Development of an Effective Treatment for A 5-Log Reduction of *Escherichia coli* in Refrigerated Pickle Products

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Abstract: Refrigerated cucumber pickle products cannot be heat processed due to the loss of characteristic sensory attributes. Typically brined refrigerated pickles contain less than 100 mM acetic acid with pH values of 3.7 to 4.0. Refrigeration (4 to 10 °C) helps to inhibit the growth of spoilage bacteria and maintain flavor, texture, and appearance of the pickles. Previous research has shown that pathogenic *Escherichia coli* strains are unusually acid resistant and survive better in refrigerated acid solutions than at higher temperatures. We found that *E. coli* O157:H7 can survive for 1 mo or longer at 4 °C in brines typical of commercial refrigerated pickles. Our objective was to develop methods to assure a 5-log reduction of pathogenic *E. coli* in these types of products, while maintaining the sensory characteristics. A novel brine formulation was developed, based on current commercial refrigerated pickle brines, which contained 25 mM fumaric acid, 5 mM benzoic acid, 70 mM acetic acid, and 342 mM (2%) sodium chloride, with a pH of 3.8. Sensory data indicate that this formulation did not affect flavor or other sensory attributes of the product, compared to traditional formulations. We achieved a 5-log reduction of *E. coli* O157:H7 at 30 °C for 1.52 ± 0.15 d, at 20 °C for 3.12 ± 0.34 d, or at 10 °C for 8.83 ± 0.56 d. Growth of lactic acid bacteria was also inhibited. These results can be used by manufacturers to assure a 5-log reduction in cell numbers of *E. coli* O157:H7 and *Salmonella* without a heat process during the manufacture of refrigerated pickle products.

Keywords: *E. coli* O157:H7, food safety, fumaric acid, refrigerated pickles, *Salmonella*

Practical Application: While refrigerated acidified vegetable products are exempt from the acidified foods regulations, we have shown that the vegetative microbial pathogens *E. coli* O157:H7 can survive for up to 1 mo in these products, given current commercial production practices. To improve the safety of refrigerated pickle products, a brine formulation with reduced acetic acid, but containing fumaric acid, was developed to assure a 5-log reduction in cell numbers of *E. coli* O157:H7 without a heat process. The formulation can be used to assure the safety of refrigerated pickled vegetables without altering sensory characteristics.

Introduction

Outbreaks of disease from *Escherichia coli* O157:H7 have been traced to several acidic foods, including juices (pH 3.5 to 4.0), fermented dairy (pH 3.9) and mayonnaise (pH 3.6 in the aqueous phase) products (Besser and others 1993; Morgan and others 1993; CDC 1996). These products have similar pH values and storage temperatures as refrigerated pickles. There has been 1 reported case of a disease outbreak from *Salmonella* Newport in half sour brined pickles, which were refrigerated but not acidified, and slowly fermented (pH 4.4) (Cook County Board of Health press release in 2010). Half sours typically have a pH above 4.0. The majority of refrigerated cucumber pickles in the current retail market are not fermented, are mildly acidified with acetic acid (70 to 100 mM, pH 3.8 to 4.0), and are not heat processed. The processing and storage temperatures for refrigerated pickle products typically range from 4 to 10 °C. Cold temperatures help prevent cucumber softening and loss of white appearance or curing. However, low temperatures have also been shown to prolong the survival of *E. coli* O157:H7 in acid and acidified foods (Zhao and others 1993; Miller and Kaspar 1994; Weagant and others 1994; Conner and Kotrola 1995; Marques and others 2001; Bachrouri and others 2002). Previous studies with the survival of *E. coli* O157:H7 and acid resistant vegetative pathogens in acetic acid solutions indicate the possibility that these organisms can survive for extended periods of time in brines typical of refrigerated pickles (Breidt and others 2007; Lu and others 2011). *E. coli* O157:H7 strains have been shown to be more acid resistant than other vegetative pathogens, such as *Salmonella* and *Listeria* under conditions typical of acidified pickle products (Breidt and others 2005, 2007). In addition, anaerobic conditions in hermetically sealed

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Table 1–Bacterial strains.

Strain ID	Strain Name	Previous ID[a]	Source
LA 70	*L. plantarum*	ATCC 14917	Laboratory strain
LA 285	*L. plantarum*	ATCC 8014	Laboratory strain
LA 445	*L. plantarum*	MOP 3	Cucumber fermentation
B 200	*E. coli* O157:H7	ATCC 43888	Laboratory strain
B 201	*E. coli* O157:H7	SRCC 1675	Apple cider
B 202	*E. coli* O157:H7	SRCC 1486	Salami outbreak
B 203	*E. coli* O157:H7	SRCC 206	Ground beef
B 204	*E. coli* O157:H7	SRCC 1941	Pork
B 206	*Salmonella* serotype Braenderup	SRCC 1093	10% salted yolk
B 207	*Salmonella* serotype Cerro	SRCC 400	Cheese powder
B 208	*Salmonella* serotype Enteritidis	SRCC 1434	Ice cream
B 209	*Salmonella* serotype Newport	SRCC 551	Broccoli with cheese
B 210	*Salmonella* serotype Typhimurium	SRCC 1846	Liquid egg
B 409	*Salmonella* serotype Newport[c]	None	Refrigerated pickles[b]

