Survival and Growth of Probiotic Lactic Acid Bacteria in Refrigerated Pickle Products

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Abstract: We examined 10 lactic acid bacteria that have been previously characterized for commercial use as probiotic cultures, mostly for dairy products, including 1 *Pediococcus* and 9 *Lactobacilli*. Our objectives were to develop a rapid procedure for determining the long-term survivability of these cultures in acidified vegetable products and to identify suitable cultures for probiotic brined vegetable products. We therefore developed assays to measure acid resistance of these cultures to lactic and acetic acids, which are present in pickled vegetable products. We used relatively high acid concentrations (compared to commercial products) of 360 mM lactic acid and 420 mM acetic acid to determine acid resistance with a 1 h treatment. Growth rates were measured in a cucumber juice medium at pH 5.3, 4.2, and 3.8, at 30 °C and 0% to 2% NaCl. Significant differences in acid resistance and growth rates were found among the 10 cultures. In general, the acid resistant strains had slower growth rates than the acid sensitive strains. Based on the acid resistance data, selected cultures were tested for long-term survival in a simulated acidified refrigerated cucumber product. We found that one of the most acid resistant strains (*Lactobacillus casei*) could survive for up to 63 d at 4 °C without significant loss of viability at 10^8 CFU/mL. These data may aid in the development of commercial probiotic refrigerated pickle products.

Keywords: acetic acid, acid resistance, lactic acid, probiotic, refrigerated pickles

Practical Application: The development of probiotic pickled vegetable products will be facilitated by identifying probiotics that can survive in acidified vegetable products with a proposed shelf life of 2 or more months. We analyzed the growth and acid resistance of 10 probiotic lactic acid bacterial cultures and found that cultures selected for acid resistance had superior long-term survival (up to 2 mo) in a simulated refrigerated probiotic vegetable product that included a fermentation step. These data may be useful to aid the development of a commercial process for the manufacture of probiotic pickles.

Introduction

Probiotics are defined by the World Health Organization as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (World Health Org. 2002). Probiotic microorganisms include species of the genera *Lactobacillus* and *Bifidobacterium*, and to date, have been used primarily in dairy applications (Collins and others 1998). Research on probiotics has increased to over 1400 publications per year, as of 2014 (McFarland 2016), with a focus on human health effects of probiotic consumption. Many studies made use of specific *Lactobacillus* or *Bifidobacterium* strains ingested in yogurt-type dairy products or in capsule form (Martinez and others 2015). Dairy products are among the most popular foods containing probiotics (Champagne and others 2005) and some studies have shown that the dairy food matrix can enhance the efficacy of probiotics (Sanders and Marco 2010). However, dairy products may be high in calories and inappropriate for some individuals for a variety of reasons, including those with vegan or lactose intolerant diets. Alternative means of providing probiotics in the human diet have been suggested, including meat products and vegetable and fruit juices (Sheehan and others 2007; Martins and others 2013; Rouhi and others 2013). While general health claims have been made concerning consumption of kimchi and other fermented vegetable products naturally containing lactic acid bacteria (LAB), the use of fermented or acidified vegetables as delivery vehicles for defined (added) probiotics remains largely uninvestigated.

Limited research on added lactic acid bacterial cultures in fermented vegetable products includes a study on the survival of *Bifidobacterium* culture in *mul*-kimchi (a type of watery kimchi made with radishes), where the culture survival was monitored for 10 d in the brine (Lee and others 1999). *Bifidobacterium* strains exhibited a 1 to 4 log decrease in 10 d after the start of fermentation. Successful fermented probiotic vegetable products may be difficult to produce due to the presence of the epiphytic microbiota carrying out the fermentation, which could out-compete an added probiotic culture, depending on relative growth rates and acid tolerance. Acidified vegetable products, which are not fermented, may also be considered as vehicles for delivery of an effective dose of selected probiotics. Acidified vegetables are defined as vegetable products to which acid or acid food ingredients are added to bring the equilibrated product pH below 4.6 (FDA 1979). For successful production of acidified probiotic vegetable products, research is needed to determine the acid tolerance and viability of probiotic LAB under conditions typical of manufacture and storage, with pH values typically below pH 4. This is because

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Table 1–Probiotic lactic acid bacteria used in this study.

Strain	ID[a]	Source[b]	Reference
Lactobacillus casei (0rla-Jonson)	LA0284	ATCC 393	Sidira and others 2010
Lactobacillus rhnmnosus (GG)	LA1134	ATCX.53103	Salminen and others 1996
Lactobacillus acidophilus (NCFM)	LA1135	ATCC700396	Sanders and Klaenhanmer 2001
Lactobacillus plantarum	LA1148	CJ ChilJedang Corp.	NA[c]
Lactobacillus plantarum	LA1198	Lyoferm Inc.	NA
Lactobacillus plantarum LM	LA1199	Lyoferm Inc.	Starovoytova and others 2007; 2009
Pediococcus acidilactici	LA1200	Biosource Flavors Inc.	Borowski and others 2009
Lactobacillus plantarum	LA1201	Biena Inc.	NA
LoLlubacillus plan laiurn	LA1202	Biosource Flavors Inc.	NA
Lactobacillus peniusus MP-10	LA1203	Univ. of Jaen, Jaen, Spain	Abriouel and others 2011

