Survival of selected lactic acid bacteria isolated from pineapple waste in refrigerated pineapple juices

Running title: Lactic acid bacteria survival in pineapple juices

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27 **ABSTRACT**

28 The aim of this research was to isolate and identify lactic acid bacteria (LAB) from
29 pineapple waste. The survival in refrigerated pineapple juices, of a selected isolate with potential
30 probiotic properties, was also studied. The 16S rRNA and *pheS* partial genes were used to identify
31 LAB, multilocus sequence typing (MLST) genes were used in order to separate strains grouping
32 with *Lactobacillus casei* and *L. paracasei* phylogenetically. Tests for survival at pH 2.0, resistance
33 to lysozyme and tolerance to bile salts were used to screen the strains for potential probiotic
34 properties. A *L. fermentum* isolate was used for the survival study. Three types of pineapple juice
35 made from pulp, a blend of pulp and peel (80:20), and peel extract were inoculated to
36 approximately 10⁶ CFU/mL with *L. fermentum* and stored at 4 °C for up to six weeks. The
37 physicochemical composition of juices, including concentration of fermentable sugars and organic
38 acids, total solids content, soluble solids content, titratable acidity and pH, was determined during
39 the survival study. Two genera and five species were identified. Pineapple juices supported the
40 survival of *L. fermentum* during refrigerated storage but the population of the bacteria decreased
41 over time regardless of the juice type. Juice made from pulp was a more suitable vehicle for the
42 survival of the selected LAB. Some of the juice physicochemical properties, including sugars and
43 organic acids content, pH and titratable acidity, varied significantly (P<0.05) during storage.
44 Further sensory studies are necessary to evaluate consumer acceptance of juices containing the
45 selected isolate.

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53 **IMPORTANCE**

54 Lactic acid bacteria (LAB), isolated from pineapple waste, were phylogenetically analyzed
 55 and characterized in regards to their tolerance to pH 2.0, lysozyme and bile salts; showing their
 56 potential as probiotic strains, if health benefits associated to their ingestion are eventually
 57 confirmed. Moreover, pineapple juice supported the survival of *Lactobacillus fermentum*, isolated
 58 from the same food matrix, during refrigerated storage at 4 °C. Among the three pineapple juices
 59 tested (pulp, pulp + peel and peel), *L. fermentum* survived better in juice made from pulp. However,
 60 significant variations were observed overtime in some of the physicochemical properties of the
 61 juices including sugars and organic acids content, pH and total titratable acidity.

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63 **KEYWORDS** Pineapple juice, cell survival, *Lactobacillus fermentum*, probiotics, molecular
 64 identification, storage

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INTRODUCTION

Probiotics are live microorganisms which, when administered in adequate amounts, confer health benefits on the host (1). They have been traditionally used for the development of functional dairy products such as fermented milk and yogurt (2). Beneficial effects associated with the ingestion of these microorganisms include maintenance of a healthy gut microbiota, improvement of the digestibility of foods, alleviation of lactose intolerance, prevention of diarrhea, control of blood cholesterol levels and hypertension, stimulation of the immune system and prevention of atopic allergies (3-5). However, consumption of milk-based beverages may be limited for certain consumers due to allergies, cholesterol diseases, dyslipidemia, and vegetarianism (6). Alternative and innovative food matrices, including fruit juices (7), are currently being investigated as substrates for novel non-dairy functional foods. Fruit juices are of particular interest as new carriers of probiotic lactic acid bacteria (LAB) due to their high concentrations of nutrients, particularly sugars, which may encourage probiotic growth. Moreover, juices do not contain starter cultures that compete with probiotics for nutrients during storage (8). The minimum concentration of live probiotic bacteria at the expiration date of a beverage should be around 7 logarithms of CFU/ml (4). Therefore, assessment of the survival of a potential probiotic strain in a selected beverage is critical in the development of new functional fruit-based drinks, even when previous studies have shown the potential of the studied microorganism as a probiotic in other food products.

Pineapple (*Ananas comosus*) ranks third in world tropical fruit production, preceded by banana and citrus (9). Costa Rica is one of the world's largest producers of the cultivar 'MD-2' (10) and it is among the top five pineapple-producing countries (Thailand, Brazil, Philippines, Costa Rica and India, in descending order) (<http://www.fao.org/statistics/>). In Costa Rica, in addition to traditional pineapple juice made from pulp, innovative beverages are produced from pineapple peels and blends of fruit peel and pulp, products that in other latitudes may be considered an agro-industrial waste. Approximately 30 % of pineapple is turned into waste during a canning operation. Pyar et al. (11) investigated the potential of pineapple waste as growth medium for *Lactobacillus*

species and found that bacterial growth on pineapple-waste culture medium was comparable to growth on Man Rogosa and Sharpe (MRS) agar. Thus, it might be expected that LAB isolated from pineapple waste may show an enhanced survival response in pineapple-derived beverages, and therefore the relevance of its isolation and identification is of particular interest. Moreover, limited information is available regarding the factors influencing probiotic survival in fruit juices (12, 13). Studying the effect of species/strain, juice composition and storage temperature and time on the survival of selected probiotic bacteria will enhance the existing knowledge related to this topic (4).

The aim of this research was to identify potential LAB strains isolated from pineapple waste and to study the survival of a selected strain in three different pineapple juices made from pulp, peels and a blend of both. The effect of juice composition on survival of the selected LAB and the changes in juice pH, total titratable acidity, soluble and total solids content, sugars content (glucose, fructose and sucrose) and organic acid concentration (citric, lactic and malic acid) during refrigerated storage of the beverages was also investigated.

RESULTS AND DISCUSSION

Bacterial strains: isolation, characterization and molecular identification. Twelve LAB morphotypes were isolated from pineapple waste, 16S rARN and *pheS* genes were amplified and sequenced (Table 1, Table S1). Two genera were identified: *Lactobacillus* spp. (11 strains) and *Weissella* spp. (one strain). The first one was the most common finding which is consistent with other reports of bacteria isolated from fruits and vegetables where *Lactobacillus* is characterized by its exceptional size and genetic diversity (14, 15), which have hindered its taxonomy (16). A clear cluster was observed comprising the costarican sequences and Genbank's selected sequences of the 16S rRNA region and *pheS* gene from bacteria (www.ncbi.com) (Fig 1). Five different species were identified: *L. parafarraginis* (2), *L. fermentum* (2) and *W. ghanensis* (1); additionally, seven isolates were grouped with *L. casei* and *L. paracasei*. However, it was not possible to separate both species considering partial genome sequences of the 16s rRNA and *pheS* genes (Fig. 1).

LAB strains have been isolated and characterized from different substrates in recent years because they potentially have probiotic properties and play an important role in the production of fermented food and beverages (17, 18). García et al. (19) for example, used the probiotic strain *L. fermentum* UCO-979C due to its potent antibacterial activity anti-*Helicobacter pylori*. Likewise, De Souza et al. (20) isolated *L. fermentum* and *L. casei* from mozzarella cheese in order to select novel and safe strains for the future development of functional fermented products; and Le and Yan (21) used probiotic strains of *Weissella* spp. to improve antioxidant activity during fermentation.

