Applied and Environmental Microbiology, Feb. 2011, p. 889–895 Vol. 77, No. 3
0099-2240/11/$12.00 doi:10.1128/AEM.02136-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Modeling the Effects of Sodium Chloride, Acetic Acid, and Intracellular pH on Survival of *Escherichia coli* O157:H7▽†

Althea M. Hosein,[1] Frederick Breidt, Jr.,[3]* and Charles E. Smith[2]

Biomathematics[1] and Department of Statistics,[2] North Carolina State University, Raleigh, North Carolina 27695, and USDA-ARS, SAA Food Science Research Unit, 322 Schaub Hall, Box 7624, North Carolina State University, Raleigh, North Carolina 27695-7624[3]

Received 9 September 2010/Accepted 16 November 2010

Microbiological safety has been a critical issue for acid and acidified foods since it became clear that acid-tolerant pathogens such as *Escherichia coli* O157:H7 can survive (even though they are unable to grow) in a pH range of 3 to 4, which is typical for these classes of food products. The primary antimicrobial compounds in these products are acetic acid and NaCl, which can alter the intracellular physiology of *E. coli* O157:H7, leading to cell death. For combinations of acetic acid and NaCl at pH 3.2 (a pH value typical for non-heat-processed acidified vegetables), survival curves were described by using a Weibull model. The data revealed a protective effect of NaCl concentration on cell survival for selected acetic acid concentrations. The intracellular pH of an *E. coli* O157:H7 strain exposed to acetic acid concentrations of up to 40 mM and NaCl concentrations between 2 and 4% was determined. A reduction in the intracellular pH was observed for increasing acetic acid concentrations with an external pH of 3.2. Comparing intracellular pH with Weibull model predictions showed that decreases in intracellular pH were significantly correlated with the corresponding times required to achieve a 5-log reduction in the number of bacteria.

Since the initial outbreak report in 1982 (37), *Escherichia coli* O157:H7 has been a serious public health concern. It has been reported that there are 20,000 infections each year in the United States (6). In the majority of the cases, the illness resolves in a week; however, in about 5% of patients, the disease progresses to hemolytic-uremic syndrome, which may result in kidney failure, neurological sequelae, and death (32). While cases of food-borne illness associated with acidified foods are rare, the FDA has expressed concern about these products, based upon disease outbreaks caused by *E. coli* O157:H7 in apple cider (3, 13) and by *Salmonella enterica* in orange juice (18). The U.S. acidified food regulation (Code of Federal Regulations, chapter 21, part 114) requires that vegetative microbial pathogens be killed and organisms of non-public heath significance cannot grow in commercial acidified vegetable products.

E. coli O157:H7 has been found to be the most acid-resistant vegetative pathogen of concern in acidified vegetables (8, 9). Jordan et al. showed that at pH 3 and 30°C, O157:H7 strains survived for up to 3 days (27). A study by Breidt et al. (8) revealed that, depending on the temperature, O157:H7 strains needed 2 to 6 days to achieve a 5-log reduction in the number of bacteria at pH 3.3 under acetic acid solutions (at 25°C and 10°C, respectively). The organism may survive even longer at refrigeration temperatures (4°C) in acidified vegetables that are not heat processed (F. Breidt, unpublished data). Therefore, it is important to quantify the effects of antimicrobials used in preventing the survival of O157:H7 in acidified vegetable products. Mathematical models of microbial inactivation of bacteria by heat, pressure, and chemicals have been extensively studied (1, 9, 15, 16, 19, 41). Traditional approaches to measuring the killing of bacteria by environmental stress use first-order kinetics (16). However, this does not account for deviations from linearity such as shoulders or tails in killing curves (31) which are evident in organic acid killing data (8).

The antimicrobial activity of organic acids is thought to be due to the ability of the undissociated acid to freely cross the cell membrane and release protons inside the cell (11, 40). The lowering of pH is opposed in the cell by removal of excess protons at the expense of ATP (5). The energy required to rid the cytoplasm of these protons drains the cell of ATP, resulting in death (2). An alternative explanation for the toxicity of organic acids is that acid anions become trapped in the cell cytoplasm and accumulate inside the cell, raising ionic strength and potentially disrupting metabolism (39). Decreasing intracellular pH results in a low transmembrane pH gradient (20) and may therefore reduce anion levels inside the cell. This may result in reduced metabolic activity, because enzymes in the cytoplasm may not function well below neutral pH. It has been suggested that *Lactobacillus plantarum* predominates in vegetable fermentations because it can tolerate a lower intracellular pH better than competing bacteria do (30). The prolonged survival of *E. coli* O157:H7 under acidic conditions has been explained mainly by the existence of acid resistance systems (24) which help to maintain a high intracellular pH, in some cases around pH 7 (42). Because of the apparent conflicting survival mechanisms, i.e., lowering the intracellular pH to prevent acid anion accumulation or maintaining a high pH to

* Corresponding author. Mailing address: USDA-ARS, SAA Food Science Research Unit, 322 Schaub Hall, Box 7624, North Carolina State University, Raleigh, NC 27695-7624. Phone: (919) 515-2979. Fax: (919) 513-0180. E-mail: Fred.Breidt@ars.usda.gov.

† Paper no. FSR10-25 of the Journal Series of the Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh.

▽ Published ahead of print on 29 November 2010.

optimize metabolic enzyme function, it is unclear how intracellular pH and cell survival are related.

