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Determination of 5-Log Pathogen Reduction Times for Heat-Processed, Acidified Vegetable Brines†‡

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ABSTRACT

Recent outbreaks of acid-resistant food pathogens in acid foods, including apple cider and orange juice, have raised concerns about the safety of acidified vegetable products. We determined pasteurization times and temperatures needed to assure a 5-log reduction in the numbers of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* strains in acidified cucumber pickle brines. Cocktails of five strains of each pathogen were (separately) used for heat-inactivation studies between 50 and 60°C in brines that had an equilibrated pH value of 4.1. *Salmonella* strains were found to be less heat resistant than *E. coli* O157:H7 or *L. monocytogenes* strains. The nonlinear killing curves generated during these studies were modeled using a Weibull function. We found no significant difference in the heat-killing data for *E. coli* O157:H7 and *L. monocytogenes* ($P = 0.9709$). The predicted 5-log reduction times for *E. coli* O157:H7 and *L. monocytogenes* were found to fit an exponential decay function. These data were used to estimate minimum pasteurization times and temperatures needed to ensure safe processing of acidified pickle products and show that current industry pasteurization practices offer a significant margin of safety.

Regulations governing acidified foods in the United States (21 Code of Federal Regulations [CFR] part 114) were established in 1979. At that time, vegetative pathogenic microorganisms were not considered a significant risk for acidified food products. The regulation was primarily designed to prevent botulism. Spore outgrowth and toxin production by *Clostridium botulinum* will not occur if the pH is maintained at or below 4.6 *(12, 23)*. Recently, however, outbreaks of *Escherichia coli* O157:H7 and several *Salmonella* serotypes have occurred in acid foods, such as apple cider and orange juice *(5, 6)*. While pathogenic microorganisms have not been found to grow in these products due to the low pH (typically below 4.0), these microorganisms may adapt to acid conditions and survive for extended periods *(11, 17, 21)*.

Recently, we have investigated the role of acetic acid in killing *E. coli* in acidified foods *(1)*. For 0.6% (100 mM) acetic acid solutions with 2% NaCl and pH values around 3.7, typical of acidified pickle products, we found that *D* values (1-log reduction times) for the death of acid-adapted *E. coli* O157:H7 can be over 40 h at 10°C, although *D* values were significantly lower at warmer temperatures and lower pH values. In a study of beef carcass wash water, a treatment with 0.2% (33.3 mM) acetic acid and a pH of approximately 3.7 showed the *E. coli* O157:H7 strain survived for up to 14 days at 15°C, while cell numbers dropped about 4 log cycles *(22)*. In that study, competitive microflora were also present and could have influenced the survival of the *E. coli* strains. A statistical analysis of several published studies showed that typical storage conditions for apple cider at refrigeration (4°C) and abuse temperatures (10°C) were not sufficient to ensure a 5-log reduction in the cell numbers of *E. coli (9)*. From these and other studies *(2, 11, 24)*, it is clear that the potential for *E. coli* to survive for extended periods in acidified vegetable products with a pH below 4 exists.

To ensure the safety of juice products, 21 CFR part 120 was established in 2001. This regulation mandates a hazard analysis critical control point (HACCP) system with a processing step designed to deliver the equivalent of a 5-log reduction in target pathogen numbers in juices. Typically a heat pasteurization process is used for juices, based on *D*- and *Z*-value data for the killing of *E. coli* O157. This organism was found to be the most heat- and acid-resistant pathogen in fruit juices *(16)*. The acidified foods regulation (21 CFR part 114) governing acidified vegetable products, however, does state that acidified foods shall be thermally processed to an extent that is sufficient to destroy microorganisms of public health significance, as well as other bacteria that can reproduce in the food. Currently, many acidified, pickled vegetable products are heat processed to ensure shelf stability and prevent the growth of spoilage microorganisms, primarily lactic acid bacteria. Until the recent juice outbreaks, there was little concern about pathogenic microorganisms surviving in these products, and, to

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TABLE 1. *Bacterial strains used in this study*

