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Research Paper

Effects of Sodium Chloride or Calcium Chloride Concentration on the Growth and Survival of *Escherichia coli* O157:H7 in Model Vegetable Fermentations

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ABSTRACT

Salt concentration has long been considered an important factor for the quality of fermented vegetable products, but the role of salts in bacterial growth and death during vegetable fermentation remains unclear. We compared the effects of various sodium chloride (NaCl) concentrations, including 1 M (6%) NaCl used in commercial cucumber fermentations and 0.34 M (2%) NaCl used in cabbage and other ready-to-eat vegetable fermentations, on the growth and death of lactic acid bacteria (LAB) of the genus *Lactobacillus* and pathogenic *Escherichia coli* (Shiga toxin–producing *E. coli,* or STEC) strains. We also investigated calcium chloride ($CaCl_2$) salt conditions. $CaCl_2$ is being used at 0.1 M (1.1%) in low-salt commercial cucumber fermentations that lack added NaCl. STEC strains have previously been shown to be among the most acid-resistant pathogens in fermented or acidified vegetables. The data showed that 1.1% $CaCl_2$, and especially 1% NaCl, had a stimulatory effect on the growth rates of STEC and LAB compared with a no-salt control, but higher NaCl concentrations decreased growth rates for STEC; to a lesser extent, LAB growth rates were also reduced. For most salt concentrations tested, maximum cell densities achieved during growth of STEC were reduced compared with those of the no-salt controls, whereas LAB mostly had cell densities that were similar to or greater than those of the no-salt controls. No consistent pattern was observed when comparing death rates with salt type or concentration for the STEC or LAB cocktails undergoing lactic acid stress (50 or 350 mM, respectively) at pH 3.2 and when comparing STEC survival in competitive culture experiments with LAB. For vegetable fermentation safety concerns, the results suggest that an important effect of salt addition is enhancement of the growth of LAB compared with STEC strains. Further research will be needed to determine factors influencing STEC survival in competition with LAB in vegetable fermentations.

HIGHLIGHTS

- NaCl and $CaCl_2$ concentrations affected LAB and STEC strains differently.
- Growth rates at 6% NaCl were reduced for STEC more than LAB in vegetable broth.
- Extent of growth was reduced for STEC versus LAB for most vegetable fermentations.
- Death rates were minimally affected by salt type or concentration with lactic acid.
- Correlations between salt and STEC die-off were inconsistent for fermentation.

Key words: Calcium; *Escherichia coli*; Microbial competition; Salt; Sodium; Vegetable fermentations

Pathogenic bacteria may be present on fresh vegetables *(38),* including cucumbers, peppers, and other vegetables that are commonly used for fermented and acidified vegetable products. During vegetable fermentations, aerobic (spoilage) microbiota, including those in *Pseudomonas, Bacillus, Serratia,* and other genera, will die off in competition with lactic acid bacteria (LAB) as acid accumulates in fermentation brines and dissolved oxygen becomes limited. Acid-resistant pathogens, such as *Escherichia coli,* may grow and survive for days or weeks in fermenting vegetables, depending on strain characteristics and acid conditions in the brine *(6)*. Shiga toxin–producing *E. coli* (STEC) strains, including O157:H7 and related serotypes, had been found to be among most acid-resistant bacterial pathogens in fermented fruit and vegetable products, including fermented cucumbers, cabbage, and apple cider *(6, 20, 41)*. In addition to pH and organic acids, sodium chloride (NaCl) is commonly considered an important safety factor affecting the die-off of microbial pathogens in fermented vegetables. However, the reported

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TABLE 1. *Bacterial strains*[a]

Strain ID	Previous ID	Genus	Source	Reference or supplier
LA471		*Lactobacillus paracasei*	Cucumber fermentation	*12*
LA023	WSO	*L. plantarum*	Olive fermentation	*11*
LA513		*L. brevis*	Cucumber fermentation	FSRU
LA516		*L. plantarum*	Cucumber fermentation	FSRU
LA445	MOP3	*L. plantarum*	Cucumber fermentation	*16*
B200	ATCC 43888	*Escherichia coli* O157:H7	Human feces (*tox*−)	ATCC
B201	SRCC 1675	*E. coli* O157:H7	Apple cider	SRCC
B202	SRCC 1486	*E. coli* O157:H7	Salami outbreak	SRCC
B203	SRCC 2061	*E. coli* O157:H7	Ground beef	SRCC
B204	SRCC 1941	*E. coli* O157:H7	Pork	SRCC
B241	28RC1	*E. coli* O157:H7	Bovine carcass	*4*

[a] ID, identification; FSRU, U.S. Department of Agriculture–Agricultural Research Service, Food Science Research Unit, Raleigh, NC; ATCC, American Type Culture Collection, Manassas VA; SRCC, Silliker Mérieux NutriSciences, Chicago, IL.

effects of NaCl on the survival of STEC in acid solutions varied from antagonistic to synergistic, based on experimental conditions *(2, 3, 10, 42)*. The literature is lacking documentation on how salt concentrations typical of vegetable fermentation brines may influence pathogen growth, death, and survival in competition with LAB during the fermentation of vegetables.

Typically, commercial cabbage fermentations, such as sauerkraut and kimchi fermentations, have around 0.34 M NaCl (2%), while commercial cucumber fermentations have around 1 M NaCl (6%) *(15, 39)*. NaCl concentrations in fermenting vegetables have been shown to influence the microbiota and quality of sauerkraut, with 2% being optimum for high-quality sauerkraut *(39)*. NaCl concentration has also been found to correlate with tissue firmness in fermented cucumbers, although tissue firmness may also be influenced by the fermentation microbiota *(36)*. NaCl has been shown to enhance the pH stability during bulk storage of fermented cucumbers *(21)*. For example, a low-salt (2.3%) cucumber fermentation in an anaerobic tank resulted in secondary (butyric acid) spoilage fermentation after several months of storage *(14)*.