Acidified vegetables typically contain 2 to 4% NaCl and various concentrations of acetic acid, up to 400 mM. There is evidence that sodium increases the acid sensitivity of *E. coli* (38), but it also aids in recovery from stress and enhances cell growth (26). We determined the effects of salt (NaCl) and acetic acid on the survival and intracellular cell physiology of *E. coli* O157:H7 and the relationship of these variables to killing kinetics. Using a Weibull model, we report a significant correlation between intracellular pH and cell survival data. In addition, we found that NaCl may have a protective effect on survival at pH 3.2 for 20 mM or lower protonated acetic acid concentrations and 4% NaCl, compared to survival at similar acid concentrations with 2% NaCl.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *E. coli* B241 (strain O157:H7, 28RC1, bovine isolate) was used in this study. This strain was chosen for its acid resistance (33). The stock culture was stored at −80°C in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI) supplemented with 20% glycerol and 1% glucose (Sigma Chemical Co., St. Louis, MO). Cultures were grown statically in 50 ml of LB broth plus 1% glucose for 18 h at 37°C to induce acid resistance. Cultures were then harvested by centrifugation (25°C, 10 min, 5,000 × *g*) and concentrated 25-fold by resuspension in 2 ml of sterile saline (8.5 g/liter NaCl). Cells were enumerated by serial dilution in 0.85% NaCl and plating on LB agar using a spiral plater (model 4000; Spiral Biotech, Inc., Norwood, MA). After 24 h of incubation at 37°C, colonies were counted using an automated plate reader (QCount; Spiral Biotech).

Acetic acid and NaCl treatments. The acetic acid (Sigma) solutions used in this study ranged from 0 mM to 60 mM protonated acid species. All acid solutions contained 20 mM D-gluconic acid sodium salt (Sigma), which functioned as a noninhibitory buffer (pKa = 3.8) (7). The ionic strength of the acid solutions (0.342, 0.684, or 1.027) was held constant by using NaCl; they were designed using pHTools software (22), a custom MATLAB routine. Additionally, the pH of the acetic acid solutions was held constant at pH 3.2 by the addition of HCl or NaOH. A final volume of 0.8 ml (for intracellular pH studies) or 1.6 ml (acid killing curves) of the acid solution in a 2-ml microcentrifuge tube contained 0.1 or 0.2 ml (respectively) of the concentrated cell suspension (as described above) and gave an initial cell concentration of approximately 5×10^9 CFU/ml. For the killing curves, 50-μl samples were removed at the indicated time intervals and immediately diluted in 0.45 ml of 0.1 M MOPS (3-morpholinopropane-1-sulfonic acid; Sigma) buffer at pH 7.0 to neutralize the acid. Cells were enumerated following serial 10-fold dilutions in 0.85% NaCl and plating on LB agar as described above.

The relationship between the log of the 5-log reduction time and acid and salt concentrations was described by the following model:

$$\log RT_5 = c_0 + c_1\, salt + c_2\, acid + c_3\, acid \times salt \tag{1}$$

Regression parameters for equation 1 were estimated by using the general linear model procedure in SAS (see below). Based on this model, a response surface was generated with the 5-log reduction times for cells exposed to 20, 30, and 40 mM acetic acid with 2%, 4%, or 6% NaCl. A total of nine measured 5-log reduction times were used to create the response surface. For the validation of this model, survival experiments were performed with cells that were exposed to 1% NaCl with 40 mM acetic acid, 2% NaCl with 60 mM acetic acid, or 4% NaCl with 60 mM acetic acid.

Intracellular pH. Intracellular pH was determined by a radioactive acid distribution method (36). *E. coli* B241 was grown and washed as previously described. All radionuclides were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). For the time course experiments, cells were incubated at 25°C with a buffered acid treatment containing 0 mM, 5 mM, or 40 mM acetic acid and either [7-^{14}C]benzoate (50 to 60 mCi/mmol) or [1,2-^{14}C]polyethylene glycol (^{14}C-PEG; 0.1 to 1 mCi/g) and ^3H_2O (1 mCi/ml) for 5, 15, 30, or 60 min. [^{14}C]benzoic acid was used to measure intracellular pH by acid partition, and ^{14}C-PEG was used to measure cell pellet-external water space. Alternatively, we used [7-^{14}C]salicylate (40 to 60 mCi/mmol) for intracellular pH measurement and D-[U-^{14}C]sorbitol (250 to 350 mCi/mmol) or [1,2-^{14}C]taurine (30 to 60 mCi/mmol) for cell-external water space measurement. For determining dry weights, the cell pellets were digested overnight in a 3 M HNO_3, dried in a vacuum system (SpeedVac Concentrator SVC 100; Savant Instruments, Inc., Farmingdale, NY), and weighed.

To measure intracellular pH, cell cultures were incubated at 25°C with the buffered acid treatments described above, containing approximately 1 μCi of [7-^{14}C]benzoic acid or ^{14}C-PEG in combination with tritiated water (^3H_2O; to measure total cell pellet volume) for 30 min. After the indicated incubation period, 500-μl samples from the acid treatment were centrifuged through a silicone oil mixture (65:35 mixture of Dow Chemical Co. types 550 and 556) in a microcentrifuge (13,000 × *g*, 15 min) at 4°C. An aliquot (100 μl) of the supernatant was removed. Cell pellets under the silicone layer were frozen for a minimum of 40 min at −80°C to solidify the silicone, and then the pellets were cut from the bottom of the tube with dog nail clippers. A detergent solution containing 50 mM Tris (Sigma) and 0.5% Tween 80 (Fisher Scientific, Pittsburgh, PA) at pH 7.0 was used to resuspend pellet samples. Pellet samples or 100 μl of the supernatant was added to 600 μl or 500 μl, respectively, of the detergent solution in a 7-ml scintillation vial (Perkin-Elmer, Waltham, MA; catalog no. 6000167). After resuspension of the cell pellet from the microcentrifuge tube fragment by vortexing, 5 ml of scintillation fluid (Scintisafe from Fisher) was added and the numbers disintegrations per minute of the ^{14}C and ^{3}H of the radionuclides were determined by dual-label counting using a scintillation counter (model LS600; Beckman Coulter, Inc., Fullerton, CA). Disintegration-per-minute values were determined from counts per minute by using ^{14}C and ^{3}H quench standards (Beckman). Intracellular cell volume was determined by subtracting the calculated pellet-external water volume from the total pellet volume, based on the number of ^{14}C-PEG (which does not enter the cell) and ^3H_2O (total pellet volume) disintegrations per minute in the pellet fraction. Intracellular pH was calculated using the intracellular/extracellular disintegration-per-minute ratio for [^{14}C]benzoate, based on the intracellular volume determined from the pellet and the supernatant ^{14}C counts with the Henderson-Hasselbalch equation, assuming an intracellular pKa for benzoic acid of 4.21.