Strain ID	Strain name	Previous ID[a]	Origin
B0195	*Listeria monocytogenes*	SRCC 529	Pepperoni
B0196	*L. monocytogenes*	SRCC 1791	Yogurt
B0197	*L. monocytogenes*	SRCC 1506	Ice cream
B0198	*L. monocytogenes*	SRCC 1838	Cabbage
B0199	*L. monocytogenes*	SRCC 2075	Diced coleslaw
B0200	*Escherichia coli* O157:H7	ATCC 43888	Laboratory strain (tox)
B0201	*E. coli* O157:H7	SRCC 1675	Apple cider
B0202	*E. coli* O157:H7	SRCC 1486	Salami outbreak
B0203	*E. coli* O157:H7	SRCC 2061	Ground beef
B0204	*E. coli* O157:H7	SRCC 1941	Pork
B0206	*Salmonella* serotype Braenderup[b]	SRCC 1093	10% salted yolk
B0207	*Salmonella* serotype Cerro	SRCC 400	Cheese powder
B0208	*Salmonella* serotype Enteritidis	SRCC 1434	Ice cream
B0209	*Salmonella* serotype Newport	SRCC 551	Broccoli with cheese
B0210	*Salmonella* serotype Typhimurium	SRCC 1846	Liquid egg

[a] SRCC strains obtained from Silliker, Inc. (Chicago, Ill.); ATCC, American Type Culture Collection (Manassas, Va.)
[b] Serotype of *Salmonella enterica.*

our knowledge, there have been no documented outbreaks of food pathogens in acidified vegetable products. Scientific data are lacking, however, to define the conditions that will assure the destruction of acid-resistant pathogens during heating in acidified vegetable products. While a target log reduction is not specified in 21 CFR part 114, we chose to determine 5-log reduction times in our studies of heat-killing pathogens in acidified vegetable brines to be compatible with the juice HACCP regulations. The objectives of this research were to define heat-processing treatments that will ensure a 5-log reduction in acid-resistant food pathogens, including *E. coli* O157:H7, *Salmonella* species, and *Listeria monocytogenes* under conditions representative of acidified pickle products.

MATERIALS AND METHODS

Preparation of pickle brines. Size 2A pickling cucumbers (about 2 cm in diameter) were used to prepare acidified (fresh pack) pickles. Approximately 640 g of cucumbers were placed in 1.4-liter jars with 640 ml of brine containing 4% sodium chloride and 0.2% calcium chloride, with a target concentration of 200 mM acetic acid. The actual acetic acid concentration was adjusted to give a pH of 4.1 after equilibration of the brine and the cucumbers, resulting in approximately 100 mM acetic and 2% sodium chloride. After filling, jars were sealed and heat processed so that the cold point of each jar was held at 74°C for 15 min to kill vegetative cells of microorganisms *(18).* The jars were then cooled by immersion in cold tap water to reduce the internal temperature to 40°C, and then they were allowed to cool to ambient temperature. The jars were stored at 25°C for at least 10 days to allow for chemical equilibration prior to removal of the brine for heat-processing experiments. Before use, the brine pH was checked and adjusted to 4.1 using 1 N NaOH or HCl, if necessary.

Apparatus. To carry out heat-killing experiments, 150 ml of filter-sterilized pH 4.1 brine was pipetted into sterile, water-jacketed (double wall) glass fermentation flasks with constant internal stirring using a magnetic stirrer. The flask consisted of a cylindrical internal (300-ml capacity) chamber surrounded by an outer isolated cylindrical jacket (ca. 100-ml capacity) with an inlet and outlet for the jacket water. Desired temperatures were maintained using a heating–cooling water bath (RTE-211, Neslab Instruments, Inc., Newington, N.H.) with an external pumping system to cycle water through the jacketed fermentation flasks. Temperatures of the brine within each flask were measured directly with sterile thermocouples (TJ36-T, Omega Technologies, Stamford, Conn.). The thermocouples were placed in the brine through flask side arms, and temperature was recorded using a data-logging apparatus (OM-3000, Omega).