In addition to NaCl, calcium chloride ($CaCl_2$) has long been used as a firming agent in pickled vegetables, including cucumbers and peppers *(13, 17)*. The effects of $CaCl_2$ on cucumber texture has been investigated *(22, 32, 34)*. However, there is some controversy about the mechanism by which calcium inhibits vegetable tissue softening *(33)*. Recently, in an effort to reduce NaCl effluent from commercial cucumber fermentation plants, a method was developed for replacing 6% NaCl (6N) with 1.1% $CaCl_2$ (1.1C) as the main salt in the fermentation *(35, 37, 40)*. The method involves the use of both $CaCl_2$ and calcium acetate in the brine without the addition of NaCl. Cucumbers fermented on a commercial scale with 1.1C, have been shown to have similar product quality to NaCl fermentations *(37, 45);* however, the effects of $CaCl_2$ versus NaCl on the competitive inhibition of acid-resistant pathogens has not been determined.

There has been a resurgence of interest in fermented vegetables in the United States, as well as an influx of traditional fermented vegetable products from Asia and other parts of the world. We investigated the effects of salt type ($CaCl_2$ or NaCl) and concentration on fermentation safety under conditions typical of commercial vegetable fermentations in the United States. For these studies, we used a cucumber juice (CJ) medium as a representative medium for fermented vegetables, because CJ has no known antimicrobial properties to hinder bacterial growth or survival. Lactobacilli were the LAB chosen for the study; these organisms are known to complete most vegetable fermentations because of the ability to grow under acid conditions *(15, 30)*. We investigated the growth and death of STEC and selected *Lactobacillus* strains under varying salt conditions in CJ medium. We also examined two competitive growth scenarios with selected *Lactobacillus plantarum* and STEC strains with different salt treatments. Our objective was to determine how $CaCl_2$ or NaCl influenced the microbial safety of vegetable fermentations.

MATERIALS AND METHODS

Preparation of CJ and salt solutions. Cucumbers (3.5 to 4 cm in diameter, obtained from commercial sources) were cut into 1-inch cubes, placed in a blender, and blended to a homogenous slurry. The slurry was frozen at –20°C for at least 18 h to help improve juice yields. After thawing to room temperature, pulp was removed by filtering the slurry through cheesecloth (bleached, grade 90; DeRoyal Textiles, Camden, SC). The slurry was then centrifuged in 250-mL aliquots (Sorvall GSA rotor, Dupont Instruments, Newton, CT) at 4,000 × *g* at 20°C for 45 min. The supernatant was refiltered using cheesecloth before filtration with a 0.2-μm-pore-size sterile bottle filter (250 mL; VWR International, Radnor, PA). The juice was diluted with deionized water containing salts to 50% final concentration (CJ) with or without the addition of salts as described later.

Culture preparation and enumeration. Five LAB strains from *Lactobacillus* and six STEC O157:H7 strains (Table 1) were grown (for approximately 16 h) independently in 5-mL broths: de Man Rogosa Sharpe (MRS) broth (Difco, BD, Sparks, MD) at 30°C and Luria-Bertani (LB) Miller formulation broth (Difco) at 37°C for LAB and STEC, respectively. Cultures of like species were combined to form inoculation cocktails. Culture cocktails were centrifuged using a Sorvall SS-34 rotor (Dupont Instruments) at 3,000 × *g* for 10 min at 10°C, and the supernatants were removed. Cells were resuspended and concentrated with 0.1 volume of saline, and aliquots were then added to CJ medium as

described later. For bacterial cell counts, samples (0.1 mL) were serially diluted using 1% morpholine–propanesulfonic acid (MOPS)–saline (0.85%) buffer at pH 7 before plating with a spiral plater (model AP5000, Advanced Instruments, Norwood, MA). Cells were plated and incubated on the appropriate agar media: MRS agar for *Lactobacillus* (48 h, 30°C) or LB agar for *E. coli* (24 h, 37°C). Colonies were counted using an automated plate counter (Q-Count, Advanced Instruments), with a limit of detection of approximately 10^2 CFU/mL. Using this method, selective enumeration of LAB and STEC from mixed-culture brines (from competitive culture experiments) was achieved, because LAB did not form countable colonies on LB agar at 37°C in 24 h, and similarly, STEC did not form colonies on MRS agar at 30°C in 48 h.

Salt effects on growth. Cell cocktails or individual strains (LAB or STEC) in 0.85% NaCl were added (4 μL of resuspended culture) to 200 μL of CJ in 300-μL microtiter plate wells (Costar 3595 flat-bottom 96-well plates, Thermo Fisher, Fair Lawn, NJ) to give an initial optical density of approximately 0.03 units above the no-cell control, typically less than 0.1 optical density at 600 nm. The CJ in the microtiter plate wells had salt concentrations that were coded as 0.55% $CaCl_2$ (0.55C), 1.1% CaCl2 (1.1C), 1% NaCl (1N), 2% NaCl (2N), 4% NaCl (4N), and 6N. The microtiter plate wells containing 204 μL of CJ with cell suspensions were overlaid with 50 μL of mineral oil (Sigma-Aldrich, St. Louis, MO) to prevent evaporation. The plates were incubated in a microtiter plate reader (model ELx808, BioTek Instruments, Inc., Winooski, VT) at a constant temperature of 30 or 37°C (as indicated later) for 24 to 48 h, until stationary phase was observed. Optical density measurements at 600 nm were automatically taken every 2 h following a 10-s shake to resuspend cells. Controls with 4 μL of sterile saline in place of cells for each medium type that was used were included in the design. Growth rates were calculated from the optical density data, which was exported from the reader software to a spreadsheet file, imported into Matlab, and then analyzed using a custom Matlab program (F.B., personal communication) that implemented the sequential processing algorithm of Breidt et al. *(7)*.