[a]ID, USDA/ARS Food Science Research Unit culture collection, Raleigh, NC.
[b]Source, culture collection source or commercial company: ATCC, American Type Culture collection, Manassas, Virginia; CJ CheilJedang Corp., Seoul, South Korea; Biosource Flavors Inc., Muskego, Wisc., U.S.A. Lyoferm Inc., Greenfield, Ind., U.S.A.; Biena Inc., St. Hyacinthe, Quebec, Canada.
[c]NA, no literature reference available.

a successful probiotic pickled vegetable product should maintain an effective dose of culture for the shelf-life of the product in an acidic medium. An effective dose may be estimated at 10^6 to 10^8 or greater colony forming units (CFU) per gram of food for a typical portion of the food product (Sanders and Marco 2010).

We investigated the growth characteristics and acid resistance of selected probiotics that have been previously characterized for use in commercial (principally diary) products. *Lactobacillus* species were primarily chosen because of the acid resistance of this genus and the dominance of *Lactobacillus* strains in vegetable fermentations. To simulate a probiotic refrigerated pickle product, we used a process that was expected to enable long-term survival of probiotics in refrigerated pickle brines adapted from the method of Pérez-Díaz and McFeeters (2011). This method was previously used for the preparation of kosher cultures in brined cucumbers that were pre-acidified with acetic acid. We used a commercial refrigerated pickle brine formulation containing approximately 100 mM acetic acid, 0.34 M sodium chloride (NaCl), and 6.9 mM (0.1%) sodium benzoate (Lu and others 2013). This brine also included approximately 25 mM glucose and fructose and other nutrients that would be in acidified cucumber pickle brines. Our objective in the current study was to characterize probiotics for growth and resistance to lactic and acetic acids, in order to determine the likelihood of successful use of these cultures for probiotic vegetable products. Our hypothesis was that cultures with superior acid resistance would exhibit long-term survival desirable for a commercial probiotic refrigerated cucumber pickle product. Cucumber juice medium was used as a generic vegetable broth medium that does not contain known antimicrobial compounds so that the results may be applied to a variety of brined vegetable products (Breidt and others 2007, 2013).

Materials and Methods

Bacterial strains and culture media

Bacterial strains used in this study are shown in Table 1. Bacteria were grown in de Man, Rogosa and Sharpe medium (MRS, Lactobacilli MRS broth or agar, BD Diagnostic Systems, Sparks, Md., U.S.A.) (deMan and others 1960) statically for 24 to 48 h at 30 °C. For enumeration, cells were serially diluted in MOPS buffer which contained 50 mM 3-[N-morpholino] propanesulfonic acid (Sigma-Aldrich, St. Louis, Mo., U.S.A.), with 0.85% NaCl at pH 7, to neutralize acid prior to plating on MRS agar with a spiral-plater (Autoplate5000, Advanced Instruments, Norwood, Mass., U.S.A.). Plates were incubated for 24 to 48 h at 30 °C, and colonies were counted using an automated plate reader (QCount, Spiral Biotech model 510, Advanced Instruments). Cucumber juice (CJ) was prepared essentially as described by Breidt and Caldwell (2011). Briefly, size 2B pickling cucumbers (approximately 3.5 to 3.8 cm in diameter) were obtained from a commercial source. The cucumbers were blended to a slurry, then frozen at –20 °C. Upon thawing, the slurry was clarified by centrifugation at 10000 × *g* in a Sorvall GSA rotor (Thermo Fisher Scientific, Waltham, Mass., U.S.A.) for 10 min, 10 °C and filtered through a 0.45 μm pore-size bottle filter (168-0045, Thermo Fisher). CJ was used undiluted or diluted 50% with water containing NaCl and organic acids as described below.

Acid resistance assay

For acid resistance determination, solutions of acetic acid (420 mM, concentrated acetic acid stock obtained from Mount Olive Pickle Company, Mount Olive, N.C., U.S.A.) or sodium L-lactate (360 mM, Sigma-Aldrich Chemistry) were prepared in saline (NaCl, at 0.85%) with 20 mM D-gluconic acid as a non-inhibitory buffer (chemicals from Sigma-Aldrich) with deionized water. Overnight cultures of cells were prepared in MRS broth at 30 °C as described above, 0.5 mL cells were centrifuged at 10000 × *g* (as above) for 10 min at room temperature. Cell pellets were suspended in an equal volume of 0.85% NaCl solution (saline) and were put into 4.5 mL of the acid solution and held for 1 h at 30 °C. Following incubation, the cell suspensions were centrifuged as described above and pellets resuspended in an equal volume of MOPS buffer to neutralize pH followed by serial dilutions in saline and plating on MRS agar. Cell counts after acid treatment were compared with counts for cells incubated in saline (as a control). Log_{10} CFU/mL reduction was determined by the difference of initial and final Log_{10} CFU/mL.