Modeling and statistical analysis. A Weibull model was used to describe the log reduction in the microbial population and to estimate the 5-log reduction in the number of bacteria. The survivors, measured in numbers of CFU per milliliter over time (hours) was described by

$$N(t) = N_0 e^{(-t/\alpha)^\beta} \text{ with } N(0) = N_0 \tag{2}$$

where $N(t)$ is the concentration of bacteria over time, N_0 is the initial bacterial load, α is the scale parameter in units of time, and β is the dimensionless shape parameter. For a β of >1, the survival curve is convex, for a β of 1, the survival curve is linear (on a logarithmic scale), and for a β of <1, the survival curve is concave. The log (base 10) of the number of survivors is thus written as follows:

$$\log N(t) = \log N_0 - \frac{1}{\ln(10)}\left(\frac{t}{\alpha}\right)^\beta \tag{3}$$

and the 5-log reduction time as a function of α and β can be written as follows:

$$RT_5 = \alpha(5\ln(10))^{1/\beta} \tag{4}$$

Statistical analysis for the Weibull model was determined using the NLIN procedure of the SAS software (SAS Institute Inc., Cary, NC). For all acid treatments, the parameters N_0, α, and β were estimated.

Solving for α in equation 4,

$$\alpha = (5\ln(10))^{1/\beta}/RT_5 \tag{5}$$

Then, the 5-log reduction time was estimated and the corresponding standard error of these estimates was determined by reparameterizing the Weibull model as follows:

$$\log N(t) = \log N_0 - 5\left(\frac{t}{RT_5}\right)^\beta \tag{6}$$

As discussed by Breidt et al. (9), the result (equation 6) is a nonlinear regression model that has additive, normally distributed errors that have equal variance. Model parameters were estimated using a nonlinear protocol (PROC NLIN) in SAS. The standard error estimates in SAS are similar to those outlined by Rawlings et al. (35).

For the radionuclide acid distribution experiments and the time course experiments, differences between the intracellular pH means were carried out using the Tukey least significant difference method ($P < 0.05$) for pairwise

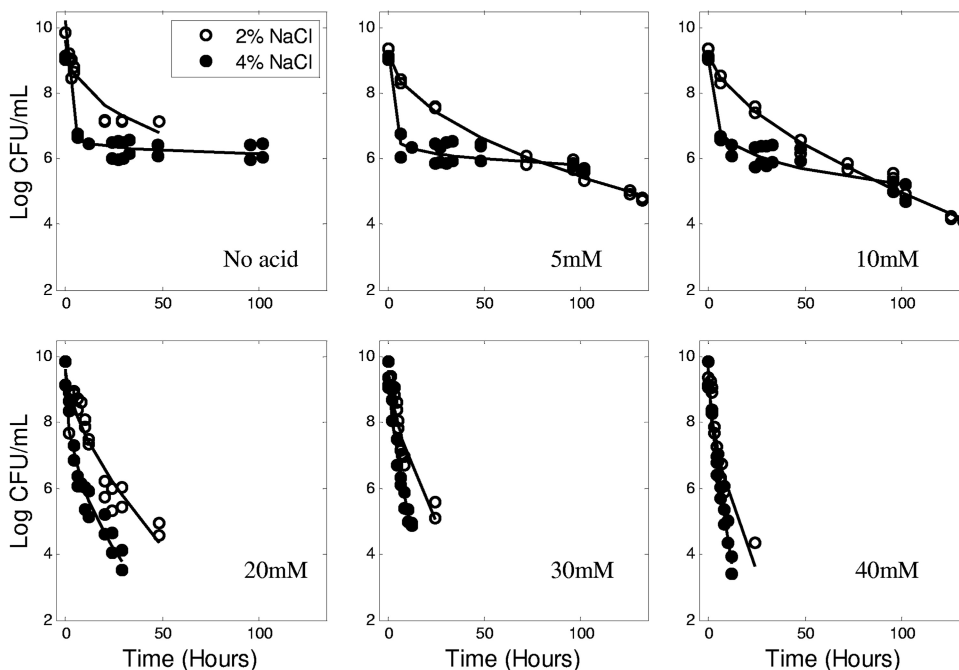

FIG. 1. Survival of *E. coli* O157:H7 exposed to 2 or 4% NaCl with 0 to 40 mM protonated acetic acid. The solid lines are Weibull prediction curves.

comparisons. The REG procedure of SAS was used for determining regression models of the 5-log reduction time, and the CORR procedure of SAS was used to determine the correlation between the 5-log reduction time and the intracellular pH.

RESULTS

Survival. The survival of *E. coli* O157:H7 exposed to 0 to 40 mM protonated acetic acid and 2 or 4% NaCl at pH 3.2 was determined (Fig. 1). A Weibull survival model was used to predict the time required to achieve a 5-log reduction in the number of bacteria (Table 1). As expected, an increase in the protonated acetic acid concentration decreased the number of survivors over time. There was no significant change in the number CFU/ml for all treatments during the first 30 min of incubation (not shown). After 60 min, survival curves were nearly linear or concave upward (Fig. 1). With greater than 20 mM protonated acetic acid, increasing the salt concentration decreased the predicted time for a 5-log reduction in the number of bacteria. With 0 to 10 mM acetic acid and 4% NaCl, there was a pronounced tailing behavior, preventing accurate predictions of the 5-log reduction time, indicating that NaCl has a protective effect on cell survival under these conditions, compared to 2% NaCl.