Equivalent length portions of the genes *nrdD*, *pgm*, *pheS*, *polA*, *mutL*, *rpoA* and *metRS* were analyzed and submitted to GenBank (Table S1) to resolve the species groups containing the isolated sequences of *L. casei* and *L. paracasei*. When compared to available sequences, a close relationship was found between our strains of *L. casei* and *L. paracasei*. Figure 2 depicts two groups of *L. casei* and *L. paracasei* strains, one containing *L. paracasei* (6710, 6711, 6712, 6714), *L. paracasei* L9 and *L. paracasei* ATCC 334 and the other comprising strains of *L. casei* and *L. paracasei* with no clear separation. It is important to mention that the taxonomic status and molecular identification of these species is still under debate (22 – 26) and is out of the scope of this paper, therefore, strains herein were named according to their closest match and phylogenetic position.

Tolerance to pH 2.0, lysozyme and bile salts. The tolerance to a pH of 2.0 of the 12 LAB isolates was evaluated. This condition simulates the phase of gastric emptying and is a strongly discriminative pH for the selection of high-acid tolerant strains (3, 27). All strains were viable after 3 h exposure to pH 2.0. However, populations were reduced (Table 2). As expected, the control samples (pH 6.0) did not show a log reduction during the time tested (data not shown). Survival was highest for *L. parafarragines* (6719) followed by *paracasei* (6710), *L. casei* (6715), and *L. fermentum* (isolates: 6702 and 6704). Overall, survival rates were greater than 80 % for most of the isolates, with the exception *L. casei* (6709) and *W. ghanensis* (6706). The high tolerance to a low pH shown by *Lactobacillus* genus was in concordance with previous reports (28), with exception

169 of the isolate of *L. casei* (6709) which showed the lowest survival response. The tolerance in acid
170 medium is one of the most important traits that could have the probiotic bacteria's (29), because
171 before they reach the gastrointestinal tract they must survive in the stomach. The average of pH
172 during human digestion is around 3.0 to 2.0 with gradients from 4.0 to 1.8 during 2 h to 3 h period
173 (30).

174 Survival after exposure to lysozyme for 30 min ranged from 77.5 to 100 % (Table 2).
175 Similar values were found after 120 min of exposure. All LAB strains were also grown in medium
176 without lysozyme and used as a control. These results confirmed the high resistance of most LAB
177 isolates to lysozyme under conditions that simulate the *in vivo* dilution by saliva that occurs after
178 juice ingestion. Bacterial resistance to lysozyme has been attributed to the peptidoglycan in the cell
179 wall, the physiological state of the cell and the lysozyme in the medium (31). Similar results were
180 found by García-Ruiz et al. (3), using LAB strains of *L. pentosaceus*, *L. casei* and *L. plantarum*, the
181 authors found survival percentages > 80 % after 120 min of incubation, with exception of some
182 strains with survival percentages around 50 %.

183 Five LAB isolates, with 90 % or higher survival at pH 2.0 and resistance to lysozyme, were
184 selected and evaluated for resistance to bile salts. According to Dunne et al. (32), human bile
185 concentrations range among 0.3 % to 0.5 %. For this reason, all the strains were evaluated on a
186 medium with 0.25 % of bile showing survival values higher than 61 % for all strains. *L. casei*
187 (6715) and both *L. fermentum* isolates showed higher values. Results are summarized in Table 2.
188 The ability to survive in the presence of bile is an important characteristic of potential probiotic
189 strains (3, 29). Mathara et al. (33) established bile tolerance limits for the selection of probiotic
190 strains. Tolerance to 0.3 % bile salts with survival above 50 % was considered good. Our results
191 were similar to ranges previously reported for *Bifidobacterium*, *Lactobacillus* strains, *P.*
192 *pentosaceus* and certain yeasts (3, 34 - 37). High bile tolerance is desirable since its benefits
193 colonization in the host gastrointestinal tract (38). In concordance with previously reports, our
194 results showed that bile survivor was not related to LAB species but it was strain-specific (27, 39).

Based on results of the three previous essays, *L. fermentum* (6702) was selected for survival studies in pineapple juices due to its enhanced survival response. *Lactobacillus* strains are usually highly resistant to acids (40), and therefore they are commonly used as probiotics. *Lactobacillus fermentum*, *L. casei* and *L. paracasei* have been reported as probiotic species. Similarly, *Weissellea* spp. have been identified as probiotics but it has also been associated with undesirable food changes and fruit deterioration (15).

Physicochemical characterization of pineapple juices during storage.

The effect of storage time on the physicochemical characteristics of the inoculated juice was significant ($P < 0.05$) for all variables except juice total solids content. These differences were significantly dependent ($P < 0.05$) on the initial *L. fermentum* population of the inoculum. Means, coefficients of determination (R^2) and probabilities are listed in Table 3. The effect of juice type on total titratable acidity was significant ($P < 0.0001$). Differences in total titratable acidity of pulp juice and pulp + peel juice were not significant ($P > 0.05$); however, the titratable acidity of both juices was significantly different ($P < 0.05$) from that of juice extracted from pineapple peels.

For the case of the soluble solids content ($^{\circ}$ Brix), a significant interaction between storage time and type of juice was found ($P = 0.006$). After six weeks of storage, the soluble solids content of pulp and peel juices was lower than the initial Brix level. This result can be explained by the presence of the metabolically active *L. fermentum* strain inoculated into the samples.

The interaction between the effects of juice type and storage time on fermentable sugars was also significant, specifically, fructose ($P < 0.0001$), glucose ($P = 0.004$) and sucrose ($P < 0.0001$). Initial concentrations of the different sugars were consistent with previously reported data for pineapple juice; sucrose was the predominant sugar, followed by relatively equal fractions of fructose and glucose (41, 42). As observed in Table 3, a general downward trend in the sugar content after six weeks of storage was found. Initial and final fructose contents were significantly different ($P < 0.05$). This difference was greater in juices made from pulp and pulp + peel. In the case

221 of peel juice, no significant differences in fructose content were observed over time ($P>0.05$), but
222 the glucose content differed significantly ($P<0.05$) after storage, possibly because of glucose
223 metabolization by *L. fermentum*. For the case of sucrose, significant differences between the juices
224 made from peel and pulp were observed.

225 The organic acids profile showed variations for the three acids analyzed (citric, malic and
226 lactic). In all cases, significant interactions between juice type and storage time were found; citric
227 ($P = 0.0496$), malic ($P<0.0001$) and lactic ($P<0.0001$). The three juices did not present significant
228 differences of citric acid at week zero. At week six, the peel juice showed significant differences
229 ($P<0,05$) when compared with juice made from pulp and pulp + peel. Citric and malic acid at week
230 6 were slightly reduced in comparison to the initial content. Lactic acid was detectable at week 6 in
231 all samples. The reduction in citric acid content may be due to metabolization by the *L. fermentum*
232 added to the juice (43). As with other members of the genus *Lactobacillus* spp., *L. fermentum* can
233 metabolize malic acid through the formation of oxaloacetate and by the malolactic enzyme (44). As
234 expected, an absence of lactic acid at week zero in all the juices was observed (41). Thus, the
235 appearance of lactic acid at week six in all samples shows clear evidence of the metabolic activity
236 of *L. fermentum* (45). Although the amounts produced were small, they were within the range
237 previously reported (4) when the survival of *L. plantarum* in pineapple juice during refrigerated
238 storage for up to six weeks was studied.