Preparation of bacterial cells. Bacteria used in this experiment consisted of cocktails of five strains each of three different genera of foodborne pathogens (Table 1). To prepare bacterial cells for the heating experiments, bacteria were grown statically at 37°C for 16 h in tryptic soy broth (Difco, Becton Dickinson, Sparks, Md.) plus 1% glucose to induce acid resistance *(4).* Each of the five cultures was grown separately. Cells were harvested by centrifugation, resuspended in 0.1 volume of sterile saline, and combined into a cocktail with approximately equal numbers of each bacterial strain. A 2-ml aliquot was added then to the 148-ml brine samples in the water-jacketed fermentation flasks to give an initial total cell count of approximately 10^8 CFU/ml. At indicated time intervals, samples were removed and immediately diluted 10-fold into ice cold saline (0.85% NaCl). Serial 10-fold dilutions were prepared as needed to enumerate surviving cells by plating with a spiral plater (Spiral Biotech, Inc., Norwood, Mass.) on nonselective agar media, tryptic soy agar (Difco, Becton Dickinson) plus 1% glucose. After 24 to 48 h incubation at 37°C, colonies were counted using an automated spiral plate counter (Q-Count, Spiral Biotech). Each time–temperature experiment was carried out using duplicate or triplicate replications with independently prepared cells.

Modeling and statistical analysis. Weibull regression models were posited for determining the mean log reduction in cell numbers and used for the estimation of 5-log reduction times as described by van Boekel *(25).* The predicted values for the log of survivors, log *S,* was given by

$$\log S = N_0 - [1/\ln 10](\tau/\alpha)^{1/\beta}$$

with the parameters of the model including the initial cell numbers (N_0) and two shape parameters (α and β). The predicted survivor

TABLE 2. *Survival of* E. coli *O157:H7 strains in pH 4.1 pickle brine at 50°C*

Time (min)	Observed[a]	Predicted[b]	Residual squared
1	8.76	8.85	0.0080
1	8.75	8.85	0.0094
1	8.74	8.85	0.0103
18	8.29	8.25	0.0013
18	8.47	8.25	0.0493
18	8.38	8.25	0.0164
30	7.96	7.65	0.1003
30	7.41	7.65	0.0570
30	7.96	7.65	0.0958
45	6.58	6.76	0.0323
45	6.50	6.76	0.0674
45	6.81	6.76	0.0329
60	5.64	5.76	0.0135
60	5.41	5.76	0.1197
60	5.74	5.76	0.0004
75	4.87	4.66	0.0437
75	4.55	4.66	0.0132
75	4.95	4.66	0.0837

[a] Observed data (log CFU per milliliter).
[b] Values predicted by the Weibull model (log CFU per milliliter).
[c] Square of the residual (error) value.

curve was plotted as log survivors versus time (τ). With the α and β parameters, the 5-log reduction times (t^*) were calculated as

$$t^* = \alpha(-\ln 10^{-5})^{\beta}$$

for statistical inference about t^*, the log S equation was reparameterized as

$$\log S = N_0 - 5(\tau/t^*)^{\beta}$$

resulting in a nonlinear regression model that has additive, normally distributed errors that have equal variance. Independent curves were fit for each temperature with each pathogen, allowing for flexibility in the initial cell counts. Least squares estimates were obtained for model parameters, along with approximate standard errors based on standard asymptotic theory for nonlinear regression models and were determined using the nonlinear protocol of SAS software (SAS Institute Inc., Cary, N.C.). The estimated 5-log reduction times (t^*) for *E. coli* O157:H7 and *L. monocytogenes* data sets from all temperatures tested were modeled as a function of increasing temperature (T) using

$$t^* = k_1\, e^{(-k_2 \times T)}$$

To estimate the dependence of reduction time on temperature and to investigate whether dependence varied between the two bacterial species, *L. monocytogenes* and *E. coli* O157:H7, an appropriate log-linear regression model was given by

$$\log t^* = \beta_0 + \beta_1 T + \beta_2 L + \beta_3 TL$$

where L is an indicator variable taking on the variable of 1 for *L. monocytogenes* and 0 otherwise. Parameters for the exponential decay function were determined using the nonlinear protocol of SAS software (SAS). Analysis of variance was carried out using the analysis of variance protocol (SAS).