Growth rates

Cell pellets obtained from overnight cultures (as described above) were suspended in 1.5 mL of CJ containing 0.37 mM (2.2%) NaCl. Cells were diluted by adding 30 μL of this cell suspension with 270 μL of CJ with 2.2% NaCl. Then 20 μL of the diluted cell suspension was added 180 μL treatment solution in microtiter plates wells (Costar 3595 flat bottom 96 well plates, Thermo Fisher) with CJ containing 0.34 mM (2%) NaCl as a final concentration at 3 different pH initial values: 5.3, 4.2, and 3.8 (adjusted with HCl). For measuring the effect of salt (1% and 2% NaCl, and no salt control) on the growth of selected strains (see below), CJ was used without pH adjustment (approximately pH 6). Inoculated microtiter plate wells (200 μL) were overlaid with 50 μL autoclaved mineral oil (Sigma-Aldrich) to prevent

evaporation, and plates were incubated in a microtiter plate reader (Bioteck Instruments, Inc. Winooski, Vt., U.S.A.) for optical density (OD) measurements at 630 nm. The initial OD values were adjusted as needed to be between 0.05 and 0.1. The plates were incubated at 30 °C for 48 h, and shaken for 30 s before readings at 1 h intervals. Growth rates were calculated using a sequential processing algorithm with Matlab software as described by Yang and others (2015).

Strain differentiation

Bacterial strains were grown in MRS broth, harvested by centrifugation and resuspended in an equal volume of saline as described above. DNA was extracted using the Ultraclean Microbial DNA Isolation kit (MO BIO Laboratories, Carlsbad, Calif., U.S.A.) as described by the manufacture's protocol. DNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Fisher) with UV wavelengths of 260 and 280 nm. Random amplified polymorphic DNA polymeras chain reaction (RAPD PCR) was then done using an oligonucleotide primer, 5′-ACGCGCCCT-3′ (Integrated DNA Technologies, Coralville, Iowa, U.S.A.) with a 25 μL RAPD PCR solution containing 13 μL Master Mix for PCR (2X). The PCR reaction and gel electrophoresis was performed as described by Plengvidhya and others (2004). The gel was photographed and banding patterns normalized based on migration distance using Bionumerics software (Version 6.6, Applied Maths, Inc., Tex., U.S.A.). A dendogram to analyze similarity of the banding patterns was constructed using the unweighted pair group arithmetic mean method and the Pearson correlation coefficient (Bionumerics).

Biochemical analysis

High performance liquid chromatography (HPLC) analysis was done using a modification of the method of McFeeters and Barish (2003). Analytes were separated on an Aminex HPX-87H column (300×7.8 mm, Bio-Rad Laboratories, Hercules, Calif., U.S.A.)

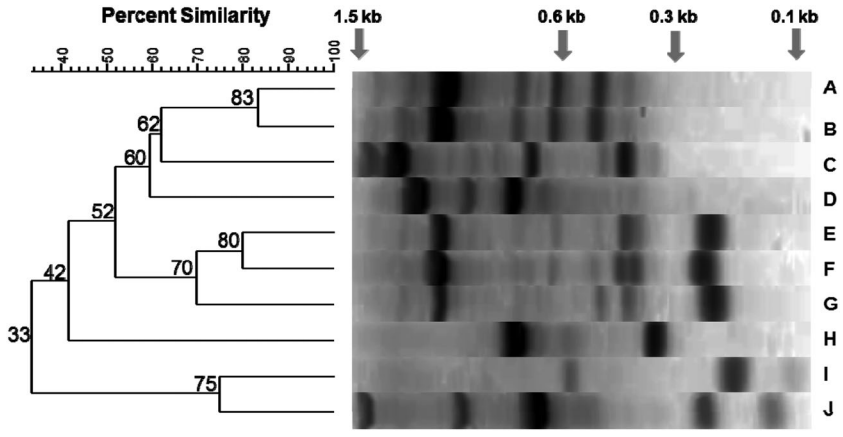

Figure 1–Dendogram of RAPD banding patterns for 10 probiotic LAB strains. Lanes: A, *L. plantarum* LA1198; B, *L. plantarum* LA1201; C, *L. pentosus* LA1203; D, *L. casei* LA284; E, *L. plantarum* LA1199; F, *L. plantarum* LA1202; G, *L. plantarum* LA1148; H, *L. rhamnosus* LA1134; I, *L. aciddophilus* LA1135; J, *P. acidilictici* LA1200. The percent similarity is shown by the numbers in the dendogram tree and the scale above. The arrows designate approximate kilobase (kb) band sizes based on 1 kb DNA ladder standards (not shown).