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240 ***Lactobacillus fermentum* survival in pineapple juices.** Survival curves for *L. fermentum*
241 (storage at 4 °C) in the different pineapple juices and the evolution of juice pH during the
242 experiment are shown in Fig. 3. Total solids and soluble solids, total titratable acidity and sugar and
243 organic acid concentrations at the beginning and the end of the survival experiment are summarized
244 in Table 3. The interaction between the nominal factors, juice type and storage time, was significant
245 ($P<0.0008$). Thus, the effect of juice type on the survival of the bacteria was dependent on the
246 storage time, and vice versa. A reduction in the initial populations of *L. fermentum* in the three

247 pineapple juices was observed during storage (Fig. 3A). This effect was less pronounced in juice
 248 made from fruit pulp, even though this beverage had a lower pH than those containing peels (Fig.
 249 3B). For juices containing peels, the cell concentration was lower than 10^7 CFU/ml after one week
 250 of storage, in contrast to pulp juice in which the cell concentration decreased below 7 logs during
 251 the second week of refrigerated storage. Bacterial counts in juices containing pineapple peel
 252 extracts did not differ significantly at week six ($P>0.05$). Worth noting, the main metabolic
 253 substrates for lactic acid bacteria are fermentable sugars (fructose, glucose and sucrose); other
 254 carbon compounds such as organic acids can be used as secondary substrates (46, 47). This suggests
 255 that survival of *L. fermentum* is limited by the content of these carbon sources in the food matrix
 256 into which it is inoculated. Pulp juice showed the major content of fermentable sugar which is
 257 coherent with the highest bacteria survival. Pulp + peel juice and peel juice showed the lowest
 258 fermentable sugars content, respectively. This results matched with a lower bacterial count and
 259 suggest the importance of sugar contain on bacterial survival. Therefore, the addition of fermentable
 260 sugars to the juice may be an option for reducing the inactivation rate of *L. fermentum* in pineapple-
 261 derived beverages. Furthermore, juice pH decreased slightly during the first two weeks before
 262 stabilizing. According to Ramos et al. (48), the presence of active and viable *L. fermentum* implies
 263 that its natural metabolic cycle is being completed. This leads to an increased production of organic
 264 acids, mainly lactic acid, causing the pH to decrease. Moreover, juice pH showed no further
 265 decrease after the second week of storage, which may be the result of the reduction of the bacteria
 266 in the juice over time (Fig. 3).

267 Tests for tolerance to pH 2.0, resistance to lysozyme and tolerance to bile salts performed
 268 showed that lactic acid bacteria isolated from pineapple waste, specifically *L. fermentum* have
 269 potential as probiotic strains, if health benefits to the host are confirmed in further studies.
 270 Moreover, pineapple juice supported the survival of *Lactobacillus fermentum* during refrigerated
 271 storage. Among the three pineapple juices tested (pulp, pulp + peel and peel), *L. fermentum*
 272 survived better in juice made from pulp. However, regardless of the juice used, significant

273 variations were found for some of the physicochemical properties of the juice, such as sugars and
274 organic acids content, pH and titratable acidity. Further sensory studies are necessary to confirm the
275 palatability of juices containing the selected isolate. Likewise, for a commercial application of this
276 isolate, the study should be reproduced using frozen or freeze-dried cultures instead of the fresh
277 cells used in our study.

278 MATERIALS AND METHODS

279 **Isolation of bacterial strains.** Twelve bacterial isolates obtained from pineapple waste from a
280 local pineapple juice manufacturing company (San Jose, Costa Rica) were tentatively identified as
281 LAB. Isolates were cultured on MRS agar (DIFCO, France) incubated at 35 ± 2 °C for 24 h in
282 anaerobic conditions and subjected to Gram staining for morphology and preliminary identification.
283 LAB cultures were maintained in 20 % (v/v) glycerol (a cryo-preserved that serves as a carrier
284 for the microorganisms) and stored at -80 °C until used.

286 **DNA extraction and PCR amplification.** DNA of LAB isolates was extracted using a
287 miniprep protocol described by Birnboim and Doly (49). Part of 16S rRNA gene (~1500 bp) using
288 the primers 27F (50) and 1492R (51) was amplified and sequenced. The reaction was performed in
289 a final volume of 25 µl and a final concentration of 1 X reaction buffer, 1.5 mM MgCl₂, 0.2 µM of
290 each primer, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Bio-Rad, USA) and 50 ng of DNA. The
291 PCR program consisted of an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of
292 40 s at 94 °C, 55 °C for 1 min and 72 °C for 1 min. The final extension step was 5 min at 72 °C.

293 In order to confirm previously results, phenylalanyl-tRNA synthase (*pheS*) gene was
294 sequenced for all the LAB isolates. The reaction was performed using primer combinations pheS-
295 21-F/pheS-22-R (52), the PCR product was amplified using iProof High-Fidelity DNA polymerase
296 (Bio-Rad, USA). Initial denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 30 s, 60
297 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C for 10 min; and holding at 4 °C. A 25 µl
298 reaction was prepared according to iProof High-Fidelity DNA polymerase and 50 ng of DNA. PCR

299 products were visualized after electrophoresis in 1 % agarose gel (high resolution) by staining with
 300 GelRed (10 000 X) (Biotum, USA). LAB isolates identify in *L. casei* and *L. paracasei* group were
 301 analyzed considering six more housekeeping genes: anaerobic ribonucleoside-triphosphate
 302 reductase (*nrdD*), alpha-phosphoglucosyltransferase (*pgm*), DNA polymerase I (*polA*), DNA mismatch
 303 repair protein (*mutL*), polymerase alpha subunit (*rpoA*) and methionyl-tRNA synthetase (*metRS*)
 304 (Table 1). PCR was performed under the same conditions that *pheS* gene. The PCR products were
 305 sent to Macrogen® (South Korea) for sequencing in both orientations. General criteria of selection
 306 were the position of the genes on the chromosome, previously reported polymorphism, and the
 307 presence of one single copy of the gene (17).