Survival in salt and acid. CJ medium was prepared with three added salts, 2N, 6N, or 1.1C, and sodium L-lactate (Aldrich Chemical Co., St. Louis, MO), which was added at 350 mM (for LAB) or 50 mM (for STEC) concentrations to allow similar log reductions, and the pH was adjusted to 3.2 with hydrochloric acid. The media (100 mL for each salt solution) were filter sterilized before use. All CJ media for these experiments were stored at 30°C for 24 to 48 h in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) with the bottle caps loose to allow gas exchange and removal of dissolved oxygen *(28)*. Aliquots (20 mL) of each CJ solution were transferred by syringe through the rubber septum of three 25-mL Vacutainer tubes (BD, Franklin Lakes, NJ). Acid concentrations were adjusted to equilibrate at the desired concentration mentioned previously after the addition of 0.2 mL of cells. Cocktails of *Lactobacillus* or *E. coli* strains were prepared as described earlier, and cells were inoculated into three replicates for each CJ-salt solution. The initial cell concentration was approximately 10^8 CFU/mL. An uninoculated control treatment was also prepared. The inoculated tubes were removed from the anaerobic chamber and incubated at 30°C (to avoid room temperature fluctuations). Sampling was done by syringe through the rubber septum so as not to introduce oxygen into the Vacutainer tube. For each of the indicated sampling time points, approximately 0.2 mL of sample was removed using 1-mL tuberculin syringes (BD), and 0.1 mL of sample was diluted immediately in 0.9 mL of 1% MOPS–saline (0.85%) buffer. Serial 10-fold dilutions were plated on MRS agar or LB agar, and plates were incubated at 30 or 37°C for 48 to 24 h, respectively. Colonies were counted after incubation using the automated plate counter as described earlier.

Competitive growth assays. Overnight cultures of LA445 (*L. plantarum*) and *E. coli* O157:H7 B200 (*tox*–) or B241 were prepared in 5 mL of MRS and LB broth at 30 and 37°C, respectively, for approximately 16 h. Cells were harvested by centrifugation (SS34 rotor at 3,000 × *g* for 10 min at 10°C) and then resuspended in 0.1 volume of CJ medium (with no salt). Using the anaerobic chamber procedure described earlier, an aliquot of 0.2 mL from each of the paired cultures (LA445 and either B200 or B241) was inoculated together into 20 mL of CJ in a 25-mL Vacutainer tube (BD) at an initial concentration of 10^4 to 5×10^6 CFU/mL. CJ was prepared as described earlier for the 6N, 2N, and 1.1C salt treatments. The Vacutainer tubes were removed from the anaerobic chamber and were incubated statically at 30°C, along with the uninoculated controls. For each of the indicated sampling time points, 1.5 to 2 mL of each sample was removed with a 3-mL syringe (BD), and 0.1 mL of each sample was immediately serially diluted in 0.9 mL of 1% MOPS–saline (0.85%) buffer. Diluted samples were plated on LB or MRS agar. The plates were incubated at 37 or 30°C for 24 or 48 h (for LB or MRS, respectively). Plates were then enumerated with a Q-Count automated spiral plate counter as describe earlier. A 1-mL aliquot of each sample was centrifuged, and the supernatant was frozen at –20°C for subsequent high-performance liquid chromatography (HPLC) analysis and measurement of pH.

Biochemical analysis. HPLC analysis for organic acids was done using a modification of the method of McFeeters and Barish *(31)*. An Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad Laboratories, Hercules, CA) was used with a Shimadzu HPLC system (Ultra Fast Liquid Chromatograph [UFLC], Shimadzu Scientific Instruments, Durham, NC) and software. Analyte separation was done at 65°C with 0.01 N sulfuric acid as the mobile phase (0.9 mL/min). For detection of acids and sugars, a refractive index detector (SPD-20A, Shimadzu) was used. The detector was standardized with lactic acid, acetic acid, and glucose and fructose solutions in the range of 100 to 0.5 mM. Brine pH values were determined using a pH meter (Accumet AB150, Fisher Scientific Co., Pittsburg, PA) with a standardized glass electrode.

Protonated acid calculations. Protonated acid concentrations were calculated from the lactic acid concentration using a derivation of the Henderson-Hasselbalch equation, based on the total acid concentrations as measured by HPLC ($mM_{total\ acid}$):

$$mM_{protonated\ acid} = mM_{total\ acid} \Big/ \left(1 + 10^{(pH - pK_{adj})}\right) \quad (1)$$

The pK_a of lactic acid was adjusted (pK_{adj}) to account for the effects salts on the ionic strength in the brines. The Davies equation was used with the constants described by Butler and Cogley *(9)*:

$$pK_{adj} = pK_a - 1.02\left(\sqrt{I} \Big/ \left(1 + \sqrt{I}\right) - 0.3I\right) \quad (2)$$

where I was the ionic strength and the unadjusted pK_a for lactic acid was 3.86. Ionic strength for the salt brines was calculated as follows:

