the technical assistance of Henry Castro, Arturo Pacheco and María Fernanda Miranda at the Food Microbiology Laboratory and Molecular Biology Laboratory from UCR. Also, the authors acknowledge M.Sc. Marlen Cordero Serrano from CIET for her technical support during the cell culture assays.

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taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology* **70**:2782–2858 DOI 10.1099/ijsem.0.004107.

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 LAB isolated from pineapple silage. *ND*, not determined. Mean values (± standard deviation, *n* = 3).

Table 3 Inhibition halos of *Salmonella enterica* and *Listeria monocytogenes* grown on culture media pre-inoculated with different LAB strains 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 4 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).

Table 5 Antibiotic resistance/susceptibility of *L. paracasei*_6714. 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. Mean values (± standard deviation, *n* = 3). Values not sharing a common letter represent significantly different values (*P* < 0.05). Notes. [a]Post-inoculation time with *Salmonella* Typhimurium.

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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 (*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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Table S1 GenBank accession numbers of 16S rRNA gene and phenylalanyl-tRNA synthase gene (*phe*S) sequences from lactic acid bacteria (LAB) isolated from pineapple peel silage.

Figure S1 Picture of plaques and the observed inhibition halos of *L. paracasei*_6712 and *L. paracasei*_6714 against *L. monocytogenes* (a, b) and *Salmonella* sp. (c, d).

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