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309 **Molecular data analysis.** The obtained sequences were assembled using the Staden
 310 program (53). Sequence similarity searches were performed using BlastN in order to compare the
 311 obtained sequences with genome complete sequences available in the databases (54). All sequences
 312 were aligned using MUSCLE (55) and deposited in the GenBank (Table S1). For phylogenetic
 313 comparison of 16S rRNA gene and *pheS* gene; 12 BAL isolates identify on this research and 33
 314 representative sequences from all the complete genome LAB available in GenBank (www.ncbi) for
 315 this species were included. A common sequence length of 1299 nucleotides (nt) for 16S rRNA gene
 316 and 420 nt of *pheS* gene were considered. For the analysis of *L. casei* and *L. paracasei* group
 317 Multilocus Typing Sequences (MTLS) of seven genes were considered and concatenated following
 318 the order. *nrdD* (734 nt), *pgm* (697 nt), *pheS* (420 nt), *polA* (731 nt), *mutL* (746 nt), *rpoA* (685 nt),
 319 *metRS* (636 nt). Sequences of seven LAB obtained on this research grouping with *L. casei* and *L.*
 320 *paracasei* and 17 LAB sequences available in GenBank (www.ncbi) were included on the study.
 321 Bayesian phylogenetic analysis was conducted using MrBayes (56). Analyses considered 10 million
 322 generations using eight chains and sampling every 2 000 generations and mixed model (57). The
 323 sequence of *L. delbrueckiae* subsp. *lactis* KTCT 3034 was included as an outgroup for
 324 phylogenetical analysis of 16S rRNA gene and *pheS* gene, while for the analysis of *L. casei* and *L.*

paracasei group *L. rhamnosus* ATCC 53103 and *L. casei* LC5 were included as outgroup.

Tolerance to pH 2.0. In order to select an acid tolerant strain for further studies, the 12 LAB isolates were exposed to pH 2.0 following the procedure of Ramos et al. (48). Lactic acid bacteria cultivated in MRS broth (DIFCO, France) at 35 ± 2 °C for 24 h were centrifuged (5000 rpm for 5 min at 24 °C) and washed two times in 0.1 % w/v peptone water, pH 7.0. Cells were resuspended in peptone water to a concentration of about 10^6 colonies forming units (CFU/mL), then centrifuged and re-suspended in MRS broth (DIFCO, France) with pH adjusted to 2.0 using 1N HCl and incubated for 3 h at 35 ± 2 °C. Samples (10 mL) obtained at time 0 and after incubation for 3 h were grown in MRS agar plates incubated in anaerobic jars for 72 h at 35 ± 2 °C.

Resistance to lysozyme. The lysozyme resistance assay was performed using the method described by Zago et al. (58) with some modifications. One mililiter of LAB cells were cultivated in MRS broth (DIFCO, France) at 30 ± 2 °C for 24 h, centrifuged at 5000 rpm for 5 min at 24 °C, washed two times in phosphate buffer (0.1 M, water pH 7.0) and 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, USA). To simulate an *in vivo* spit test, bacterial suspensions (10^8 CFU/mL) were inoculated into 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) in the presence of 100 mg/L of lysozyme (Sigma Aldrich, USA). Bacterial suspensions in SES without lysozyme were included as controls. The survival rate was calculated as the percentage of CFU/mL after 30 and 120 min relative to the CFU/mL at time zero. The microbial population was determined by cell counts in MRS agar. Assays were carried out in triplicate. Serial dilutions were done in 0.1 % sterile peptone water. Plates were incubated for 72 h at 35 °C.

Tolerance to bile salts. Isolates with a survival rate of 90 % or higher after exposure to pH 2.0 were evaluated for tolerance to bile salts. The method described by García-Ruiz et al. (3) was

utilized with minor modifications. Strains grown overnight were inoculated (2 % v/v) independently into MRS broth with 0.25 % bile salt (w/v) (Sigma-Aldrich, USA). Cultures were incubated in tilted tubes at 35 ± 2 °C for 24 h at 250 rpm in a rotatory benchtop incubated shaker (Lab Companion model SI-600R, Jeio Tech Company, South Korea). LAB plate counts in MRS were determined and compared to a control culture (sample without bile salts). Results were expressed as the percentage of growth (CFU/mL) compared to the control. The assay was conducted in triplicate and each sample was plated in duplicate.

Pineapple juices. Aseptic pineapple (*Ananas comosus*, cultivar MD-2) juice extracted from fruit pulp and two pineapple juice concentrates, one made from pineapple peels and the other from a blend of pineapple peel and pulp extracts (80:20, respectively), were obtained locally (San Jose, Costa Rica). The concentrates were reconstituted with distilled water to about 12° Brix. Reconstituted juices were pasteurized and hot filled at 83 °C into clean glass containers to ensure commercial sterility. Containers were immediately sealed and inverted for at least 5 min to sterilize the headspace and metal lids. The hot fill step for the juices made from concentrate was performed to obtain shelf stable samples and therefore avoid the presence of unwanted microbiota that would interfere with quantification of the selected LAB during the survival study. To verify the absence of background microbiota, three replicate samples of each juice were plated on plate count agar (OXOID Thermo Scientific Fisher, UK) and acidified potato-dextrose agar (pH 3.5) (OXOID Thermo Scientific Fisher, UK) and incubated at 35 ± 2 °C for 24 h and at 25 ± 2 °C for 5 days, respectively, to determine total plate counts as well as mold and yeast counts.

Physicochemical characterization of juices. Juice pH was measured with a 450 pH/ion Meter (Corning, USA). Total titratable acidity, expressed in grams of citric acid per 100 g of juice, was measured with a Titrando 836 System (Metrohm, Switzerland). Total solids content was determined by the vacuum oven method (59). The soluble solids content was measured using an

Abbe refractometer model NAR-4T (ATAGO, Japan). The concentration of sugars (sucrose, glucose and fructose) and organic acids (citric, malic and lactic) was assessed by High Performance Liquid Chromatography (HPLC, Shimadzu, Japan). To determine sugar concentrations, standard solutions (Sigma-Aldrich, USA) and samples were injected onto the HPLC unit provided with a refractive index detector (RID) and a Zorbax Carbohydrate Analysis column (150 mm x 4.6 mm ID, 5 µm particle size; Agilent Technologies, USA). Samples were resolved using a blend of acetonitrile and purified water (75:25 v/v) at a 1.2 mL/min flow rate in an isocratic run for 10 min at 30 °C. For the assessment of the concentration of organic acids, the HPLC unit was coupled with a photodiode array (PDA, model SPF-20^a, Shimadzu) detection system and a Hi-plex H column (300 mm x 7.8 mm, 8 µm particle size; Agilent Technologies, USA). A solution of 0.0045 M H₂SO₄ was used as the mobile phase at a 0.6 ml/min flow rate in an isocratic run for 20 min at 50 °C. A detection wavelength of 210 nm was used. All physicochemical analyses were performed in triplicate.

***Lactobacillus fermentum* survival in pineapple juices**

Culture preparation. *Lactobacillus fermentum* (6702) isolated from pineapple waste from the same juice manufacturing company where juices were obtained was grown in MRS (DIFCO, France) broth and incubated at 225 rpm in a rotatory benchtop incubated shaker (Lab Companion model SI-600R, Jeio Tech Company, South Korea) at 35 ± 2 °C for 24 h. LAB cultures were preserved in 1.5 ml cryovials containing 30 % glycerol and stored at -20 °C until used. The inoculum concentration was approximately 10⁹ CFU/ml. The exact initial counts were determined in triplicate after inoculating the juice samples used in the survival experiment.