Effects of acid and salt on 5-log reduction time. A predictive model was developed using data collected from cells exposed to 20 to 40 mM acetic acid and 2 to 6% NaCl (Fig. 2). The following model was appropriate to predict the 5-log reduction time based on these acid and salt concentrations:

$$\log RT_5 = 2.57^{****} - 1.49salt^{***} - 29.37acid^{**} + 22.63(acid \cdot salt)^{*} \quad (7)$$

Parameters that were statistically significant are denoted with four stars ($P < 0.0001$), three stars ($P = 0.0008$), two stars ($P = 0.0017$), or one star ($P = 0.0196$). The coefficient of determination (R^2) for the equation was 0.9830. Validations for the predictive model (equation 7) were performed using three selected salt and acid combinations, and the results of these studies are illustrated in Fig. 2. The predictive model for the three validation experiments underestimated the time required for a 5-log reduction in the number of bacteria.

Intracellular pH. To establish which radionuclides are appropriate for measuring intracellular pH, we compared [^{14}C]benzoate with [^{14}C]salicylate (Fig. 3). A two-way analysis of variance was performed to compare the radioactive acid used to measure pH and acetic acid concentration, where the acetic acid concentration was found to be more significant than the choice of radionuclide. Radioactively labeled sorbitol (34), inulin (29), taurine (20), and PEG (17) were used for cell pellet-external water volume measurements. [^{14}C]sorbitol, [^{14}C]inulin, and [^{14}C]taurine gave inconsistent results, including negative calculated cell volumes (data not shown). ^{14}C-PEG, however, consistently gave cell volumes of approximately 1 μl/mg cell dry weight (data not shown).

For intracellular pH measurements, all treatments showed a trend of declining pH with exposure time (Fig. 4). No significant change in the viable cell count was observed for all treatments during the first 30 min of acid incubation time. After 1 h of incubation, the largest decline in cell numbers was 0.36 log with 4% NaCl and 40 mM protonated acetic acid (data not shown). For the 4% NaCl treatment at pH 3.2 with no acetic acid, the intracellular pH decreased more than 1 pH unit, from greater than pH 6.5 to less than pH 5.5 during the 1 h of incubation. With 40 mM acetic acid for both 2% and 4% NaCl, the intracellular pH values showed a decrease of only about 0.5 pH unit, ranging from approximately pH 6.0 to pH 5.5, when the incubation time in-

TABLE 1. Weibull model parameters and 5-log reduction estimates for the acid-salt treatments used in this study

NaCl concn (%) and protonated acetate concn (mM)	N_0 (SE)[a]	α (SE)[b]	β (SE)[c]	RT5 (SE)[d]
2				
0	9.61 (0.25)	0.50 (0.58)	0.41 (0.20)	194.92 (70.70)
5	9.22 (0.13)	1.54 (0.59)	0.52 (0.43)	173.49 (10.46)
10	9.18 (0.14)	2.58 (0.83)	0.62 (0.52)	131.52 (6.49)
20	9.57 (0.35)	0.94 (0.70)	0.63 (0.39)	44.95 (6.08)
30	9.59 (0.30)	0.58 (0.38)	0.63 (0.41)	27.91 (3.99)
40	9.64 (0.37)	0.19 (0.15)	0.54 (0.35)	17.16 (3.10)
4				
0	10.22 (1.84)	4.28E-25 (2.85E-23)	0.04 (0.03)	ND[e]
5	9.33 (0.39)	8.26E-11 (1.04E-9)	0.08 (0.03)	ND
10	9.07 (0.23)	6.88E-4 (1.48E-3)	0.18 (0.03)	ND
20	9.61 (0.30)	0.07 (0.05)	0.43 (0.05)	20.33 (3.22)
30	9.57 (0.26)	0.28 (0.16)	0.65 (0.45)	12.39 (1.20)
40	9.56 (0.26)	0.32 (0.14)	0.71 (0.53)	9.65 (0.81)
6				
10	9.04 (0.26)	0.01 (0.02)	0.37 (0.08)	9.33 (1.79)
20	9.05 (0.18)	0.001 (0.004)	0.28 (0.07)	9.31 (2.77)
30	9.42 (0.30)	0.004 (0.006)	0.33 (0.07)	6.90 (1.40)
40	9.37 (0.32)	0.09 (0.071)	0.56 (0.09)	7.29 (0.95)

[a] N_0, initial bacterial count in CFU/ml.
[b] α, Weibull scale parameter in hours.
[c] β, Weibull dimensionless shape parameter.
[d] RT5, 5-log reduction time in hours.
[e] ND, not determined.

creased from 5 to 60 min. Interestingly, only the 5 mM protonated acid treatment with 2% NaCl resulted in no significant change in the intracellular pH during the 60-min incubation.

Intracellular pH and survival. Increasing the concentration of protonated acetic acid decreased the intracellular pH (Fig. 5) with a linear trend ($R^2 = 0.99$) for the 2% NaCl treatment. The 5-log reduction time predicted by the Weibull model showed a similar trend for the 2% NaCl treatment, with the time decreasing from 195 h to 17 h (Table 1 and Fig. 5; $R^2 = 0.90$). With 2% NaCl data, the coefficients of determination for the relationship between (i) the intracellular pH and the calculated intracellular acetate anion concentration and (ii) the 5-log reduction time were 0.95 and 0.89, respectively (data not shown). For the 4% NaCl treatment, however, the relationship between protonated acetic acid and intracellular pH and 5-log reduction time was not linear. Between 0 and 10 mM protonated acid, the intracellular pH increased from 5.7 to 6 (Table 2 and Fig. 5), and as the protonated acid concentration increased from 10 to 40 mM, the intracellular

