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

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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. 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.

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† 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, 2RBCI, harrington) 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 rpm). Bacterial cells were enumerated by direct serial dilution in 0.85% NaCl and plating on LB agar using a spiral plater (model 4000: Spiral Biotech, Inc., Northwood, 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 acetic acid species. All acid solutions contained 20 mM Na-gluconic acid sodium salt (Sigma), which functioned as a concentrated 25-fold by resuspension in 2 ml of sterile saline (8.5 g/liter NaCl). Cultures were then harvested by centrifugation (25°C, 10 min, 5,000 rpm). Bacterial cells were enumerated by direct serial dilution in 0.85% NaCl and plating on LB agar using a spiral plater (model 4000: Spiral Biotech, Inc., Northwood, MA). After 24 h of incubation at 37°C, colonies were counted using an automated plate reader (QCount; Spiral Biotech).

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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·salt)*

(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 [14C]benzoate with [14C]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. [14C]sorbitol, [14C]inulin, and [14C]taurine gave inconsistent results, including negative calculated cell volumes (data not shown). [14C]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-

![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.](http://aem.asm.org/)
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 pH decreased 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.

**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)$^a$ | $\alpha$ (SE)$^b$ | $\beta$ (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.

$b$ $\alpha$, Weibull scale parameter in hours.

$c$ $\beta$, Weibull dimensionless shape parameter.

$d$ RT5, 5-log reduction time in hours.

$e$ ND, not determined.

**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).
pH declined to 5.7. The estimated $\alpha$ and $\beta$ 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 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% ($10^6$ 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...
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$^+$ transporters 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,

| NaCl concn (%) | 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 Forneca for help with high-performance liquid chromatography analyses. This work was partially supported by a grant from the Pickle Packers International.

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