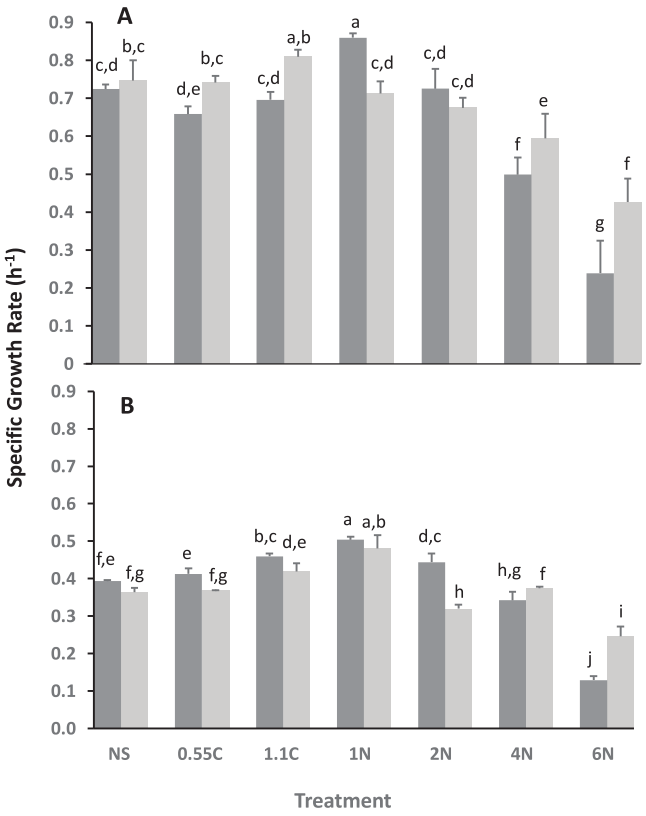

FIGURE 1. *Effect of salt on growth rate at 30 and 37°C in CJ medium. The growth rates of a cocktail of six STEC strains (dark gray bars) and five LAB strains (light gray bars) were measured at 37°C (A) and 30°C (B). Treatments were labeled on the* x *axis as NS, no added salt; 0.55C, 0.55% $CaCl_2$; 1.1C, 1.1% $CaCl_2$; 1N, 1% NaCl; 2N, 2% NaCl; 4N, 4% NaCl; and 6N, 6% NaCl. Lowercase letters indicate significant differences (*$P < 0.05$*), and error bars indicate the standard deviations.*

$$I = 1/2 \sum \left(i^2 \times c\right) \quad (3)$$

where i was the charge on an ion in solution and c was the concentration of an ion in solution.

Modeling and statistics. Three or more independent replications were used for all experiments, and microtiter plates had four repeated measures for each replicate, with the exception of individual strain growth rate measurements, which had one replication and 12 repeated measures. Modeling of killing curves was done using the second-order polynomial regression function in Microsoft Excel (Redmond, WA). Analysis of variance was carried out using statistical analysis software (SAS; SAS Institute, Cary, NC). 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

Effect of salt on growth. To determine how STEC and LAB strains were affected by various salt conditions, microtiter plate assays were used to generate growth rate data at 37 and 30°C (Fig. 1). Under optimum growth conditions for STEC at 37°C in CJ medium (Fig. 1A), treatment 1N had a significant increase in the growth rate compared with the no-salt control (0.86 and 0.72 h^{-1}, respectively) for STEC. However, as NaCl concentration increased to 6% (treatment 6N), the mean specific growth rates of *E. coli* were found to decreased to 0.24 h^{-1}. Mean STEC growth rates for the $CaCl_2$ treatments of 0.67 h^{-1} (0.55C) and 0.70 h^{-1} (1.1C) were not significantly different from that of the no-salt control (0.72 h^{-1}). The mean STEC and LAB growth rates were similar to each other at 37°C for the no-salt control, and LAB strains showed no significant difference between the no-salt control and the $CaCl_2$ treatments at this temperature. LAB growth rates were significantly greater than STEC rates for all salt treatments with the exception of 1N and 2N; however, LAB growth rates declined to 0.59 and 0.42 h^{-1} for treatments 4N and 6N (Fig. 1A), respectively.

At 30°C, representative of commercial cucumber fermentations (Fig. 1B), similar trends were seen for STEC growth as at 37°C, although growth rates were 0.2 to 0.3 h^{-1} slower for each treatment. Unlike the 37°C data, however, LAB growth rates were similar to or slower than those for STEC strains for all treatments except 4N and 6N. The mean LAB growth rate was approximately twice the STEC rate (0.13 compared with 0.25 h^{-1}) with treatment 6N, typical of most commercial brine fermentations. For both 37 and 30°C experiments, treatment 1N had the highest growth rate for STEC, including the no-salt control, indicating that this concentration of NaCl was stimulatory to growth for both species.

The individual growth rates for each of the STEC strains in the cocktail were compared at 37 and 30°C for selected treatments: no salt, 1.1C, 2N, and 6N (Fig. 2). The data showed that the growth rates of each strain were similar to each other with the exception of strain B200, which had slower average growth rates than the other strains for all conditions except for 2N at 37°C. As was apparent for the strain cocktails, treatment 6N, the typical salt concentration of commercial cucumber fermentations, inhibited growth of STEC, and treatments 1.1C and 2N were similar to the no-salt controls.

The maximum optical density achieved at the stationary phase at 37 and 30°C was significantly higher for LAB compared with STEC for all treatments, with the exception of treatment 0.55C at 30°C (Fig. 3). The magnitude of the difference was approximately twofold or less for most treatments, including 1.1C, 2N, and 4N, and was greater than threefold for treatment 6N at both 37 and 30°C. The stimulatory effect observed with growth rate with treatment 1N (Fig. 1) was also apparent for maximum optical density data, where treatment 1N had the highest values of STEC and LAB optical densities for both temperatures, comparing each species separately.

Survival in salt brines. To determine the survival of LAB and STEC strains in CJ medium with lactic acid at pH 3.2, we used three added salt conditions representative of vegetable fermentations: 6N, 2N, and 1.1C. For the *L. plantarum* cocktail, approximately 350 mM lactic acid or greater was needed to obtain the desired log reduction (4 to 5 log CFU/mL) in cell counts within 48 h (Fig. 4). With reduction of the pK_a of lactic acid resulting from the ionic