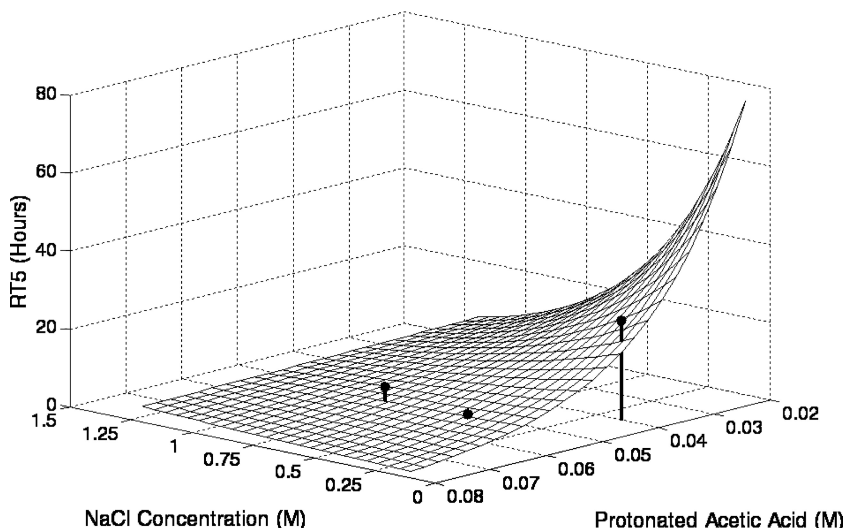

FIG. 2. Response surface plot created by using equation 7 (see text) and showing 5-log reduction times (RT5) as influenced by the protonated acetic acid concentration and the NaCl concentration. Several measured 5-log reduction times are also shown (black circles).

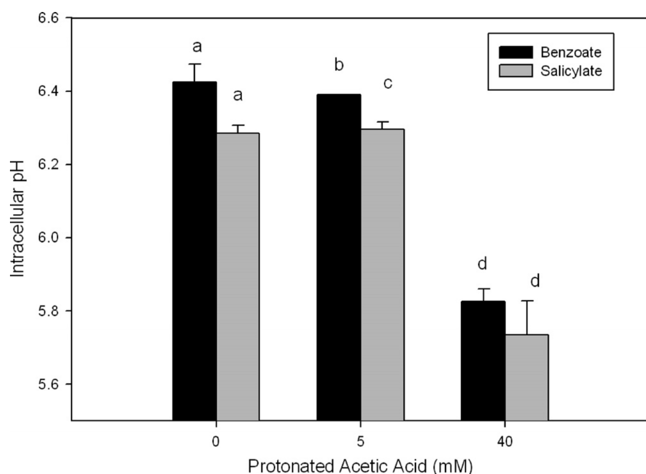

FIG. 3. Intracellular pH of *E. coli* O157:H7 exposed to 2% salt and incubated at 25°C for 30 min with a buffered acid treatment and either [^{14}C]benzoate or [^{14}C]salicylate. The data are mean values of two independent replicates; errors bars show standard deviations. Letters within the same protonated acetic acid concentration indicate no difference ($P > 0.05$).

pH declined to 5.7. The estimated α and β parameters of the Weibull model both approached 0 for survival curves when the protonated acid concentrations were below 10 mM with 4% NaCl, and a 5-log reduction time could not be reliably predicted (Table 1). This corresponded to survival curves with an extended tailing behavior (Fig. 1). With 4% NaCl and protonated acetic acid concentrations above 10 mM, the 5-log reduction times declined from 20 to 10 h, which is similar to the trend seen with the 2% NaCl treatments (Fig. 5).

DISCUSSION

Acetic acid and NaCl are the primary barriers to survival of acid-resistant pathogens in many acidified foods (7). For acidified vegetables with pH values above 3.3, a heat process is required to ensure safety, but products below pH 3.3 rely on acetic acid for a 5-log reduction in cell numbers of vegetative microbial pathogens (8). A 5-log reduction (the standard used by the FDA for process filings) of *E. coli* O157:H7 strains in pickled vegetable brines may take up to 6 days, depending on the temperature (8). Little is known about the intracellular physiology of *E. coli* strains undergoing the acid stress typical of acidified foods. We examined the effects of NaCl, acetic acid, and intracellular pH on the survival of *E. coli* O157:H7 at pH 3.2, which is typical of non-heat-processed acidified food products. These products can have up to 400 mM acetic acid; however, we used 0 to 40 mM protonated acetic acid for our studies because this allowed accurate measurements of intracellular pH. At higher acetic acid concentrations, the lethal effects of the acid treatments prevented accurate measurements of intracellular pH because of the accumulation of dead cells. With 0 to 40 mM protonated acetic acid, nonlinear killing kinetics were observed. No significant decrease in the number of CFU/ml was observed during the first 30 min of incubation for all treatments (data not shown). After the first 30 to 60 min, survival behavior was concave (up) or nearly log linear. As in