RESULTS

For the prepared cucumber brines, the measured pH varied from 3.94 to 4.12 prior to adjustment. The acetic acid concentration after equilibration of the brine and cucumbers was found to vary between 0.4 and 0.6% (data not shown). The data from three independent experiments with five *E. coli* O157:H7 strains held in pH 4.1 pickle brine at 50°C are shown in Table 2. The temperature was maintained with less than 1°C variation from the desired set temperature (data not shown). The equation describing the fitted regression line for these data was

$$\log S = 8.858 - 5(\tau/85.356)^{1.356}$$

and the observed and predicted data are shown in Figure 1. Plots of residuals against fitted values did not reveal any inhomogeneity of variance on the log scale (data not shown).

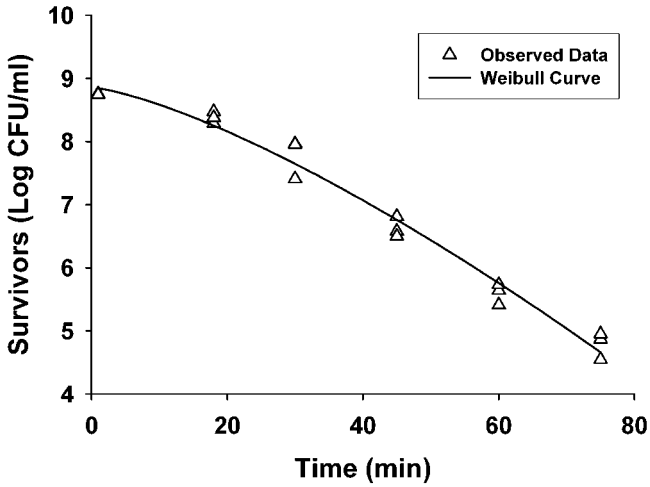

FIGURE 1. *Survival of cells at 50°C in pH 4.1 pickle brine. Data from three independent replications show the numbers of surviving cells from a cocktail of five* E. coli *O157:H7 strains (triangles) over time. The equation for the fitted regression line for* E. coli *O157:H7 strains was log* S $= 8.858 - 5(\tau/85.356)^{1.356}$.

Data were also collected using the same cocktail of *E. coli* strains over a range of temperatures between 50 and 60°C. These temperatures were chosen to allow accurate measurement of the killing curves, which ranged from 75 to 4 min for the range (50 to 60°C, respectively). For every data set, the Weibull regression provided a good fit, with the simple reparameterized three-parameter model always explaining $R^2 \geq 99\%$ of the variation in log survivors over time (Table 3).

In addition to the *E. coli* data, cocktails of the five *L. monocytogenes* strains were subjected to heating at three temperatures (54, 58, and 60°C) in the pH 4.1 pickle brine. An analysis of variance test determined that there was no difference between the *E. coli* O157:H7 and the *L. monocytogenes* 5-log reduction time data using the log-linear regression model, i.e., $\beta_2 = \beta_3 = 0$. An F ratio test of 0.051 was observed (on df = 2,5) indicating no difference ($P = 0.98$). Cocktails of five *Salmonella* strains were also subjected to heating treatments in the pH 4.1 pickle brine at 52 and 56°C. The *Salmonella* strains were found to be less heat resistant than *E. coli.* The 5-log reduction times for the cocktail of *Salmonella* strains were 15.9 min (52°C) and 3.8 min (56°C), compared with 61.2 and 17.1 min for the

TABLE 3. *Model data for* E. coli *O157:H7 including Weibull parameters and statistics*

Temp (°C)	N_0[a] (SE), min	β[b] (SE)	t^*[c] (SE)	U_{99}[d]
50	8.86 (0.12)	1.36 (0.12)	85.36 (2.30)	92.14
52	9.23 (0.27)	1.14 (0.15)	64.75 (3.55)	74.80
54	8.43 (0.08)	2.56 (0.25)	29.21 (0.62)	31.45
56	9.40 (0.86)	0.64 (0.26)	20.02 (4.40)	34.31
58	8.87 (0.13)	1.26 (0.08)	7.26 (0.15)	7.68
60	8.90 (0.27)	1.08 (0.15)	2.95 (0.13)	3.33

[a] Estimate value and standard error for the initial cell number.
[b] Estimated value and standard error for the parameter.
[c] Estimated value and standard error for the 5-log reduction time.
[d] Upper 99% confidence interval t^*.