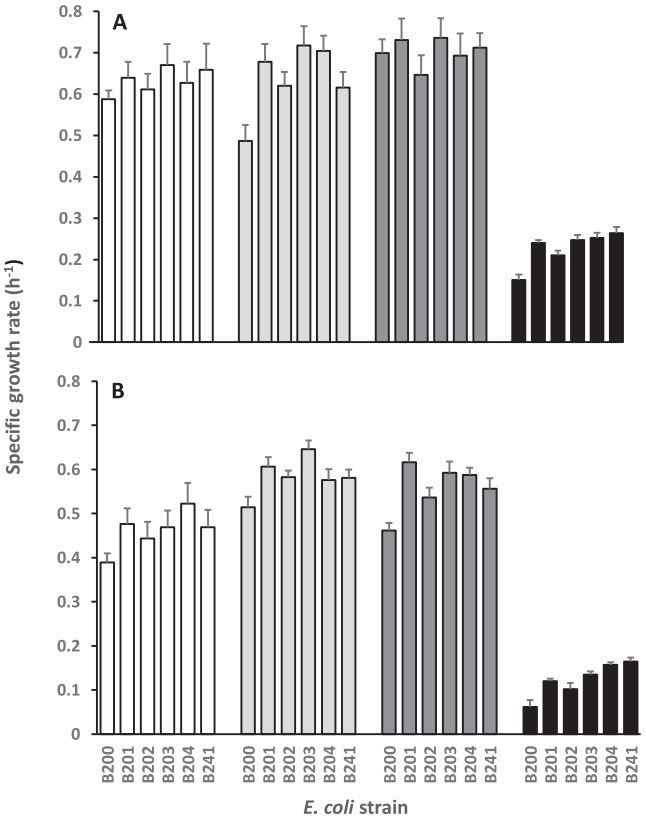

FIGURE 2. *Effect of salt on growth rate at 30 and 37°C in CJ medium. The growth rates of individual STEC strains at 37°C (A) or 30°C (B) were measured in 50% CJ containing no added salt (clear bars), treatment 1.1C (1.1% $CaCl_2$, light gray bars), treatment 2N (2% NaCl, dark gray bars), or treatment 6N (6% NaCl, black bars). Error bars represent the standard deviations.*

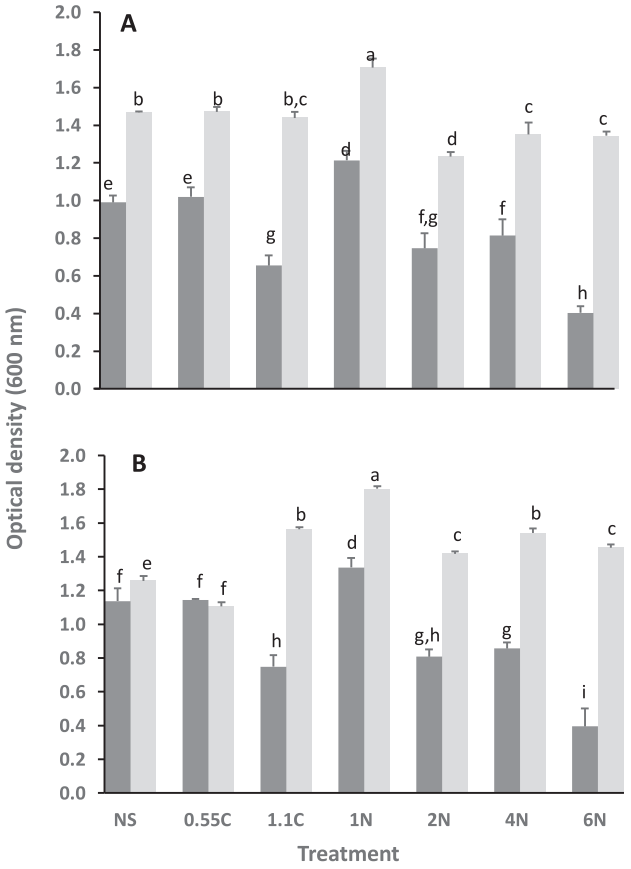

FIGURE 3. *Effect of salt on the maximum culture optical density at 30 and 37°C in CJ medium. The maximum optical density resulting from growth at 600 nm or a cocktail of six STEC strains (dark gray bars) or five LAB strains (light gray bars) was measured at 37°C (A) and 30°C (B). Treatments were labeled on the* x *axis as NS, no added salt; 0.55C, 0.55% $CaCl_2$; 1.1C, 1.1% $CaCl_2$; 1N, 1% NaCl; 2N, 2% NaCl; 4N, 4% NaCl; and 6N, 6% NaCl. Lowercase letters indicate significant differences (*$P < 0.05$*), and error bars indicate the standard deviations.*

strength of the solutions, the protonated acid concentration resulting from the added 350 mM lactate was 270, 253, and 249 mM for treatments 6N, 2N, and 1.1C, respectively. Cell concentrations were reduced from 8.5 log CFU/mL to between 2.7 and 2.5 log CFU/mL for treatments 6N and 2N, respectively (Fig. 4), with no statistically significant difference in the cell counts. However, with treatment 1.1C, cell counts (which had a protonated acid concentration similar to the 2N treatment) were only reduced to 4.2 log CFU/mL. The difference between the calcium and the sodium salt treatments was statistically significant at 48 h, indicating that the calcium treatment increased the survival of LAB strains compared with the lactic acid treatments with NaCl. For *E. coli* strains, CJ with 50 mM lactic acid was used at pH 3.2 with three added salts, resulting in protonated lactic acid concentrations of 37.2, 35.4, and 35.5 mM for the 6N, 2N, and 1.1C treatments, respectively. At 18 h, cell counts for 6N and 1.1C treatments were reduced from 8.3 to 3.0 and 3.2 log CFU/mL, respectively (Fig. 5). For the 2N treatment, cell counts were reduced to only 3.9 log CFU/mL at 18 h, a significant increase over both the 6N and the 1.1C treatments.

Bacterial competition assays. Bacterial competition experiments were conducted with LA445 (a commercial cucumber fermentation isolate) in competition with B200, which had the slowest growth rate among the strains tested, and B241, which was among the fastest-growing STEC strains. These strains were previously found to have similar resistance to lactic acid stress (F.B., personal communication). For B200 in competition with LA445, the B200 cell numbers reached a maximum of between 10^8 and 10^9 CFU/mL at 24 h after inoculation for the 1.1C and 2N treatments, with initial cell concentrations of around 10^4 CFU/mL (Fig. 6). However, for treatment 6N, no growth of B200 was apparent. At 24 to 32 h, B200 cell numbers were in decline, with the 2N treatment having the highest surviving numbers (8.58 ± 0.07 log CFU/mL), followed by the 1.1C treatment (4.3 ± 0.91 log CFU/mL). By 48 h, B200 cell counts for all treatments were below the level of detection (Fig. 6). The lactic acid concentrations at 48 h were 26.64 ± 0.45, 21.72 ± 9.66, and 26.71 ± 4.76 mM and the pH values for the mixed cultures at 48 h were 3.45 ± 0.06, 4.07 ± 0.09, and 3.94 ± 0.03 for 6N, 2N, and 1.1C, respectively (Table 2). Protonated lactic acid concentrations calculated from the lactic acid values were 16.84, 10.04, and 8.25 mM (for 6N, 2N, and 1.1C, respectively). At the end of the fermentation