Experimental design. Survival of *L. fermentum* (6702) in three pineapple juices made from pineapple pulp, peel extract, and a blend of pulp juice and peel extract was evaluated using the procedure reported by Nualkaekul and Charalampopoulos (4) with minor modifications. Samples

of 100 mL of the juices were independently inoculated with 0.07 mL of the selected LAB targeting an initial concentration between 10^7 and 10^8 UFC/mL. The inoculated juices were stored at 4 °C for up to 6 weeks. Samples were collected weekly and analyzed for pH, soluble solids content (expressed in °Brix) and LAB counts. To determine the count of viable LAB cells, 0.1 mL of juice was spread onto MRS agar (DIFCO, France) and incubated in anaerobic jars at 35 ± 2 °C for 72 h before counting. The survival experiments were performed in triplicate for each condition, using independent inocula for each of the replicates. To verify the absence of background microbiota, three replicate control samples of each juice were plated on plate count agar and acidified (pH 3.5) potato-dextrose agar and incubated at 35 ± 2 °C for 24 h and at 25 ± 2 °C for 5 days, respectively, to determine total plate and mold and yeast counts. The sugar content (glucose, fructose and sucrose), organic acid concentration (malic, lactic and citric), total solids content and total titratable acidity (expressed as percentage of citric acid in 100 mL of juice) of the juices were determined at the beginning and the end of the experiment.

The effect of storage time at 4 °C on the physicochemical properties of pineapple juices inoculated with a selected strain of *L. fermentum* was evaluated. A full factorial design with three levels for type of juice (pulp, pulp + peel and peel) and two levels for storage time (week 0 and week 6) was constructed. The initial LAB count was included in the model as an independent variable. In addition, the effects of storage time and juice type on the survival of *L. fermentum* in pineapple juices refrigerated at 4 °C were examined. A full factorial design with three juice types (pulp, peel + pulp and peel) and seven storage times (from week 0 to week 6) was used. The population of this microorganism was used as the response.

Statistical analysis. To analyze the physicochemical properties of the juices and the survival of *L. fermentum* over time, two- and three-way analyses of variance (ANOVA), Tukey's honestly significant difference, and Student's t tests for means comparisons were performed using JMP version 11 (SAS Institute Inc., USA). Differences were considered significant at a *P* value of <

429 0.05.

430

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437

438 Reference

- 439 1. **Food and Agriculture Organization of the United Nations & World Health Organization**
440 **(FAO/WHO)**. 2002. Guidelines for the evaluation of probiotics in food
441 http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf
- 442 2. **Sah BNP, Vasiljevic T, McKechnie S, Donkor, ON**. 2015. Effect of refrigerated storage on
443 probiotic viability and the production and stability of antimutagenic and antioxidant peptides in
444 yogurt supplemented with pineapple peel. *J Dairy Sci* **98**: 5905–5916.
- 445 3. **García-Ruiz A, González de Llano D, Esteban-Fernández A, Requena T, Bartolomé B,**
446 **Moreno-Arribas MV**. 2014. Assessment of probiotic properties in lactic acid bacteria isolated from
447 wine. *Food Microbiol* **44**:220-225.
- 448 4. **Nuallkaekul S, Charalampopoulos D**. 2011. Survival of *Lactobacillus plantarum* in model
449 solutions in fruit juices. *Int J Food Microbiol* **146**: 111-117.
- 450 5. **Shukla M, Kumar Jha Y, Admassu S**. 2013. Development of probiotic beverage from whey and
451 pineapple juice. *J Food Proces Technol* **4**:2-4.
- 452 6. **Perricone M, Bevilacqua A, Altieri C, Sinigaglia M, Corbo MR**. 2015. Challenges for the
453 production of probiotic fruit juices. *Beverages* **1**:95-103.

- 454 **7. Rúa J, López-Rodríguez I, Sanz J, García-Fernández MC, del Valle MP, García-Armesto**
455 **MR.** 2018. Improving functional properties of “Piel de Sapo” melon juice by addition of a *Lippia*
456 *citriodora* natural extract and probiotic-type lactic acid bacteria. Food Sci Technol **96**:75–81.
- 457 **8. Garcia M, Vidal T, Tibério AL, Rodrigues S.** 2013. Sonicated pineapple juice as substrate for
458 *L. casei* cultivation for probiotic beverage development: Process optimisation and product stability.
459 Food Chemistry **139**:261–266.
- 460 **9. Nadzirah KZ, Zainal S, Noriham A, Normah I, Siti Roha AM, Nadya H.** 2013. Physico-
461 chemical properties of pineapple variety N36 harvested and stored at different maturity stages. Int
462 Food Res J **20**:225-231.
- 463 **10. León R, Kellon D.** 2012. Characterization of ‘MD-2’ Pineapple Planting Density and
464 Fertilization Using a Grower Survey. HortTech **22**:644-650.
- 465 **11. Pyar H, Liong MT, Peh HH.** 2014. Potentials of pineapple waste as growth medium for
466 *Lactobacillus* species. Int J Pharm Pharm Sci **1**:142-145.
- 467 **12. Kaprasob R, Kerdchoechuena O, Laohakunjita N, Sarkarb D, Shetty K.** 2017.
468 Fermentation-based biotransformation of bioactive phenolics and volatile compounds from cashew
469 apple juice by select lactic acid bacteria. Process Biochem **59**:141–149.
- 470 **13. Park JB, Lim SH, Sim HS, Park JH, Kwon HJ, Nam HS, Kim MD, Baek HH, Ha SJ.** 2017.
471 Changes in antioxidant activities and volatile compounds of mixed berry juice through fermentation
472 by lactic acid bacteria. Food Sci Biotechnol **26**:441-446.
- 473 **14. Dicagno R, Cardinalo G, Minervini G, Antonielli L, Rizzello C, Ricciuti P, Gobbetti M.**
474 **2010.** Taxonomic structure of the yeasts and lactic acid bacteria microbiota of pineapple (*Ananas*
475 *comosus* L. Merr.) and use of autochthonous starters for minimally processing. Food Microbiol
476 **27**:381-389.
- 477 **15. Torino M, Font G, Mozzi F.** 2015. Biopolymers from lactic acid bacteria: novel applications
478 in foods and beverages. Front Microbiol **6**:1-9.
- 479 **16. Zheng J, Ruan L, Sun M, Gänzle M.** 2015. A genomic view of Lactobacilli and Pediococci