[a]SRCC strains obtained from Silliker, Inc. (Chicago, IL.); ATCC American Type Culture Colleciton (Manassas, VA).
[b]Obtained from Cook County Dept. of Public Health, Chicago IL.

pickle jars may prevent the oxidative stress on acid injured cells (Stim-Herndon and others 1996; Kreske and others 2008; Bearson and others 2009), and thereby aid the survival of vegetative pathogens in pickle products.

In this study, we have found that *E. coli* O157:H7 strains can survive for up to 1 mo in commercial refrigerated pickle brines containing acetic and benzoic acids under anaerobic conditions. Our objective was to develop an effective treatment to ensure a 5-log reduction of *E. coli* O157:H7 for refrigerated pickles without heat processing, so as to maintain the sensory attributes of refrigerated pickle products. Previous research has indicated that fumaric acid is a more effective bactericidal agent against *E. coli* O157:H7 than acetic acid under anaerobic conditions (Podolak and others 1996; Comes and Beelman 2002; Kondo and others 2006; Lu and others 2011). The bacteriostatic effect of fumaric acid against lactic acid bacteria, which can potentially spoil refrigerated pickles, has also been reported previously (Cofran and Meyer 1970; Ough and Kunkee 1974; Pérez-Díaz and McFeeters 2010; Pérez-Díaz 2011). Currently, manufacturers must rely solely on the prevention of contamination of cucumbers or brines with vegetative pathogens to assure the safety of refrigerated pickles. We show that fumaric acid can be used in refrigerated pickles to assure a 5-log reduction of acid resistant *E. coli* O157:H7 without requiring a heat treatment, and with little or no impact on product acceptability.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are shown in Table 1. Frozen stocks of these cultures were stored at –80 °C in Luria Bertani (LB) broth for *E. coli* O157:H7 and *Salmonella enterica* strains. *Lactobacillus plantarum* strains were stored in Lactobacilli deMan Rogosa and Sharpe (MRS) broth supplemented with 16% glycerol (Sigma-Aldrich Chemical Co. Inc., St. Louis, Mo., U.S.A.). LB broth and MRS broth and agar (15 g/L for plate media) were purchased from Becton, Dickinson and Co. (Fairlawn, N.J., U.S.A.; Le Pont de Claix, France).

E. coli O157:H7 and *Salmonella* strains were statically cultured at 37 °C in LB broth supplemented with 1% glucose overnight (17 h) to induce acid resistance (Buchanan and Edelson 1996). The individual strains were combined, and then harvested by centrifugation at 5000 × *g* for 10 min at 25 °C. The cell pellets were washed with sterile saline (0.85% sodium chloride [NaCl]) then resuspended with 0.1 volume of saline to make a 5-strain *E. coli* O157:H7 cocktail or 6-strain cocktail of *Salmonella,* with an initial cell count of approximately 10^9 to 10^{10} CFU/mL. *L. plantarum* strains were statically cultured at 30 °C in MRS medium for 24 h. Three strain *L. plantarum* cocktails were prepared following the same cell harvesting procedures described above, using MRS broth for growth at 30 °C. For acid treatments (below), the strain cocktails were diluted 100-fold for an initial cell concentration of 10^7 CFU/mL. Experiments with the cell cocktails were done using 3 independent replicates. Protonated acid concentrations were calculated using pH tools, a Matlab program (Dr. Dan Dougherty, Michigan State Univ.) or similar Matlab algorithms (F. Breidt, unpublished), adjusting pKa values for ionic strength (0.342) and temperature (10, 20, or 30 °C). Organic acids and other chemicals were purchased from Sigma Chemical Co.

Fermentation inhibition with fumaric acid

The inhibitory effects of fumaric acid on the growth of *L. plantarum* was determined in simulated brines (pH 4.0) containing 50% cucumber juice, 2% NaCl, 5 mM benzoic acid, and 15 to 35 mM fumaric acid (as indicated). Glass pickle jars (235 mL, 8 oz) were filled with 220 mL of brine containing fumaric acid, and vacuum sealed with commercial lug caps fitted with a rubber septum to allow inoculation and sampling using sterile syringes. The lids were heated in boiling water for 10 s and immediately used to cap the filled jars. An aliquot of 1 mL *L. plantarum* cell suspension was then injected into each jar through the septum using a 1 mL syringe resulting in an initial cell concentration of around 10^5 CFU/mL. Inoculated jars were incubated at different temperatures (10, 20, and 30 °C). Brine samples (1.0 mL) were aseptically taken from pickle jars using sterile syringes, centrifuged as described above, resuspended and diluted in sterile saline to estimate bacterial cell populations by measuring optical density at 600 nm. For biochemical analysis (high performance liquid chromatography [HPLC]), samples (approximately 1 mL) were spun in a microcentrifuge (12000 × *g*, 1 min., Marathon 16KM, Fisher Scientific, Pittsburgh, Pa., U.S.A.) decanted, and the supernatants frozen until assayed (see below).