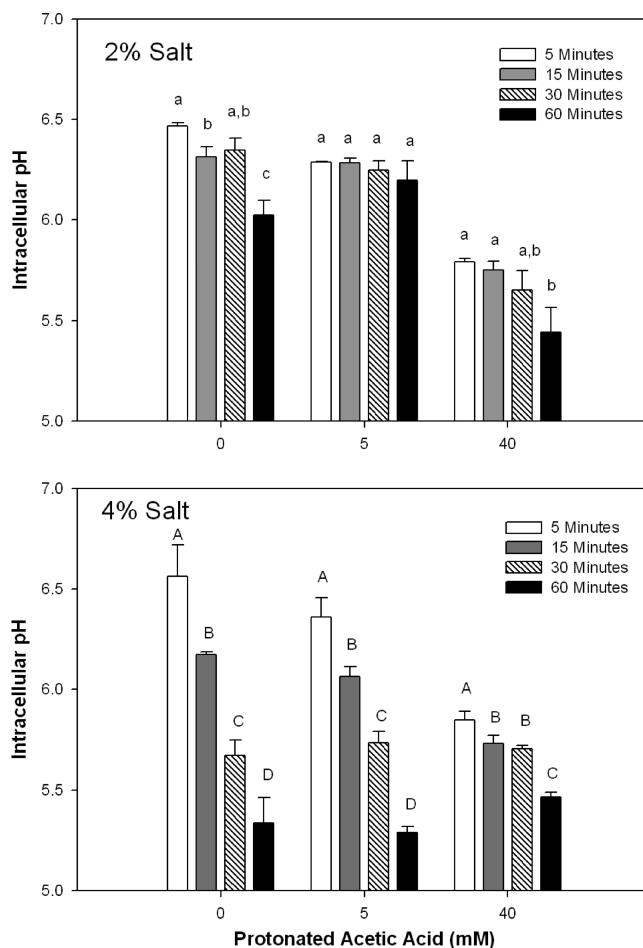

FIG. 4. Intracellular pH of *E. coli* O157:H7 exposed to no acetic acid or 5 or 40 mM protonated acetic acid in the presence of 2 or 4% salt at 25°C. Cells were incubated with acid treatment for 5 min (white bars), 15 min (gray bars), 30 min (striped bars) or 60 min (black bars). The extracellular pH was 3.2. Data are mean values from three independent replicated experiments. Error bars represent the standard deviations of these replicates. Identical letters over error bars within the same acid treatment indicate that there is no significant difference ($P \geq 0.05$) between incubation times.

previous studies (9, 19, 25, 41), a Weibull model was used to describe the survival curves. *E. coli* O157:H7 exposed to 4% NaCl and less than 20 mM acetic acid had survival curves with a remarkable tailing behavior. Under these conditions, approximately 0.1% (1×10^6 CFU/ml) of the initial population remained resistant to the acid solution and survived for over 100 h with little change in cell numbers (Fig. 1). As discussed by Jordan et al. (27), these data emphasize the need for nonlinear models that predict conditions for eliminating pathogens from foods where surviving subpopulations of viable cells can potentially cause disease outbreaks.

Intracellular pH measurements showed that pH decreased for most acid treatments during the first 60 min of incubation (Fig. 4). To correlate intracellular pH with cell survival, we chose 30 min; during this time, no significant change in cell number occurred. After 60 min, a decrease of 0.36 $\log_{10}$ CFU/ml was seen for the 4% NaCl treatment with 40 mM acetic acid. Therefore, the 30-min time was chosen to correlate

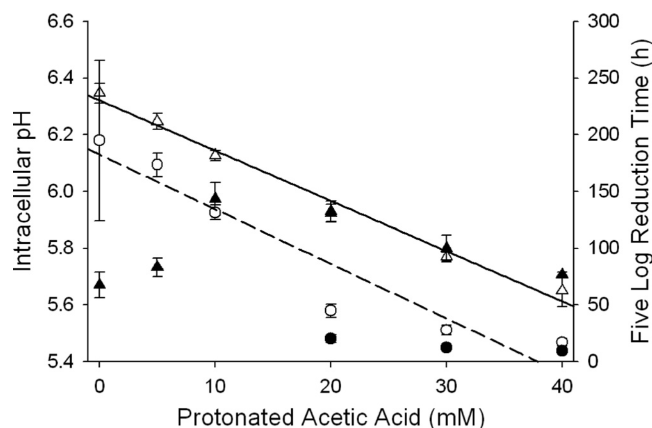

FIG. 5. Intracellular pHs (triangles) and estimated 5-log reduction times (circles) for 2% NaCl (open symbols) and 4% NaCl (filled symbols) treatments. The lines represent linear regression of the protonated acid concentration and the intracellular pH (solid line, R^2 = 0.99) or 5-log reduction time (dashed line, R^2 = 0.90) for the 2% NaCl treatment only. The error bars represent the standard errors of three independent replications. Note that only three data points for 5-log reduction time were predicted for the 4% NaCl treatment.

intracellular pH changes with subsequent cell survival. The two radionuclide compounds used, [^{14}C]benzoic and [^{14}C]salicylic acid, have different pKa values (4.2 and 3.0, respectively) but gave similar intracellular pH measurements. Similar results were observed by Russell (39), who found no apparent difference in the intracellular pH determination when using radioactively labeled benzoate or acetate. For our studies, we chose [^{14}C]benzoate for intracellular pH measurements because the pKa of this acid was closer to the measured intracellular pHs.

Increasing the acetic acid concentrations decreased cell survival at a given NaCl concentration, which is consistent with previous literature (21). However, the data in Fig. 1 show that with protonated acid concentrations of 10 mM or less, long-term survival (between 50 and 100 h) was better with 4% NaCl that with 2% NaCl. At higher protonated acetic acid concentrations (Fig. 2), survival decreased as NaCl and protonated acetic acid concentrations increased. Modeling the 5-log reduction time with acid concentrations of 20 mM or greater (equation 7) underestimated the 5-log reduction time and would therefore not be appropriate for food safety applications without modification. However, the model did show that 98% of the variability could be explained by NaCl concentration, acetic acid concentration, and the interaction between the salt and acid concentrations. The consequences of salt relative to the survival of *E. coli* have been investigated (12, 14, 23, 26). Chapman et al. have shown that with *E. coli* SERL 2 in the presence of sucrose at pHs ranging from 3.2 to 4, increasing NaCl concentrations (1 to 3%, wt/wt) increased the time required for a 3 $\log_{10}$ reduction, resulting in a protective salt effect (14). A similar observation was reported for growing *E. coli* O157:45, where, in combination with acid, NaCl conferred a protective effect against the bactericidal acid pH (12). However, the protective salt effect was not observed in our study when the protonated acid concentration was 20 mM or greater (Fig. 1). Our results suggest that a protective salt effect is only apparent with low-acid (10 mM or less) conditions and 4% NaCl. At these salt concentrations, our intracellular pH measurements did not follow the same trend as with 2% NaCl, being lower than expected, as described below. Casey and Condon reported that the protective salt effect may be a result of the increased osmolarity or may be due to increases in the cytoplasmic pH of the cells (12). At 4% NaCl and less than 20 mM acetic acid, the observed tailing behavior was correlated with a lower intracellular pH 30 min after the start of incubation in the acid solution (Fig. 5 and Table 2), possibly reducing the accumulation of the acid anion and cell death. Additional research to examine the function of Na^+ antiporters at low concentrations of acetic acid may help with understanding protective salt behavior.