- 480 demonstrates that phylogeny matches ecology and physiology. Appl Environ Microbiol. **81**:7233–
481 7243.
- 482 17. **Cai H, Rodríguez BT, Zhang W, Broadbent JR, Steele JL.** 2007. Genotypic and phenotypic
483 characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests
484 frequent recombination and niche specificity. Microbiology **153**:2655–2665.
- 485 18. **Ouattaraa HD, Ouattaraa HG, Drouxb M, Reverchon S, Nasser W, Niamke SL.** 2017.
486 Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: Species diversity and
487 citrate lyase production. Int J Food Microbiol 256:11–19
- 488 19. **García A, Navarro K, Sanhueza E, Pineda S, Pastene E, Quezada M, Henríquez K,**
489 **Karlyshev A, Villena J, González C.** 2018. Characterization of *Lactobacillus fermentum* UCO-
490 979C, a probiotic strain with a potent anti-*Helicobacter pylori* activity. Electron J Biotechnol **25**:75-
491 83.
- 492 20. **de Souza, BMS, Borgonovi TF, Casarotti, SN, Todorov SD, Barretto AL.** 2018.
493 *Lactobacillus casei* and *Lactobacillus fermentum* strains isolated from mozzarella cheese: probiotic
494 potential, safety, acidifying kinetic parameters and viability under gastrointestinal tract conditions.
495 Probiotics & Antimicro Prot <https://doi.org/10.1007/s12602-018-9406-y>
- 496 21. **Le B, Yang SH.** 2018. Isolation of *Weissella* strains as potent probiotics to improve antioxidant
497 activity of salted squid by fermentation. J Appl Biol Chem **61**(1):93-100.
- 498 22. **Dicks LM, Du Plessis EM, Dellaglio F, Lauer E.** 1996. Reclassification of *Lactobacillus casei*
499 subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev.,
500 designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the
501 name *Lactobacillus paracasei*. Int J Syst Evol Microbiol **46**:337-340.
- 502 23. **Dobson CM, Chaban B, Deneer H, Ziola B.** 2004. *Lactobacillus casei*, *Lactobacillus*
503 *rhamnosus*, and *Lactobacillus zeae* isolates identified by sequence signature and immunoblot
504 phenotype. Can J Microbiol **50**:482-488.
- 505 24. **Cai H, Thompson R, Budinich MF, Broadbent JR, Steele.** 2009. Genome sequence and
506 comparative genome analysis of *Lactobacillus casei*: Insights into their niche-associated evolution.

Genome Biol Evol. **1**:239–257

25. **Diancourt L, Passet V, Chervaux C, Garault P, Smokvina T, Brisse S.** 2007. Multilocus sequence typing of *Lactobacillus casei* reveals a clonal population structure with low levels of homologous recombination. Appl Environ Microbiol. **73**(20):6601–6611.

26. **Kang J, Chung W-H, Lim T-J, Whon T-W, Lim S, Nam Y-D.** 2017. Complete genome sequence of *Lactobacillus casei* LC5, a potential probiotics for atopic dermatitis. Front Immunol **8**:413.

27. **Lacerda-Ramos C, Thorsen L, Freitas R, Jespersen L.** 2013. Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. Food Microbiol **36**:22-29.

28. **Tripathi M, Giri S.** 2014. Probiotic functional foods: survival of probiotics during processing and storage. J Funct Foods **9**:225–241.

29. **Hernández-Alcántara AM, Wachter C, Goretti Llamas M, López P, Pérez-Chabela ML.** 2018. Probiotic properties and stress response of thermotolerant lactic acid bacteria isolated from cooked meat products. Food Sci Technol **91**:249–257.

30. **Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E.** 2006. Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int Dairy J **16**:189–199.

31. **Cunningham FE, Proctor VA, Goetsch SJ.** 1991. Egg-white lysozyme as a food preservative: an overview. World Pollution Sci J **47**:141–163.

32. **Dunne CO, Mahony L.** 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation *in vivo* findings. Am J Clin Nutr **73**: 386-392.

33. **Mathara JM, Schillinger U, Guigas UC, Franz C, Kutima MP, Mbugua SK, Shin HK, Holzapfel WH.** 2008. Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk products in Kenya. Int J Food Microbiol **126**:57-64.

34. **Delgado S, O’Sullivan E, Fitzgerald G, Mayo B.** 2008. *In vitro* evaluation of the probiotic

570 properties of human intestinal *Bifidobacterium* species and selection of new probiotic candidates. J
571 Appl Microbiol **104**:119–1127.

572 35. **Hui Y, Chen F, Nollet L.** 2010. Handbook of fruit and vegetable flavors. Wiley, Estados
573 Unidos.

574 36. **Jensen H, Grimmer S, Naterstad K, Axelsson L.** 2012. *In vitro* testing of commercial and
575 potential probiotic lactic acid bacteria. Int J Food Microbiol **153**:216-222.

576 37. **Turchi B, Mancini S, Fratini F, Pedonese F, Nuvoloni R, Bertelloni F, Ebani VV, Cerri D.**
577 2013. Preliminary evaluation of probiotic potential of *Lactobacillus plantarum* strains isolated from
578 Italian food products. World J Microbiol Biotech **29**:1913–1922.

579 38. **Luo Y, Ma BC, Zou LK, Cheng JG, Cai YH, Kang JP, Li B, Gao XH, Wang P, Xiao JJ.**
580 2012. Identification and characterization of lactic acid bacteria from forest musk deer feces. African
581 J Microb Res **6**:5871-5881.

582 39. **Maldonado NC., Ruiz CS, Otero MC, Sesma F, Nader-Macías ME.** 2012. Lactic acid
583 bacteria isolated from young calves e characterization and potential as probiotic. Res Vet Sci **92**:
584 342e349.

585 40. **Tannock G.** 2004. A special fondness for lactobacilli. Appl Environ Microbiol **70**:3189-3194.

586 41. **Haruenkit R.** 2004. Analysis of sugars and organic acids in pineapple, papaya and star fruit by
587 HPLC using an aminex HPx-87 H column. King Mongkut's Agric J **22**:11-18.

588 42. **Siti A, Zainal S, Noriham A, Nadzirah K.** 2013. Determination of sugar content in pineapple
589 waste variety N36. Int Food Res J **20**:1941-1943.

590 43. **Pereira GV, Miguel MG, Ramos CL, Schwan RF.** 2012. Microbiological and
591 physicochemical characterization of small-scale cocoa fermentations and screening of yeast and
592 bacterial strains to develop a defined starter culture. Appl Environ Microbiol **78**(15): 5395-405.

593 44. **Matthews A, Grimaldi A, Walker M, Bartowsky E, Grbin P, Jiranek. V.** 2004. Lactic acid
594 bacteria as a potential source of enzymes for use in vinification. Appl Environ Microbiol **70**: 5715–
595 5731.