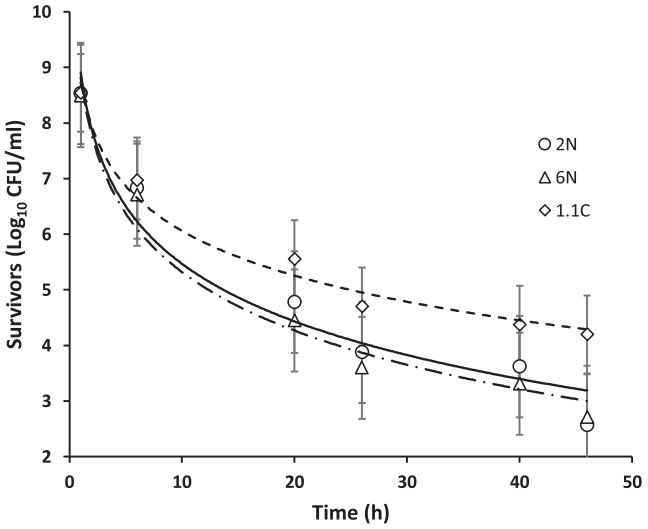

FIGURE 4. *Survival of LAB strains with lactic acid in CJ medium with varying salts. Cell survival (log CFU per milliliter) was measured in CJ (pH 3.2, 30°C) with 350 mM lactic acid: treatment 2N, 2% NaCl (circles, solid line); treatment 6N, 6% NaCl (triangles, dot-dash line); or treatment 1.1C, 0.1% $CaCl_2$ (diamonds, dash line). Lines represent fitted data from three independent measurements using polynomial regression as described. Standard deviations for the replicates are indicated by the error bars.*

experiment at 56 h, glucose and fructose remained present at 8 mM or greater for all treatments (data not shown).

For the mixed-culture experiments with LA445 and B241, the initial B241 cell count was approximately 5×10^6 CFU/mL, and maximum cell counts of approximately 10^8 log CFU/mL were obtained within 8 h (Fig. 7) for 2N and 1.1C. Similar to the B200 experiment, little or no growth

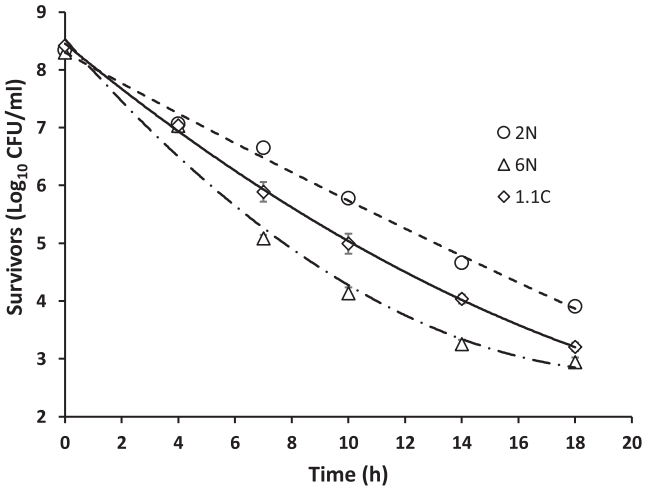

FIGURE 5. *Survival of STEC strains with lactic acid in CJ medium with varying salts. Cell survival (log CFU per milliliter) was measured in CJ (pH 3.2, 30°C) with 50 mM lactic acid: treatment 2N, 2% NaCl (circles, solid line); treatment 6N, 6% NaCl (triangles, dot-dash line); or treatment 1.1C, 0.1% $CaCl_2$ (diamonds, dash line). Lines represent fitted data from three independent measurements using polynomial regression as described. Standard deviations for the replicates are indicated by the error bars.*

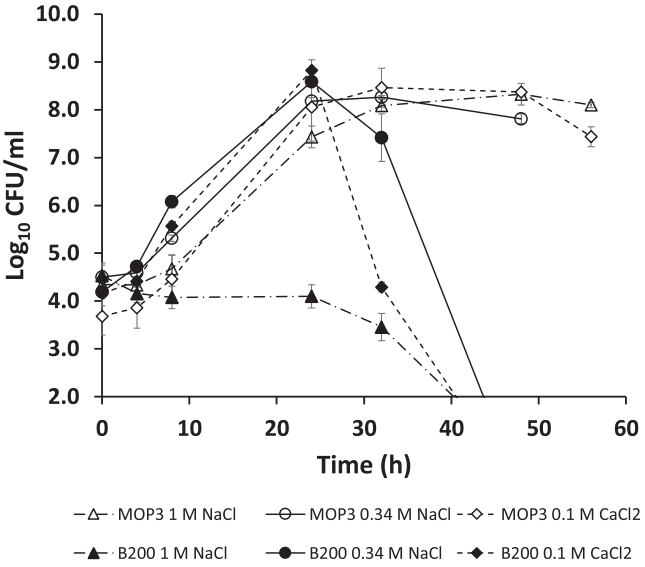

FIGURE 6. *Effect of salts on the competitive growth and death of* E. coli *B200 with* L. plantarum *LA445. The growth and death of B200 (filled symbols) or LA445 (open symbols) in five-strain cocktails were measured in CJ medium at 30°C: treatment 2N, 2% NaCl (circles); treatment 6N, 6% NaCl (triangles); and treatment 1.1C, 1.1% $CaCl_2$ (diamonds). Standard deviations for three replications are indicated by the error bars.*

was seen with B241 for the 6N treatment. By 32 h, B241 was reduced to near the level of detection, 2.18 ± 3.78 log CFU/mL for the 6N treatment, and B241 had 5.01 ± 0.57 and 6.16 log CFU/mL for 1.1C and 2N, respectively. By 48 h, B241 was below the level of detection for the 2N treatment with a lactic acid concentration of 41.91 ± 4.24 mM, corresponding to 16.67 mM protonated acid (with