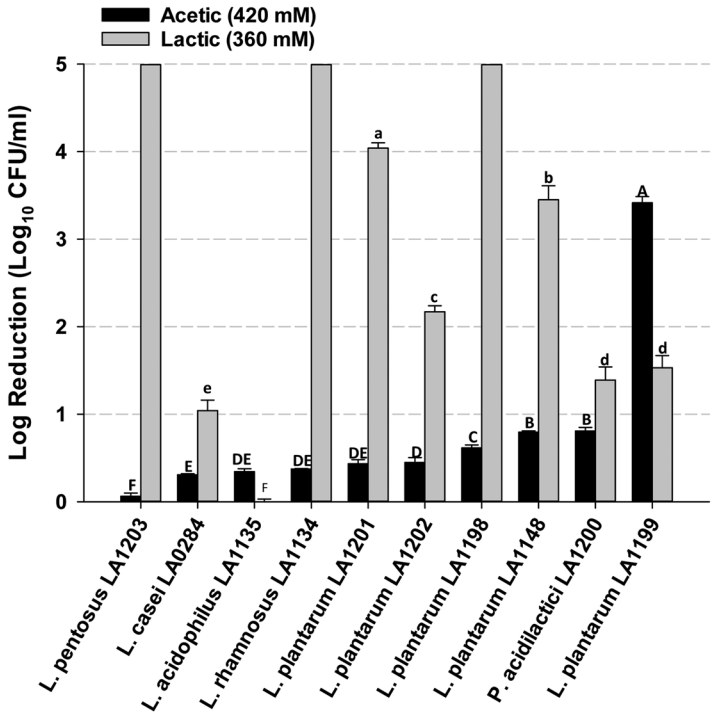

Figure 2–Acid sensitivity of probiotics to lactic and acetic acid solutions at 30 °C. The light gray bars represent $\log_{10}$ CFU/mL reduction with 360 mM lactic acid, and the dark gray bars represent $\log_{10}$ CFU/mL reduction with 420 mM acetic acid. The error bars represent the standard error for 3 or more measurements. Bars at the indicated detection limit had had too few colonies to count. The grouped bars were sorted by the acetic acid data. The upper case letters (acetic acid data) and lower case letters (lactic acid data) represent statistical significance for differences within a treatment ($P < 0.05$).

using a Shimadzu HPLC system (UFLC, Shimadzu Scientific Instruments, Durham, N.C., U.S.A.) with accompanying software. Separation was performed at 65 °C using 0.01 N H_2SO_4 for the mobile phase with a flow rate of 0.9 mL/min. An ultraviolet light detector (210 nm, RID-10A, Shimadzu) was used for quantification of malic, succinic, lactic, propionic, butyric, and benzic acids. In addition, acetic acid, glucose, fructose, ethanol, and glycerol were quantified using a refractive index detector (SPD-20A, Shimadzu) that was connected in series. Brine pH values were determined using a pH meter (Accumet AB150, Fisher Scientific Co., Pittsburg, Pa., U.S.A.).

Survival in simulated refrigerated pickle brine

Selected cell cultures, consisting of 2 acid resistant strains(LA284, LA1135) and 2 acid sensitive strains (LA1198, and LA1203) were grown overnight in 10 mL MRS broth. LA1135

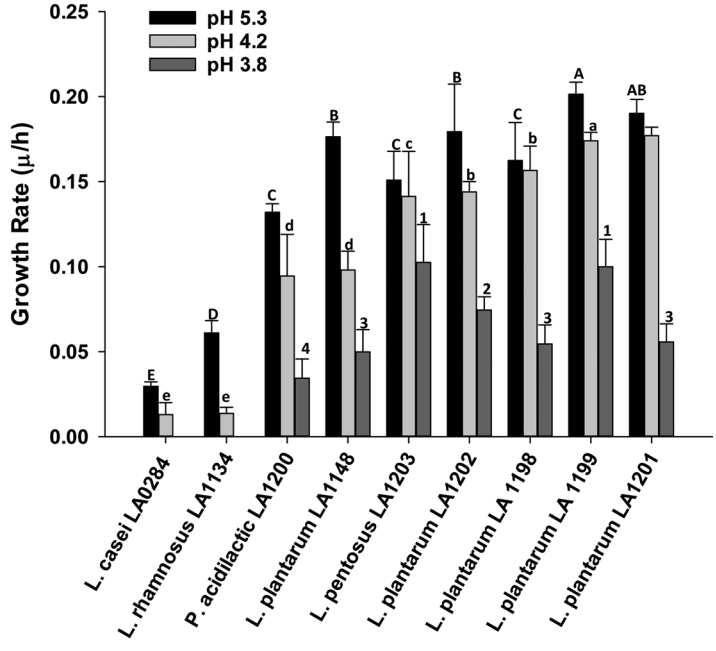

Figure 3–Specific Growth rate (μ, h^{-1}) data for the probiotic cultures in CJ containing 2% NaCl at 30 °C for 3 initial pH values. The bar color indicates the pH value: dark gray. pH 5.3; light gray, pH 4.2; medium gray, pH 3.8. The error bars indicate the standard error for 3 or more measurements. The bars for each strain were sorted by pH 4.2 growth rates. The upper case letters (pH 5.3 data), lower case letters (pH 4.2 data) and numbers (pH 3.8 data) represent statistical significance for differences within a treatment ($P < 0.05$).