E. coli O157:H7 strains (respectively). For this reason, only the data from the most heat- and acid-resistant organisms (*E. coli* and *L. monocytogenes*) were used to fit a model of the 5-log reduction times (Fig. 2).

Inspection of the 5-log reduction time data in Figure 2 suggested that the 5-log reduction times (t^*) for *E. coli* O157:H7 and *L. monocytogenes* decayed exponentially with increasing temperature. With the two-parameter exponential decay function (as described above) for these data, the model coefficients were found to be $k_1 = 4.8601 \times 10^7$ and $k_2 = 0.264$. The model explained 98% of the variance, and a plot of the predicted reduction time values was overlaid on the data as shown in Figure 2. Predicted 5-log reduction times for temperatures above 60°C (for which heat-killing curves could not be generated) were then calculated using the model parameters (Table 4). For temperatures between 65 and 80°C, the predicted 5-log reduction times ranged from 1.72 (0.56) to 0.033 (0.022) min (with the asymptotic standard errors in parentheses), respectively. A validation test at 65°C found that the bacterial cell numbers were reduced to below the level of detection, greater than a 5-log reduction, after 1 min, which was somewhat less than the model prediction of 1.7 min.

DISCUSSION

To prepare acid-adapted cells, we cultured bacterial strains statically in glucose to induce the acid tolerance response. Previous research has shown that *E. coli*, *L. monocytogenes*, and *Salmonella* strains have inducible acid resistance *(4, 10, 14, 15)* and that undissociated acetate can specifically trigger acid-resistance mechanisms in *E. coli* O157:H7 grown under anaerobic conditions *(8)*. Once acid resistance is induced, cells may survive at pH values as low as pH 2.0 for up to 6 h at 37°C *(8)*. A glutamate-dependent acid-resistance system has been found to be one of the most effective acid-resistance systems in *E. coli (3, 15)*. We had previously demonstrated that vegetable brine components (including glutamic acid) may increase the survival of *E. coli* O157:H7 under acid conditions *(1)*. In this report, we used brine from acidified cucumbers, which had been stored to allow equilibration of acids, sugars, and other diffusible brine components, including glutamic and other amino acids *(7)*. The brine used in these studies is typical of acidified pickle products. Garlic, spices, dill, and other commonly used ingredients were not added, however, to avoid the possibility that increased killing rates for the bacteria would result. Based upon discussions with processors and our own observations, pH 4.1 was selected as a reasonable upper pH limit used for commercial pickled vegetable products. Higher pH values are typically not used because of the

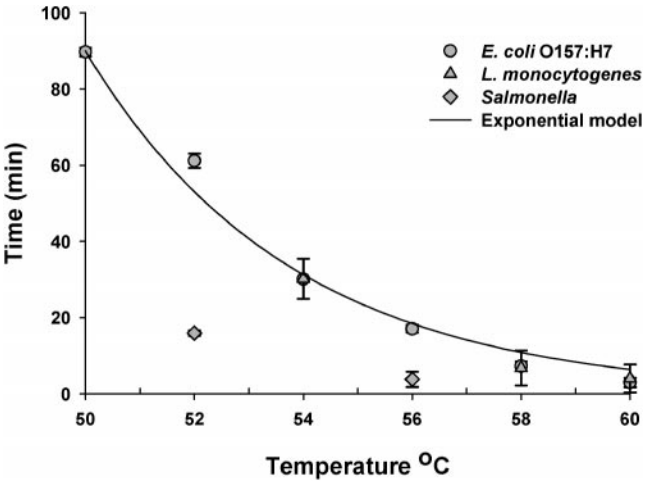

FIGURE 2. *Five-log reduction times for* E. coli, L. monocytogenes, *and* Salmonella *strains in pH 4.1 pickle brine at selected temperatures. The calculated 5-log reduction times based on the Weibull model for* E. coli *(circles),* L. monocytogenes *(triangles), and* Salmonella *(diamonds) with the standard error (error bars). Only the* E. coli *and* L. monocytogenes *data were used for the exponential decay model (line). The* R^2 *value for the model was 0.98.*