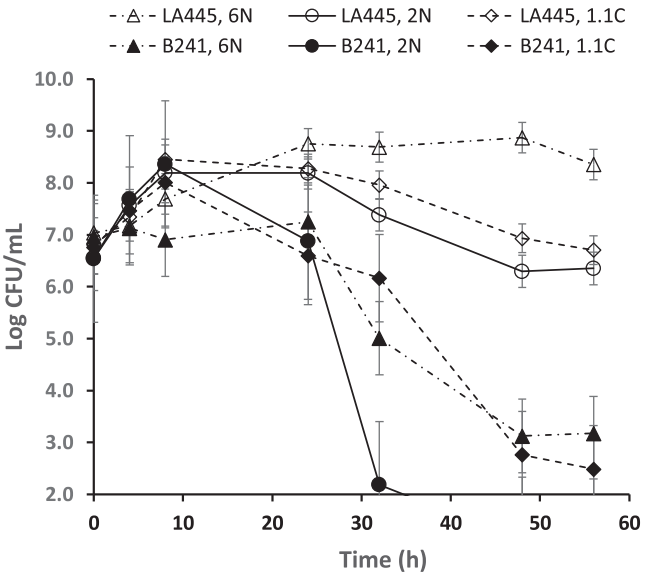

FIGURE 7. *Effect of salts on the competitive growth and death of* E. coli *B241 with* L. plantarum *LA445. The growth and death of B241 (filled symbols) or LA445 (open symbols) in five-strain cocktails were measured in CJ medium at 30°C: treatment 2N, 2% NaCl (circles); treatment 6N, 6% NaCl (triangles); and treatment 1.1C, 1.1% $CaCl_2$ (diamonds). Standard deviations for three replications are indicated by the error bars.*

TABLE 2. *Organic acids and pH from bacterial competition*

		B200 and LA445			B241 and LA445		
Treatment	Time (h)	Lac, mM (SD)[a]	pH (SD)[b]	Prot, mM[c]	Lac, mM (SD)	pH (SD)	Prot, mM
6N (6% NaCl)	0	BDL[d]		BDL	BDL	5.20 (0.04)	BDL
	4	BDL		BDL	BDL	5.16 (0.09)	BDL
	8	BDL		BDL	11.22 (2.32)	5.07 (0.05)	0.42
	24	6.04 (0.95)	4.55 (0.19)	0.68	41.76 (5.04)	3.53 (0.05)	23.91
	32	17.02 (0.86)	3.83 (0.08)	6.84	44.73 (5.28)	3.45 (0.05)	27.75
	48	26.64 (0.45)	3.45 (0.06)	16.48	51.79 (6.63)	3.35 (0.03)	34.76
	56	29.14 (1.35)	3.40 (0.03)	18.70	57.67 (5.90)	3.32 (0.03)	39.48
2N (2% NaCl)	0	BDL	5.58 (0.30)	BDL	BDL	5.34 (0.09)	BDL
	4	BDL	5.58 (0.30)	BDL	BDL	5.34 (0.08)	BDL
	8	BDL	5.59 (0.28)	BDL	19.24 (2.11)	4.55 (0.20)	1.90
	24	12.79 (4.08)	4.63 (0.06)	1.07	38.56 (4.45)	3.78 (0.14)	15.13
	32	21.72 (9.66)	4.07 (0.09)	5.37	39.67 (5.56)	3.79 (0.07)	15.27
	48	26.33 (10.40)	3.81 (0.07)	10.04	41.91 (4.24)	3.77 (0.10)	16.67
	56	ND[d]	ND	ND	41.72 (1.74)	3.75 (0.12)	17.06
1.1C (1.1% $CaCl_2$)	0	BDL	5.48 (0.11)	BDL	BDL	5.04 (0.09)	BDL
	4	BDL	5.52 (0.10)	BDL	BDL	4.89 (0.22)	BDL
	8	BDL	5.53 (0.09)	BDL	22.31 (5.66)	4.15 (0.26)	4.82
	24	13.29 (6.75)	4.72 (0.22)	0.91	40.28 (7.55)	3.59 (0.13)	20.22
	32	17.06 (9.21)	4.57 (0.31)	1.61	35.38 (6.83)	3.53 (0.05)	18.84
	48	26.71 (4.76)	3.94 (0.03)	8.25	42.68 (4.59)	3.56 (0.03)	22.16
	56	26.73 (4.87)	3.975 (0.08)	7.80	40.51 (10.88)	3.45 (0.15)	23.42

[a] Lac, mean lactic acid concentration ($n = 3$).
[b] pH, mean pH ($n = 3$).
[c] Prot, protonated lactic acid from mean values, with pK_a adjusted for ionic strength.
[d] BDL, below detection limit; ND, not determined.

mean values). Although the lactic acid concentrations for the 2N and 1.1C treatments at 48 h were within 1 mM of each other, the pH values differed (3.77 ± 0.10 and 3.56 ± 0.03), resulting in protonated lactic acid concentrations of 17.06 and 23.42 mM, respectively. B241 cells remained viable (between 10^2 and 10^4 CFU/mL) for up to 56 h for the 6N and 1.1C treatments. The lactic acid concentrations (Table 2) for 6N and 1.1C treatments at 56 h were 57.67 ± 5.90 and 40.51 ± 10.88 mM, corresponding to 39.48 and 23.42 mM protonated acid (from mean values), respectively. Sugars (glucose and fructose) remained for all treatments at 56 h with 3.6 mM or greater (data not shown).