By examining the intracellular pH under selected conditions (2% and 4% NaCl and 0, 5, 10, 20, and 40 mM protonated acetic acid), we sought to identify how internal cell physiology is altered by the external environment and how those changes may lead to cell death. The relationship between the intracellular pH and survival of *E. coli* has been previously considered (10, 28, 40). In the presence of lactic acid, lowering the intracellular pH of *E. coli* was not sufficient to cause cell death (28). Brudzinski and Harrison (10) examined the acid tolerance response of *E. coli* O157:H7 when it is exposed to acetic acid at various temperatures and pHs and concluded that cell death may have resulted from a reduction in the intracellular pH. As reviewed by Jordan et al. (27), several studies have attempted to indentify the factors contributing to increased acid tolerance in *E. coli*. Diez-Gonzalez and Russell found that intracellular acetic acid accumulation in *E. coli* O157:H7 was limited by the ability of the organism to lower its intracellular pH (21).

For all of the 2% NaCl treatments, we found that a lower intracellular pH correlated (R^2 = 0.95; not shown) with a decrease in the observed 5-log reduction time, suggesting that lowering the intracellular pH reduced survival. For the same data set, increasing the intracellular acetate anion concentration,

TABLE 2. Intracellular pHs and total intracellular acetic acid concentrations for the acid-salt treatments used in this study

NaCl concn (%) and protonated acetate concn (mM)	Calculated extracellular acetate anion concn (mM)[a]	Intracellular pH (SE)[b]	Calculated intracellular acetate anion concn (mM)[c]
2			
0	0.00	6.35 (0.03)	0.00
5	0.78	6.25 (0.03)	154.57
10	1.02	6.13 (0.02)	233.22
20	3.13	5.93 (0.04)	298.67
30	4.64	5.77 (0.02)	306.49
40	4.28	5.65 (0.06)	312.34
4			
0	0.00	5.67 (0.05)	0.00
5	0.36	5.73 (0.03)	47.17
10	0.71	5.97 (0.05)	162.50
20	1.82	5.92 (0.03)	289.20
30	3.63	5.80 (0.05)	328.24
40	4.31	5.71 (0.01)	353.20

[a] Calculated from the total protonated acetic acid concentration using high-performance liquid chromatography.
[b] Mean intracellular pH from three independent replicates.
[c] Calculated using the Henderson-Hasselbalch equation.

based on the protonated acid concentration and calculated from the intracellular pH data (Table 2), was also found to correlate with a decrease in survival ($R^2 = 0.89$, not shown). At 4% NaCl and 0 to 10 mM protonated acetic acid, the intracellular pH increased, and prolonged survival of a subpopulation of cells was apparent from the data shown in Fig. 1. Enhanced survival of *E. coli* O157:H7, compared to pH effects, was previously reported for these salt and acid conditions (4). However, the intracellular pH value with 4% NaCl and 5 mM protonated acetic acid was similar to the intracellular pH with 2% NaCl and 40 mM protonated acid (around pH 5.8), although the corresponding cell survival data were very different (Fig. 1). The relationships among the intracellular pH, acetic acid anion concentration, and cell survival remain unclear. Future work may include separating live and dead cell populations for intracellular pH measurements of the surviving cells at different times of incubation with acid solutions. It is likely that other factors, including the transmembrane motive force, which is determined by proton flux, may also help elucidate the relationship between the internal physiology of *E. coli* O157:H7 and cell death.

ACKNOWLEDGMENTS

We thank Roger F. McFeeters for helpful discussions of this work, Sandra Parker for excellent secretarial assistance, and Donald Fornea for help with high-performance liquid chromatography analyses.

This work was partially supported by a grant from the Pickle Packers International Inc.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or North Carolina Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable.