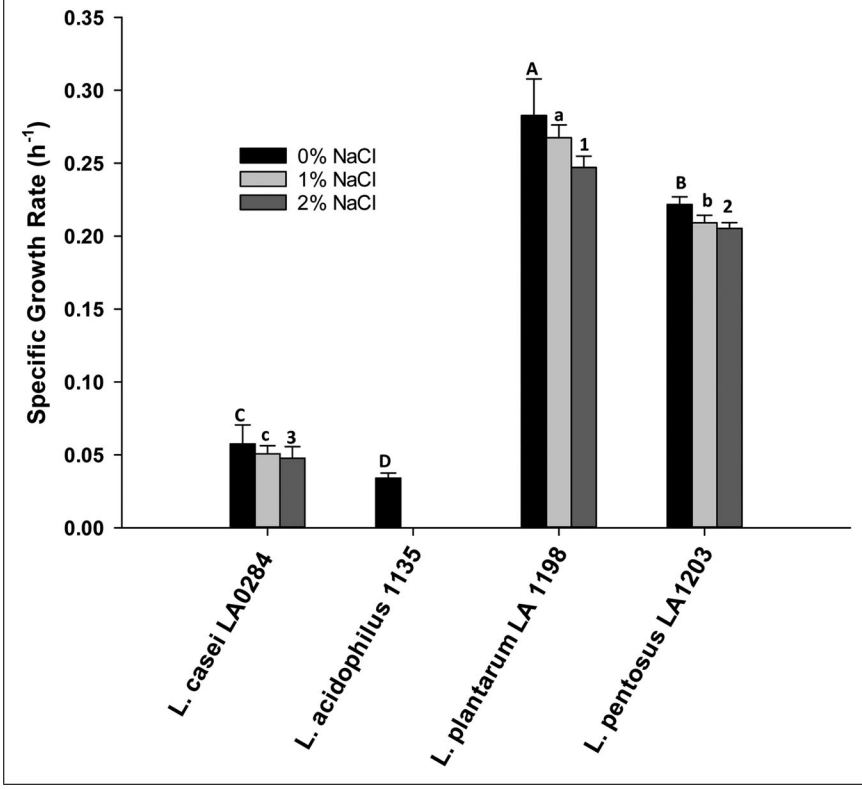

Figure 4–Specific Growth rate (h^{-1}) data for selected probiotic cultures in CJ at pH 6, 37 °C with 3 salt levels values. The bar color indicates the salt concentration value: black, no salt; light gray, 1% NaCl; dark gray, 2% NaCl. The error bars indicate the standard error for 3 or more measurements. The upper case letters (no salt data), lower case letters (1% NaCl) and numbers (2% NaCl) represent statistical significance for differences within a treatment ($P < 0.05$).

was grown anaerobically with 2 consecutive overnight cultures, to encourage better growth. Cells were harvested by centrifugation and resuspended in an equal volume of saline as described above. An aliquot of each culture (9 mL) was added (separately) to 81 mL of 50% CJ, resulting in 90 mL of a cell suspension at 10^7 to 10^8 CFU/mL. The CJ brine (CJB) solution was based on a commercial brine formulation for refrigerated pickles, with approximately 100 mM acetic acid, 6 mM sodium benzoate, and 2% NaCl, at pH 4.3. The 4×90 mL cultures were incubated in an anaerobic chamber at 30 °C for 48 h. Aliquots of the cultures (10 mL), were then aseptically dispensed into vacutainer tubes using sterile 10 mL syringes. The tubes were removed from the anaerobic chamber, and replicate ($n = 3$) tubes of each of the 4 cultures were incubated at 4 °C or 14 °C for up to 63 d. Culture samples (1 mL) were removed with a syringe through the vacutainer tube septum and plated on MRS agar at the indicated time intervals following serial dilutions in saline.