DISCUSSION

In vegetable fermentations, bacterial competition between LAB and other microbiota is based on competition for nutrients (free sugars) and production of antimicrobial fermentation end products (organic acids). LAB produce lactic acid and acetic acid from glucose and fructose, which will diffuse into the brine from vegetables undergoing fermentation. As pH falls below the pK of the acids, the protonated acids formed may freely cross the cytoplasmic membranes of bacteria. This results in lowering of internal cell pH and accumulation of acid anion, which is trapped within bacterial cells *(8, 43, 44)*. To oppose this drop in intracellular pH, bacterial cells can remove excess protons at the expense of cellular energy (ATP) *(1, 5)*. However, in vegetable fermentations, a large pH difference between the cell cytoplasm and the fermentation brine may result in the accumulation of molar quantities of organic acid anion (typically lactate or acetate) because of the large difference between internal and external pH *(43)*. It has been proposed that LAB predominate in vegetable fermentations because of their ability to lower internal pH more than other organisms *(30)* and therefore reduce delta pH. However, it remains unclear how salts present in vegetable fermentation brines may influence microbial ecology and acid resistance.

Acid-resistant bacterial pathogens, and STEC in particular, may use a strategy similar to that of LAB to survive in vegetable fermentations by lowering internal cell pH; however, several lines of evidence suggest that increasing cell pH may be important for STEC survival in the presence of organic acids. Casey and Condon *(10)* showed that NaCl increased survival of STEC in a lactic acid containing rich medium at pH 4.2 by increasing internal pH. Similarly, Lee and Kang *(25)* showed that NaCl increased the time needed for a 1-log reduction (*D*-values) by approximately twofold in laboratory media containing 0.25% acetic acid when 3% NaCl (approximately 0.52 M) was added to the acid medium. This effect of NaCl has also been seen in both laboratory and cucumber-based media *(2, 27)*. Further study has shown that the NaCl effect on STEC survival in acetic acid correlated with an increase in internal cell pH and a less negative charge balance (delta psi) across the bacterial membrane *(26)*. Hosein et al. *(19)* also found a linear relationship between internal cell pH and 5-log reduction times for STEC with 0.34 M (2%) NaCl, and protonated acid concentrations of up to 20 mM increased internal pH and aided cell survival. However, these studies

did not address the influence of salts on the outcome of competition between LAB and STEC that are important to fermentation safety.

A complicating factor in studies of organic acid sensitivity of STEC is that dissolved oxygen in the test medium has been shown to significantly increase acid killing of STEC *(24)*. Oxygen is present in liquids at sea level at concentrations of approximately 5 to 10 mg L^{-1}. The oxygen effect on acid killing may be due to inactivation at low pH (below pH 7) of cellular enzymes that detoxify oxygen radicals. Kreske *(23)* found evidence that the correlation between reduced cellular pH and survival of *E. coli* under selected organic acid stress conditions may result from reduced catalase activity in cell extracts as pH drops from neutral to pH 5 *(23)*. Although further research may be needed to support this hypothesis, it may explain the differences in cell survival seen with increasing intracellular pH because of NaCl addition as described earlier. The mechanism by which NaCl may increase intracellular pH remains unclear. Sodium/hydrogen (Na^+/H^+) antiport mechanisms for pH regulation have been proposed for *E. coli,* but Na^+/H^+ antiporter proteins have mostly been shown to function at alkaline, not acidic, pH values *(29)*. To avoid complications potentially because of dissolved oxygen effects with our experiments, we preincubated our CJ medium in an anaerobic chamber and used Vacutainer tubes to help excluded oxygen from the medium during competitive growth and acid-killing experiments.

Salt is commonly added to vegetable fermentations at concentrations between 2 and 6%, corresponding to our 2N and 6N treatments. We found relatively small differences were seen with death rates of STEC in response to salt type or concentration. However, growth rates did correlate with salt concentration, although little effect was seen with 1.1C. In general, we found that growth rates decreased as NaCl increased (Figs. 1 and 2). Treatment 1N increased significantly in growth rate compared with the same medium without NaCl for both LAB and STEC. It is possible that the stimulation of growth we observed with 1N was related to enhanced transport or metabolic effects because of a Na^+/H^+ antiport mechanism or Na^+ effects on transport of nutrients *(18, 46)*; further research may be needed to determine the reason for this effect.

During competitive growth and death of two selected STEC strains and *L. plantarum* LA445 (isolated from a commercial vegetable fermentation) under anaerobic conditions, the die-off of the STEC strains did not follow a consistent pattern with regards to salt condition or initial cell concentrations (Figs. 6 and 7). As expected, the 6N treatment resulted in little or no evident growth in both experiments (Figs. 6 and 7), consistent with the lower growth rates for STEC with this treatment (Figs. 1 and 2) and the lower maximum cell density achieved (Fig. 3). This treatment did result in long-term survival with strain B241 in the competition experiment (even though acid concentrations were higher than with the B200 experiment), along with the 1.1C treatment. With B241, the 2N treatment had the most rapid apparent death rate (Fig. 7), but with the mixed-culture cocktails, treatment 2N resulted in the greatest survival at 18 h. Because of these disparate results and the many variables that may affect competitive growth of bacteria in vegetable fermentations *(15),* we concluded there was no consistent pattern of salt effects on cell survival during competitive growth of STEC and LAB with our data.

For the conditions of our study, we found that NaCl conditions typical of cabbage or other low-salt ready-to-eat vegetable fermentations (2N) did not significantly affect the growth rates of LAB or STEC, although the maximum cell density achieved by STEC strains was significantly less than in the absence of salt. However, this was not true for the conditions typical of $CaCl_2$ commercial cucumber fermentations (treatment 1.1C). The salt conditions used in typical NaCl commercial cucumber fermentations (treatment 6N) reduced both LAB and STEC growth rates and further reduced the maximum cell density achieved by STEC in growth experiments. Survival of STEC in mixed-culture fermentation experiments with *L. plantarum* LA445, a commercial cucumber fermentation isolate, showed no consistent pattern relating salt concentration and acid killing between STEC cultures or with acid-killing experiments. We conclude from these data that NaCl may function in vegetable fermentations principally to aid LAB growth, primarily in terms of the maximum cell densities achieved. Because of differences due to bacterial strain and initial cell count levels, further work will be needed to determine factors influencing survival of STEC in vegetable fermentations.

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