REFERENCES

1. **Albert, I., and P. Mafart.** 2005. A modified Weibull model for bacterial inactivation. Int. J. Food Microbiol. **100:**197–211.
2. **Axe, D. D., and J. E. Bailey.** 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherichia coli*. Biotechnol. Bioeng. **47:**8–19.
3. **Besser, R. E., et al.** 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA **269:**2217–2220.
4. **Bjornsdottir, K., F. Breidt, Jr., and R. F. McFeeters.** 2006. Protective effects of organic acids on survival of *Escherichia coli* O157:H7 in acidic environments. Appl. Environ. Microbiol. **72:**660–664.
5. **Booth, I. R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49:**359–378.
6. **Boyce, T. G., D. L. Swerdlow, and P. M. Griffin.** 1995. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N. Engl. J. Med. **333:**364–368.
7. **Breidt, F., Jr., J. S. Hayes, and R. F. McFeeters.** 2004. Independent effects of acetic acid and pH on survival of *Escherichia coli* in simulated acidified pickle products. J. Food Prot. **67:**12–18.
8. **Breidt, F., J. S. Hayes, and R. F. McFeeters.** 2007. Determination of 5-log reduction times for food pathogens in acidified cucumbers during storage at 10 and 25°C. J. Food Prot. **70:**2638–2641.
9. **Breidt, F., Jr., J. S. Hayes, J. A. Osborne, and R. F. McFeeters.** 2005. Determination of 5-log pathogen reduction times for heat-processed, acidified vegetable brines. J. Food Prot. **68:**305–310.
10. **Brudzinski, L., and M. A. Harrison.** 1998. Influence of incubation conditions on survival and acid tolerance response of *Escherichia coli* O157:H7 and non-O157:H7 isolates exposed to acetic acid. J. Food Prot. **61:**542–546.
11. **Brul, S., and P. Coote.** 1999. Preservative agents in food. Mode of action and microbial resistance mechanism. Int. J. Food Microbiol. **50:**1–17.
12. **Casey, P. G., and S. Condon.** 2002. Sodium chloride decreases the bacteriocidal effect of acid pH on *Escherichia coli* O157:H45. Int. J. Food Microbiol. **76:**199–206.
13. **CDC.** 1997. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider—Connecticut and New York, October 1996. MMWR Morb. Mortal. Wkly. Rep. **46:**4–8.
14. **Chapman, B., N. Jensen, T. Ross, and M. Cole.** 2006. Salt, alone or in combination with sucrose, can improve the survival of *Escherichia coli* O157 (SERL 2) in model acidic sauces. Appl. Environ. Microbiol. **72:**5165–5172.
15. **Chen, H.** 2007. Use of linear, Weibull, and log-logistic functions to model pressure inactivation of seven foodborne pathogens in milk. Food Microbiol. **24:**197–204.
16. **Chen, H., and D. G. Hoover.** 2003. Pressure inactivation kinetics of *Yersinia enterocolitica* ATCC 35669. Int. J. Food Microbiol. **87:**161–171.
17. **Cook, G. M.** 2000. The intracellular pH of the thermophilic bacterium *Thermoanaerobacter wiegelii* during growth and production of fermentation acids. Extremophiles **4:**279–284.
18. **Cook, K. A., et al.** 1998. Outbreak of *Salmonella* serotype Hartford infections associated with unpasteurized orange juice. JAMA **280:**1504–1509.
19. **Coroller, L., I. Leguerinel, E. Mettler, N. Savy, and P. Mafart.** 2006. General model, based on two mixed Weibull distributions of bacterial resistance, for describing various shapes of inactivation curves. Appl. Environ. Microbiol. **72:**6493–6502.
20. **Diez-Gonzalez, F., and J. B. Russell.** 1997. The ability of *Escherichia coli* O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid. Microbiology **143:**1175–1180.
21. **Diez-Gonzalez, F., and J. B. Russell.** 1997. Effects of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and acetate on *Escherichia coli* O157:H7 and K-12: uncoupling versus anion accumulation. FEMS Microbiol. Lett. **151:**71–76.
22. **Dougherty, D. P., E. R. Da Conceicao Neta, R. F. McFeeters, S. R. Lubkin, and J. F. Breidt.** 2006. Semi-mechanistic partial buffer approach to modeling pH, the buffer properties, and the distribution of ionic species in complex solutions. J. Agric. Food Chem. **54:**6021–6029.
23. **Entani, E., M. Asai, S. Tsujihata, S. Tsujihata, and M. Ohta.** 1998. Antibacterial action of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157:H7. J. Food Prot. **61:**953–959.
24. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129–5135.
25. **Janssen, M., et al.** 2007. Individual and combined effects of pH and lactic acid concentration on *Listeria innocua* inactivation: development of a predictive model and assessment of experimental variability. Appl. Environ. Microbiol. **73:**1601–1611.
26. **Jordan, K. N., and K. W. Davies.** 2001. Sodium chloride enhances recovery and growth of acid-stressed *E. coli* O157:H7. Lett. Appl. Microbiol. **32:**312–315.
27. **Jordan, K. N., L. Oxford, and C. P. O'Bryne.** 1999. Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increased acid tolerance. Appl. Environ. Microbiol. **65:**3048–3055.
28. **Jordan, S. L., et al.** 1999. Augmentation of killing of *Escherichia coli* O157 by combinations of lactate, ethanol, and low-pH conditions. Appl. Environ. Microbiol. **65:**1308–1311.
29. **Kroll, R. G., and I. R. Booth.** 1981. The role of potassium transport in the generation of a pH gradient in *Escherichia coli*. Biochem. J. **198:**691–698.
30. **McDonald, L. C., H. P. Fleming, and H. M. Hassan.** 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. **56:**2120–2124.
31. **McKellar, R. C., and X. Lu.** 2004. Modeling microbial responses in food. CRC Press, Boca Raton, FL.
32. **Mead, P. S., and P. M. Griffin.** 1998. *Escherichia coli* O157:H7. Lancet **352:**1207–1212.
33. **Oh, D.-H., et al.** 2009. *Escherichia coli* O157:H7 strains isolated from environmental sources differ significantly in acetic acid resistance compared with human outbreak strains. J. Food Prot. **72:**503–509.
34. **O'Sullivan, E., and S. Condon.** 1997. Intracellular pH is a major factor in the induction of tolerance to acid and other stresses in *Lactococcus lactis*. Appl. Environ. Microbiol. **63:**4210–4215.
35. **Rawlings, J. O., S. G. Pantula, and D. A. Dickey.** 1998. Applied regression analysis: a research tool, 2nd ed. Springer Verlag, New York, NY.
36. **Riebeling, V., R. K. Thauer, and K. Jungermann.** 1975. The internal-alkaline pH gradient, sensitive to uncoupler and ATPase inhibitor, in growing *Clostridium pasteurianum*. Eur. J. Biochem. **55:**445–453.
37. **Riley, L. W., et al.** 1983. Hemorrhagic colitis associated with a rare Escherichia coli serotype. N. Engl. J. Med. **308:**681–685.
38. **Rowbury, R. J., M. Goodson, and T. J. Humphrey.** 1994. Sodium chloride induces an NhaA/NhaR-independent acid sensitivity at neutral external pH in *Escherichia coli*. Appl. Environ. Microbiol. **60:**1630–1634.
39. **Russell, J. B.** 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. **73:**363–370.
40. **Salmond, C. V., R. G. Kroll, and I. R. Booth.** 1984. The effect of food preservatives on pH homeostasis in *Escherichia coli*. J. Gen. Microbiol. **130:**2845–2850.
41. **Virto, R., D. Sanz, I. Alvarez, S. Condon, and J. Raso.** 2006. Application of the Weibull model to describe inactivation of *Listeria monocytogenes* and *Escherichia coli* by citric and lactic acid at different temperatures. J. Sci. Food Agric. **86:**865–870.
42. **Warnecke, T., and R. T. Gill.** 2005. Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. Microb. Cell Fact. **4:**25.