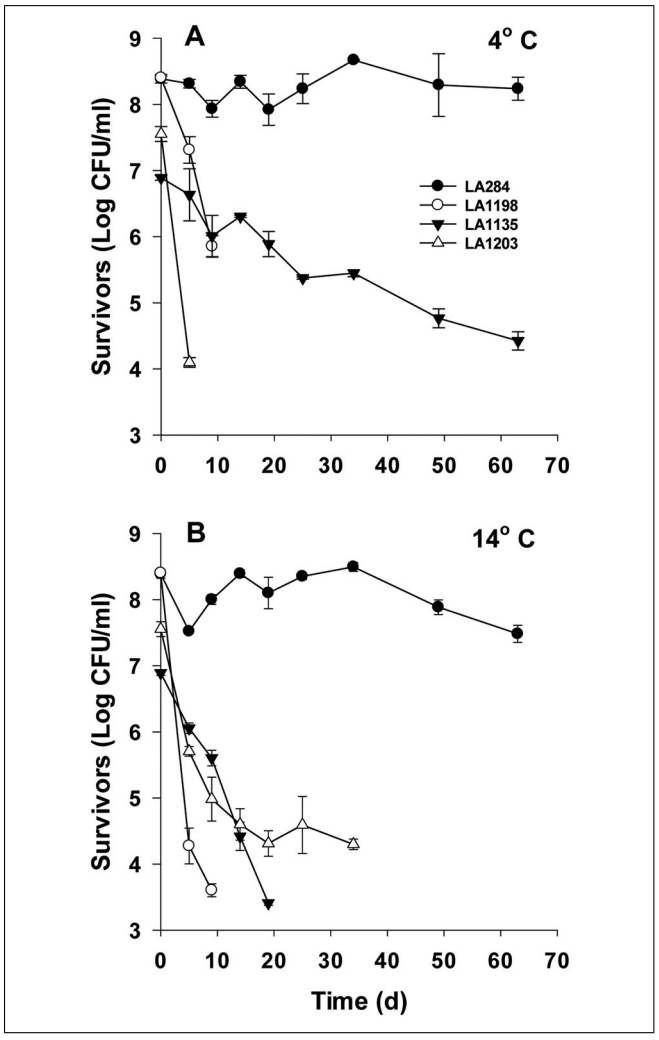

Figure 5–Survival of selected probiotic cultures. Survival was determined at 2 temperatures, 4 °C (A) and 14 °C (B). The solid fill symbols represent cultures selected on the basis of acid resistance, and the open symbols were cultures selected as acid sensitive: filled circles, *L. casei* LA284; filled downward triangle, *L. acidophilus* LA1138, open circle, *L. plantarum* LA1198; and upward open triangle *L. pentosus* LA1203. The error bars represent the standard error of 3 or more measurements. The dashed line indicates the temperature shift from 30 °C to the temperature indicated in the graph (at 2 d).

Statistical analysis

Three or more replications were used for all experiments. Analysis of variance was carried out using statistical analysis software (SAS, SAS Institute, Cary, N.C., U.S.A.). The Waller-Duncan k-ratio *t* test was used to analyze the significance differences for growth rate and log reduction data at the 0.05 probability level.

Results and Discussion

The genetic relatedness of the 10 probiotic LAB as determined by RAPD is shown in Figure 1. RAPD is a rapid and efficient method for strain differentiation that has been used for LAB from vegetable fermentations (Power 1996; Plengvidhya and others 2004). Strain differences were apparent between species, although similarities within a species were evident. Two *Lactobacillus plantarum* strains, LA1198 and LA1201, had 83% similar banding patterns, and the remaining 3 *L. plantarum* strains, LA1199, LA1202, and LA1148 were 70% to 80% related by the random prime banding patterns. Among the remaining species, *Pediococcus acidilactici* LA1200, *L. acidophilus* LA1135 were similar (75%) to each other but different from the other LAB species (33%). These data indicate that the strains from different sources were genetically distinct from each other, and replicate strains were not present among the probiotic cultures studied.

To determine the acid resistance of the probiotics, the 10 strains were subjected to acid challenge with both lactic and acetic acids, because these acids are present in fermented and acidified vegetable products, and influence flavor, microbiota, and safety (Fleming and others 2001; McFeeters 2004; Breidt 2006). For this acid resistance assay, we used acid concentrations that were found to result in a measurable $\log_{10}$ reduction values within 1 h of acid treatment. For lactic acid (Figure 2), 360 mM acid resulted in a greater than 5-$\log_{10}$ reduction for 3 of the strains at pH 3.2, *L. rhamnosus* LA1134, *L. pentosus* LA1203, and *L. plantarum* LA1198. *L. acidophilus* LA1135 had no detectable reduction, and *L. casei* LA284 had approximately 1 $\log_{10}$ reduction, with the remaining strains showing between a 1.3 and 3.4 $\log_{10}$ CFU/mL reduction. Results for acetic acid with 360 mM (data not shown) indicated that there was no appreciable log reduction for any of the strains, except *L. plantarum* LA 1148 (0.2 $\log_{10}$ reduction), so the acetic acid concentration used in the assay was increased. With 420 mM acetic acid (Figure 2), most strains had $\log_{10}$ reduction values of less than 1 $\log_{10}$ while *L. plantarum* LA1199 had approximately a 3.3 $\log_{10}$ reduction. Two cultures, *L. casei* LA284 and *L. acidophilus* LA1135 were resistant to both acid conditions tested, showing approximately 1 $\log_{10}$ reduction or less. These strains had no significant difference in acetic acid resistance. Interestingly, *L. pentosus* LA1203 was one of the least resistant to lactic acid (>5-$\log_{10}$ reduction) and was also among the most resistant to acetic acid (<0.1 $\log_{10}$ reduction) for the acid conditions tested. Similarly the strain most sensitive to acetic acid, *L. plantarum* LA1199, was among the 3 strains most resistant to lactic acid. It is unclear why there were acid-specific differences in log reduction values, and this will be the subject of future research.

Growth rates of the 10 strains were examined using a microtiter plate method with CJ containing 2% NaCl at 3 different starting pH values (5.3, 4.2, and 3.8) (Figure 3). No growth was observed for *L. acidophilus* LA1135 for any pH, and strains *L. casei* LA284 and *L. rhamnosus* LA1134 had no measurable growth with an initial pH 3.8. In general, decreasing initial pH reduced the measured specific growth rate, although 3 strains had no statistically significant difference in growth rate between initial pH values of 5.3

Table 2–Biochemical analysis of CJB fermented by selected cultures.