TABLE 4. *Predicted 5-log reduction times for* E. coli *O157:H7,* Salmonella, *and* L. monocytogenes *for temperatures above 60°C in acidified pickle brines*

Temp (°C)	t^*[a] (SE)	U_{95}[b]
61	4.94 (1.14)	7.64
62	3.79 (0.97)	6.08
63	2.91 (0.81)	4.83
64	2.24 (0.67)	3.83
65	1.72 (0.56)	3.04
66	1.32 (0.46)	2.40
67	1.01 (0.38)	1.90
68	0.78 (0.31)	1.50
69	0.60 (0.25)	1.19
70	0.46 (0.20)	0.94
71	0.35 (0.16)	0.74
72	0.27 (0.13)	0.58
73	0.21 (0.11)	0.46
74	0.16 (0.09)	0.36
75	0.12 (0.07)	0.28
76	0.09 (0.05)	0.22
77	0.07 (0.04)	0.18
78	0.05 (0.03)	0.14
79	0.04 (0.03)	0.11
80	0.03 (0.02)	0.09

[a] Estimate value and standard error for the 5-log reduction time, including five times the standard error as described in the text.
[b] Upper 95% confidence interval for t^*.

proximity to pH 4.6, the cutoff value above which *C. botulinum* may grow and produce toxin.

In previous studies of the survival of *E. coli* O157:H7 in acidified foods, we used *D*-value determinations to report acid-killing rates. In this study combining heat and acid treatments on the survival of food pathogens in vegetable brines, we found that nonlinear kinetics were typically seen (Fig. 1; data not shown). The Weibull model has been described in several reports as being well adapted for food pathogen inactivation studies *(20, 25).* One drawback of using the nonlinear models for microbial survival data is that this complicates the statistical analysis of the derived killing kinetics values, including calculated 5-log reduction times. By using a reparameterized model for determining the 5-log reduction time estimates, the standard error and confidence intervals were simply determined using the SAS NLIN procedure.

We have found that acidified pickle products contain between 100 and 400 mM acetic acid and have final equilibrated pH values between 3.2 and 4.0. For some of these products, vegetative cells of acid-resistant food pathogens may survive for extended periods (≥2 weeks) in simulated pickle brines *(1).* Current industry practice is to carry out a pasteurization step for most acidified cucumber pickle products, with the primary goal being to inhibit spoilage by lactic acid bacteria or fermentative yeasts. These practices have historically used pasteurization times and temperatures significantly greater than those described above *(18).* Until recently, there have been no serious concerns about the survival of vegetative microbial pathogens in these products.

We found the thermal resistance of *E. coli* O157:H7 and *L. monocytogenes* to be identical under the conditions of the assay. *Salmonella* strains were significantly less heat resistant. Novak and Yuan *(19)* found three strain cocktails of *E. coli* O157:H7 and *L. monocytogenes* were similarly affected by heat and ozone treatments on meats. A study with ground beef comparing *Salmonella, L. monocytogenes,* and *E. coli* five-strain cocktails showed that *Salmonella* was more heat sensitive than *E. coli* or *L. monocytogenes,* which were also similar in *D* values *(13).* Mazzotta *(16)* found that *Salmonella* was more heat sensitive than *L. monocytogenes* or *E. coli* O157:H7, and the *E. coli* O157:H7 strains were the most heat resistant of these pathogens in fruit juices.

This study has defined conditions needed to assure a 5-log reduction for acid-sensitized food pathogens in brined, acidified vegetable products. Our studies were limited to acidified cucumber brines, which had an equilibrated pH of 4.1. For the times and temperatures commonly used for processing acidified vegetable products (typically between 70 and 80°C and for 5 to 15 min), we found 5-log reduction times were less than 1.7 min (at 65°C) with pH 4.1 brine. Therefore, these pasteurization processes exceed conditions necessary for killing acid-resistant pathogens. These studies may have application to a variety of acidified foods; however, due to the limited scope of our experiments (acidified cucumber brines at pH 4.1), further work will be needed to determine conditions needed to ensure safe processing for other acidified vegetable products.

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