- 596 45. **Kandler O.** 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek J*
597 *Microbiol and Serol.* **49**:209-224.
- 598 46. **Foschino R, Picozzi C, Galli A.** 2001. Comparative study of nine *Lactobacillus fermentum*
599 bacteriophages. *J Appl Microbiol* **91**:394-403.
- 600 47. **Dan T, Liu W, Song Y, Xu H, Menghe B, Zhang H, Sun Z.** 2015. The evolution and
601 population structure of *Lactobacillus fermentum* from different naturally fermented products as
602 determined by multilocus sequence typing (MLST). *BMC Microbiol* **15**:107.
- 603 48. **Ramos CL, Thorsen L, Schwan RF, Jespersen L.** 2013. Strain-specific probiotics properties
604 of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from
605 Brazilian food products. *Food Microbiol* **36**:22-29.
- 606 49. **Birnboim HC, Doly J.** 1979. A rapid alkaline procedure for screening recombinant plasmid
607 DNA. *Nucleic Acids Res* **7**:1513–1523.
- 608 50. **Edwards U, Rogall T, Blöcker H, Emde M, Bötter EC.** 1989. Isolation and direct complete
609 nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal
610 RNA. *Nucleic Acids Res* **17**:7843–7853.
- 611 51. **Weisburg W.G, Barns SM, Pelletier DA, Lane DJ.** 1991. 16S ribosomal DNA amplification
612 for phylogenetic study. *J Bacteriol* **173**:697-703.
- 613 52. **Naser SM, Thompson FL, Hoste B, Gevers D, Dawyndt P, Vancanneyt M, Swings J.** 2005.
614 Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus*
615 species based on *rpoA* and *pheS* genes. *Microbiology* **151**: 2141–2150
- 616 53. **Bonfield JK, Smith KF, Staden R.** 1995. A new DNA sequence assembly program. *Nucleic*
617 *Acids Res* **23**:4992-4999.
- 618 54. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search
619 tool. *J Mol Biol* **215**:403-410.
- 620 55. **Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S.** 2013. MEGA6: Molecular
621 evolutionary genetics analysis version 6.0. *Mol. Biol. Evol* **30**:2725-2729.

- 622 56. **Huelsenbeck JP, Ronquist F, Nielsen R, Bollback J.** 2001. Bayesian inference of phylogeny
623 and its impact on evolutionary biology. *Science* **294**:2310–2314.
- 624 57. **Ronquist F, Huelsenbeck JP.** 2003. MrBayes 3: Bayesian phylogenetic inference under mixed
625 models. *Bioinformatics* **19**:1572–1574.
- 626 58. **Zago M, Fornasari ME, Carminati D, Burns P, Suárez V, Vinderola G, Reinheimer J,**
627 **Giraffa G.** 2011. Characterization and probiotic potential of *Lactobacillus plantarum* strains
628 isolated from cheeses. *Food Microbiol* **28**:1033–1040.
- 629 59. **Association of Official Analytical Chemists (AOAC).** 2005. Official Method of analysis
630 AOAC 925.45: Loss on Drying (Moisture) in Sugars, 18 ed. Association of Official Analytical
631 Chemists: Washington, DC.

632

633 **Figure legends**

634 **Fig1** Phylogenetic tree for equivalent partial 16S rRNA gene sequences (1299 nucleotides (nt)) (A)
 635 and phenylalanyl-tRNA synthase (*pheS*) gene (420 nt) (B) of lactic acid bacteria's (LAB) isolated
 636 from pineapple waste and comparison with sequences available in databases from other parts of the
 637 world. Sequences were aligned (using MUSCLE) and the phylogenetic tree was estimated by using
 638 Bayesian inference. Numbers above branches represent the Bayesian posterior probabilities. The
 639 bar indicates substitutions per site. Sequences corresponding to those present in Costa Rica are
 640 shown in bold letters. The phylogenetic trees were rooted using an equivalent sequence from *L.*
 641 *delbrueckii* subsp. *lactis* KTCT 3034.

642

643 **Fig 2** *L. casei* and *L. paracasei* phylogenetic tree considering the concatenated sequences of lactic
 644 acid bacterias (LAB) isolated from pineapple waste. Partial gene of anaerobic ribonucleoside-
 645 triphosphate reductase (*nrdD*) gene, DNA mismatch repair protein (*mutL*) gene, DNA polymerase I
 646 (*polA*) gene, methionyl-tRNA synthetase (*metRS*) gene, phosphoglucomutase (*pgm*), polymerase
 647 alpha subunit (*rpoA*) and phenylalanyl-tRNA synthase (*pheS*) gene, were included. Comparison
 648 with sequences available in databases from other parts of the world was done. Sequences were
 649 aligned (using MUSCLE) and the phylogenetic tree was estimated by using Bayesian inference.
 650 Numbers above branches represent the Bayesian posterior probabilities. The bar indicates
 651 substitutions per site. Sequences corresponding to those present in Costa Rica are shown in bold
 652 letters. The phylogenetic trees were rooted using an equivalent sequence from *L. rhamnosus* ATCC
 653 53103 and *L. casei* LC5.

654

655 **Fig 3** Evolution of the (a) *L. fermentum* population and (b) juice pH (mean $\pm$ standard deviation, $n =$
 656 3) in pineapple juices (from pulp, peels and a blend of pulp and peels) during juice storage at 4 °C
 657 for up to six weeks. Values that do not share a common letter represent significantly different values

658 (P<0.05) based on Tukey's analysis of multiple comparisons.

TABLE 1 Primers used for molecular identification of lactic acid bacteria (LAB) isolated from pineapple waste residuals

Primer name	Primer sequence (F/R)	Location ^a
27F/1492R	5'- AGA GTT TGA TCC TGG CTC AG -3'	259 513...261 026
	5'- ACG GCT ACC TTG TTA CGA CTT -3'	
<i>pheS</i> -21-F/ <i>pheS</i> -22-R	5'- CAYCCNGCHCGYGAYATGC -3'	1 670 081...1 670 575
	5'- CCWARVCCRAARGCAAARCC -3'	
<i>nrdD</i> -F/ <i>nrdD</i> -R	5'- GCTTGAAGCGTGATTTAGCC -3'	129 746...130 540
	5'- ACATTCGATCGCCAATTGTT -3'	
<i>pgm</i>	5'- AGGCATTTGCTGCTCCTATG-3'	927 617...928 409
	5'- GGGATCAGTCGCGATTAAGA-3'	
<i>polA</i> -F/ <i>polA</i> -F	5'- TTATCATGTGGCCGAACAAA -3'	1 702 740...1 703 578
	5'- GTTTGCGTCAAAGTCTGCAA -3'	
<i>multL</i> -F/ <i>multL</i> -R	5'- ATCGGCAACATTAAGCAACC -3'	2 225 886...2 226 701
	5'- GATGACGCCCATTTGGATAAC -3'	
<i>rpoA</i> 21F/ <i>rpoA</i> 23R	5'- ATGATYGARTTTGAAAAACC -3'	2 458 334...2 459 129

	5'- ACHGTRTTTRATDCCDGCRCG -3'	
<i>metRS</i> -F/ <i>metRS</i> -R	5'- CGGTATTTTGCCAGCCTTTA -3'	2 586 141...2 586 862
	5'- CATTTCGCCTTTTAGCTTGC -3'	

^aCoordinates of the primers sequence template for the complete genome of strain *L. paracasei* ATCC 334 (GenBank accession no. CP000423).