Culture	Time (d)[b]	Temp. (°C)[c]	pH[d]	Concentration [mM][e]			
				Acetic	Lactic	Glucose	Fructose
NA[a]	0	NA	4.2	97.49 ± 0.20	–	24.5 ± 0.10	31.3 ± 0.10
LA0284	5	4	3.9	92.5 ± 0.29	25.7 ± 1.07	14.6 ± 0.43	25.7 ± 0.17
LA0284	63	4	3.8	87.8 ± 0.29	43.7 ± 2.74	12.9 ± 0.53	19.2 ± 0.37
LA0284	5	14	3.9	92.6 ± 0.21	29.7 ± 1.37	12.9	24.8 ± 0.28
LA0284	63	14	3.5	88.3 ± 0.10	65.7 ± 1.70	–	12.4 ± 0.95
LA1135	5	4	4.1	92.9 ± 0.50	9.2 ± 1.09	20.3 ± 0.46	28.6 ± 0.25
LA1135	63	4	4.2	88.3 ± 0.06	8.8 ± 0.96	18.7 ± 0.40	26.4 ± 0.16
LA1135	5	14	4.1	92.7 ± 1.20	10.4 ± 1.01	19.9 ± 0.47	28.3 ± 0.21
LA1135	19	14	4.2	82.1 ± 0.09	12.1 ± 1.17	22.1 ± 3.13	29.1 ± 2.78
LA1198	5	4	3.4	93.6 ± 0.81	101.7 ± 1.31	–	–
LA1198	9	4	3.5	84.1 ± 1.64	89.3 ± 0.71	–	–
LA1198	5	14	3.4	92.6 ± 0.44	101.0 ± 0.72	–	–
LA1198	9	14	3.4	83.8 ± 1.68	89.1 ± 1.06	–	–
LA1203	5	4	3.4	93,5 ± 0.31	100.0 ± 0.69	–	–
LA1203	5	14	3.4	93.7 ± 0.44	101.9 ± 0.84	–	–
LA1203	34	14	3.5	88.5 ± 0.23	90.5 ± 0.48	–	–

[a]NA, Not applicable (analysis of broth prior to fermentation).
[b]d, Time in days since inoculation (including 48 h fermentation at 30 °C).
[c](°C), temperature of refrigerated storage in °C.
[d]pH of the CJB, standard deviation was at or below 0.1 pH units for all measurements.
[e][mM], Concentration of each metabolite in mM units ± standard deviation, the dashed lines (–) indicate values below the limit of detection (<5 mM for 1:10 dilution of samples).

or 4.2 (*L. plantarum* strains LA1201, LA1202, LA1198, and *L. pentosus* 1203) (Figure 3, data not shown).

Based on the results for acid resistance and growth rates, 2 of the most acid resistant strains, *L. acidophilus* LA1135 and *L. casei* LA284, and 2 of the most acid sensitive strains, *L. plantarum* LA1198, and *L. pentosus* LA1203 were selected for further analysis. We re-examined the growth of these 4 strains, at 37 °C, because *L. acidophilus* strains are known to prefer temperatures of 37 °C or greater for growth (Medvedova and others 2016), and because no growth was observed for this strain at 30 °C with 2% NaCl with this strain. For this experiment, the effect of NaCl concentration on growth was also considered, and the data shown in Figure 4. *L. acidophilus* LA1135 was found to grow slowly (0.04 u, h^{-1}) in CJ only in the absence of added NaCl. There was little difference in growth rates based on NaCl for the other 3 strains, with only a 0.05 difference in growth rate between 0% and 2% NaCl for *L. casei* LA284. However, for the 2 salt treatments and no salt controls, growth rates varied significantly between strains with the 2 acid resistant strains having the lowest growth rates for all treatments.