TABLE 2 Tolerance to a pH of 2.0, lysozyme and bile salts of the LAB isolates (mean $\pm$ standard deviation, $n = 3$)

LAB strain	Tolerance to pH 2.0 (%Survival)	Resistance to lysozyme (% of survival)		Resistant to bile at 0.25 % (% of survival)
		t ₃₀	t ₁₂₀	
<i>L. casei</i> _6709	45.0 $\pm$ 0.03	100 $\pm$ 2	99.4 $\pm$ 0.7	ND ^a
<i>L. casei</i> _6713	88.0 $\pm$ 0.2	100 $\pm$ 1	99 $\pm$ 1	ND
<i>L. casei</i> _6715	93.8 $\pm$ 2.1	97 $\pm$ 5	96 $\pm$ 3	74 $\pm$ 5
<i>L. fermentum</i> _6702	92.2 $\pm$ 0.6	101 $\pm$ 4	99 $\pm$ 2	78 $\pm$ 7
<i>L. fermentum</i> _6704	90.0 $\pm$ 0.7	100.0 $\pm$ 0.6	101 $\pm$ 3	78 $\pm$ 4
<i>L. paracasei</i> _6710	95.5 $\pm$ 1.7	100.0 $\pm$ 0.6	100 $\pm$ 1	61 $\pm$ 9
<i>L. paracasei</i> _6711	85.0 $\pm$ 7.3	100.0 $\pm$ 0.2	98.78 $\pm$ 0.02	ND
<i>L. paracasei</i> _6714	80.0 $\pm$ 1.1	98 $\pm$ 4	99 $\pm$ 5	ND
<i>L. paracasei</i> _6712	84.1 $\pm$ 0.4	100 $\pm$ 2	100 $\pm$ 6	ND
<i>L. parafarraginis</i> _6717	86.4 $\pm$ 0.1	77.5 $\pm$ 0.1	75.8 $\pm$ 0.2	ND
<i>L. parafarraginis</i> _6719	99.7 $\pm$ 0.1	97.2 $\pm$ 0.2	88.7 $\pm$ 0.3	67 $\pm$ 7
<i>W. ghanensis</i> _6706	77.6 $\pm$ 2.1	100 $\pm$ 5	98 $\pm$ 3	ND

^a ND, not determined

TABLE 3 Physicochemical characterization of pineapple juices used to study *L. fermentum* survival at time zero and after six weeks of storage at 4 °C

Variable		R ^{2a}	P ^b	Juice	Control (non-inoculated) ^{cd}	Week 0 ^c	Week 6 ^c
Total solids content (g/ 100 g juice)		0.17	> 0.05	pulp	12.50 ± 0.30 ^a	12.90 ± 0.2 ^a	12.70 ± 0.2 ^a
				pulp + peel	13.00 ± 1.00 ^a	13.00 ± 0.5 ^a	12.80 ± 0.6 ^a
				peel	13.60 ± 0.70 ^a	12.90 ± 0.9 ^a	13.40 ± 0.5 ^a
Soluble solids (Brix)		0.90	< 0.006	pulp	13.00 ± 0.00 ^a	13.0 ± 0 ^a	11.60 ± 0.005 ^b
				pulp + peel	13.30 ± 0.30 ^a	13.50 ± 0.3 ^a	12.80 ± 0.3 ^a
				peel	13.30 ± 0.30 ^a	13.10 ± 0.5 ^a	11.40 ± 0.3 ^b
Total acidity (g citric acid/100 g juice)	titratable	0.81	< 0.0001	pulp	0.51 ± 0.01 ^b	0.50 ± 0.005 ^b	0.51 ± 0.02 ^b
				pulp + peel	0.47 ± 0.006 ^b	0.48 ± 0.01 ^b	0.49 ± 0.01 ^b
				peel	0.55 ± 0.03 ^a	0.54 ± 0.02 ^a	0.57 ± 0.03 ^a
Fructose (g/100 mL juice)		0.10	< 0.0001	pulp	4.35 ± 0.50 ^{ab}	4.65 ± 0.1 ^a	2.61 ± 0.04 ^{de}
				pulp + peel	3.32 ± 0.40 ^{γδ}	3.63 ± 0.1 ^{βγ}	2.69 ± 0.4 ^{δe}
				peel	2.38 ± 0.1 ^ε	3.42 ± 0.3 ^{γδ}	2.84 ± 0.3 ^{γδe}
Glucose (g/100 mL juice)		0.87	< 0.004	pulp	2.59 ± 0.03 ^{γδ}	2.05 ± 0.06 ^ε	2.27 ± 0.2 ^{δe}
				pulp + peel	3.16 ± 0.2 ^{ab}	3.35 ± 0.1 ^a	2.76 ± 0.2 ^{βγδ}
				peel	2.63 ± 0.4 ^{γδ}	2.85 ± 0.1 ^{αβγ}	2.44 ± 0.2 ^{γδe}
Sucrose (g/100 mL juice)		0.90	< 0.0001	pulp	6.94 ± 0.3 ^b	6.77 ± 0.3 ^b	8.59 ± 0.1 ^a
				pulp + peel	6.47 ± 0.7 ^b	7.08 ± 0.1 ^b	6.21 ± 0.2 ^{βγ}
				peel	6.29 ± 0.4 ^b	6.59 ± 0.2 ^b	5.28 ± 0.4 ^γ
Citric acid		0.71	< 0.0496	pulp	0.32 ± 0.05 ^{αβγ}	0.37 ± 0.01 ^{αβ}	0.28 ± 0.06 ^{βγ}

(mg/100 mL juice)				pulp + peel	$0.302 \pm 0.002^{\beta\gamma}$	$0.305 \pm 0.002^{\beta\gamma}$	$0.27 \pm 0.03^{\gamma}$
				peel	$0.41 \pm 0.02^{\alpha}$	$0.34 \pm 0.04^{\alpha\beta\gamma}$	$0.34 \pm 0.02^{\alpha\beta\gamma}$
Malic	acid	0.97	<0.0001	pulp	$0.23 \pm 0.01^{\alpha}$	$0.253 \pm 0.008^{\alpha}$	$0.204 \pm 0.006^{\beta}$
(mg/100 mL juice)				pulp + peel	$0.170 \pm 0.003^{\gamma\delta}$	$0.177 \pm 0.003^{\beta\gamma}$	$0.145 \pm 0.004^{\delta\epsilon}$
				peel	$0.15 \pm 0.02^{\gamma\delta\epsilon}$	$0.100 \pm 0.005^{\epsilon}$	$0.13 \pm 0.01^{\epsilon}$
Lactic	acid	1.00	< 0.0001	pulp	ND ^{c\gamma}	ND ^{\gamma}	$0.099 \pm 0.004^{\alpha}$
(mg/100 mL juice)				pulp + peel	ND ^{\gamma}	ND ^{\gamma}	$0.0703 \pm 0.0009^{\beta}$
				peel	ND ^{\gamma}	ND ^{\gamma}	$0.094 \pm 0.009^{\alpha}$

^a R², determination coefficient

^b P, probability

^c The values correspond to average $\pm$ standard deviation ($n = 3$). Values for juices in the same quadrant of physico-chemical property that do not share a common symbol represent significantly different values ($P < 0.05$) based on Tukey's analysis of multiple comparison.

^d The control sample corresponds to non-inoculated juice for the study of survival at zero time and after six weeks.

^e ND, not detected (Detection limits: ≤ 15.28 mg/mL; a value of 0 is assigned for statistical analysis).

A

B

A **B**