A pair of acid resistant (*L. acidophilus* LA1135 and *L. casei* LA284) and of acid sensitive strains (*L. pentosus* LA1203 and *L. plantarum* LA1198) were inoculated into CJB and incubated anaerobically for 48 h at 30 °C, following the procedure similar to that of Pérez-Díaz and McFeeters (2011), which we previously found may result in long-term (>1 mo) survival of a probiotic *Lactobacillus* strain (data not shown). After growth in the anaerobic chamber for 2 d, the cultures were transferred into sterile vacutainer tubes (under anaerobic conditions), followed by refrigeration for up to 63 d (Figure 5A and B). As expected, *L. acidophilus* LA1135 did not show evidence of growth in CJB (with 2% NaCl), and had little or no change in cell numbers (approximately $\log_{10}$ 7) from the initial inoculum value. However, this culture showed only an approximate 2 log reduction after 63 d at 4 °C. At 14 °C LA1135 cell counts were below the limit of detection after 20 d (Figure 5B). The other acid resistant culture, *L. casei* LA284 reached $\log_{10}$ 8.2 during growth, and had no significant reduction in cell number for up to 63 d at 4 °C. This high concentration $\log_{10}$ 8 CFU/mL of cells present after 63 d would likely allow a commercial probiotic pickle product to meet standards required for effective probiotic products of $\log_{10}$ 6 to $\log_{10}$ 8 CFU/mL in a food product (Sanders and Marco 2010), although further work would be needed with commercial products to determine consumption levels for probiotics in the products. Interestingly, this *L. casei* strain was previously found to survive in yogurt at concentrations greater than 6-log CFU/mL for more than 1 mo at 4 °C (Sidira and others 2013), and another *L. casei* strain (*L. casei* DN-114 001) was found have to survive with greater than 6-log CFU/mL in fruit juice for up to 12 wk at 4 °C (Sheehan and others 2007). At 14 °C LA284 had approximately a 1 log reduction during the same time (Figure 5, to approximately $\log_{10}$ 7.5CFU/mL for 63 d). The cultures previously determined to be acid sensitive (as shown in Figure 2), however, showed reduced survival in comparison to LA284 at 4°, with both cultures being below the level of detection (less than log 2.5 CFU/mL with the spiral plater) within 14 d. At 14 °C LA1198 was below detection level within 14 d. LA1203, however, had a 3.2 log reduction within 20 d, then remained essentially unchanged ($\log_{10}$ 4.5 CFU/mL) for the next 15 d. The cell counts for LA1203 were below the level of detection by 49 d at 14 °C.

The chemistry of the CJB was analyzed at selected times (before fermentation, 5 d after inoculation and after the cells were below the limit of detection) for each of the cultures in the long-term survival assay (Table 2). The initial CJB contained approximately 100 mM acetic acid and 2% NaCl, similar to commercial formulations as described (Fleming and others 2001; Lu and others 2013). As expected, benzoate concentration did not change during incubation (data not shown). Benzoate was included at 0.1% to be compatible with commercial refrigerated pickle formulations. The glucose and fructose concentrations in the brine prior to fermentation were 25 and 31 mM, respectively. Following the temperature shift (after fermentation for 48 h at 30 °C) to 4 or 14 °C, the 2 acid sensitive cultures (LA1198 and LA1203) had no detectable sugar remaining after 5 d and had produced

approximately 100 mM lactic acid (Table 2). The acid resistant cultures, however (LA284 and LA1135), had between 12 and 20 mM glucose and approximately 25 to 29 mM fructose remaining after 5 d, depending on refrigerated incubation temperature (4 or 14 °C, respectively), which was expected because of the negligible growth rate (Figure 4). The reduced acid compared to the acid sensitive cultures likely aided in the extended survival of the acid resistant cultures during long-term (63 d) incubation. At 4 °C sugar concentrations continued to be reduced for LA284 to below the detection limit for glucose and 12.4 mM fructose, respectively. Correspondingly, the pH for LA284 showed a small decline (a decrease of 0.1 pH units) at 4 °C and the lactic acid concentration increased from 26 to 66 mM at 14 °C with a decrease to pH 3.5 (Table 2). LA1135 had between 8 and 10 mM lactic acid produced during fermentation and showed little or no evidence of metabolic activity after fermentation between 5 and up to 63 d of incubation. As expected, the pH of the brine remained similar to the initial pH of the medium (pH 4.2). For the acid sensitive strains (LA1198 and LA1203) lactic acid concentration increased to about 100 mM with a corresponding drop in pH to 3.5, consistent with a complete fermentation of the sugars. Both lactic and acetic acid decreased somewhat (10 mM or less) during incubation up to 34 d, and the pH remained at 3.4 to 3.5 after the temperature shift.

Conclusions

Probiotic pickled vegetable may be desirable for consumers who are intolerant to dairy based foods, and they are, in general, lower in calories than dairy products. An ideal refrigerated shelf life for a probiotic pickle product would likely be between 2 and 4 mo, assuming cell numbers of the probiotic culture could be maintained at sufficiently high enough levels, estimated at $\log_{10}$ 6 to $\log_{10}$ 8 CFU/mL, in the product. We have developed methods for selecting cultures for probiotic pickle products. We used a novel process that involved inoculating a commercial refrigerated pickle brines with $\log_{10}$ 7 CFU/mL of a probiotic culture followed by a fermentation period of 48 h, then 2 mo storage at refrigeration temperatures. Previously we have found that this method resulted in the survival of a probiotic culture in commercial refrigerated pickles (refrigerated dill pickle spears) for greater than 1 month (data not shown). Two acid resistant cultures selected from this study were found to survive in simulated brine (CJB) for up to 63 d at 4 °C, with 1 culture (*L. casei* LA284) having little or no loss in viability at greater than $\log_{10}$ 8 CFU/mL. It is likely that the relatively slow rate of fermentation by the acid resistant culture resulted in reduced production of lactic acid and higher pH (and therefore enhanced survival) compared to the acid sensitive cultures. The data show the *L. casei* LA284 may be effective as a probiotic culture for the process described above. Additional research includes methods for extending shelf life of a refrigerated probiotic pickle products not involving fermentation, and will be presented in a companion paper by Cauley and others, (unpublished).

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