Survival of *E. coli* O157:H7 in refrigerated pickle brines

Brine was obtained by decanting the cover liquor from the jars of 4 commercial refrigerated pickle brands (1.4 L, 46 oz). The jars were obtained directly from manufacturers or purchased at local retail markets. The brines were filter-sterilized and stored at –20 °C for up to 2 mo before use. Biochemical analysis (described below) were done to determine NaCl content, pH, and the concentrations of organic acids and sugars. The brine samples were incubated for 24 h under anaerobic conditions to remove dissolved oxygen as previously described (Lu and others 2011). To determine effective treatments for a 5-log reduction of *E. coli* O157:H7 cells, cell suspensions were prepared as described above and inoculated (approximately 10^8 CFU/mL) into the brine samples (3 mL) supplemented with fumaric acid (0, 15, 25, and 35 mM) at pH 3.8, and held at 10, 20, and 30 °C in 5 mL Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, N.J., U.S.A.). The Vacutainer tubes were used to simulate the atmospheric conditions

in vacuum-sealed refrigerated pickle jars. An aliquot of 0.3 mL of the *E. coli* O157:H7 cocktail was injected through the Vacutainer tube septum (3.3 mL total volume). Three or more replicate experiments were done for each treatment, and similar replicate experiments were done with simulated refrigerated pickle brines (50% cucumber juice), containing 2% salt, 70 mM acetic acid, 5 mM benzoic acid and 25 to 35 mM fumaric acid.

For microbial analysis, 1 mL brine samples were aseptically removed from the Vacutainer tubes using sterile syringes. Samples were immediately neutralized by 1:10 dilution (0.1 mL into 1 mL) in pH 7 buffer of 0.1 M 3-*N*-morpholino-propanesulfonic acid (MOPS, Sigma Aldrich Chemical Co.) containing 0.85% NaCl. Sampling and dilution procedures were done in an anaerobic chamber (COY anaerobic chambers, Grass Lake, Mich., U.S.A.) to prevent oxidative damage to the acid injured cells. Neutralized and diluted samples were then taken out of the chamber, and plated on LB agar plates using a spiral plater (Model 4000, Spiral Biotech, Inc., Norwood, Mass., U.S.A.). Plates were incubated in anaerobic jars (Gaspak^{TM} 100 system, BD) at 37 °C for 24 h, and colonies were counted with an automated plate reader (Q-count, Spiral Biotech Inc.).

Chemical analyses

The concentrations of fermentable sugars and organic acids in brine samples were determined by HPLC analysis using a 30-cm HPX-87H column (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) as described by McFeeters and Barish (2003). In brief, the column was heated to 65 °C and eluted with 0.03N sulfuric acid at a flow rate of 0.9 mL/min. A Thermo Separations UV6000 diode array detector (Spectra System Thermo Scientific, Waltham, Mass., U.S.A.) was used at 210 nm to measure organic acids. A Waters model 410 refractive index detector (Waters Corp., Millipore Corp., Billerica, Mass., U.S.A.) connected in series with the diode array detector was used to measure glucose, fructose and ethanol. External standardization of the detectors was done using 4 concentrations of either sugar or organic acid standards. The NaCl content of commercial brine samples was determined by titration using standardized silver nitrate $AgNO_3$ solution (0.1711 N) (Fajans and Hassel 1923). Measurement of pH was done with a Fisher Scientific accumet AR25 pH meter (Fisher Scientific), and the brine pH was adjusted as needed using 6 N hydrochloric acid HCl or 3N sodium hydroxide NaOH.

Sensory analysis

Consumer acceptance tests for refrigerated pickles containing either 0 or 25 mM food grade fumaric acid (kosher certified, #W248800, Sigma-Aldrich) were conducted using custom manufactured products, prepared by a commercial pickle company. The acetic acid concentration was reduced by approximately 20% (90 to 74 mM) in the formulation with fumaric acid to generate equivalent sour taste (Da Conceicao Neta and others 2007), based on the calculated total protonated forms of fumaric and acetic acids (with Matlab, as described above) at the target pH of 3.7. Consumers ($n = 104$, aged 18 or above) were asked to evaluate the control and fumaric acid containing product using a 9-point hedonic scale for various sensory attributes including overall impression, flavor, and texture.

Statistical methods

Data were analyzed using SigmaPlot software (Version 10, Systat Software, Inc., San Jose, Calif., U.S.A.). The 5-log reduction times were estimated from linear and nonlinear functions of the regression coefficients for each die-off model of *E. coli* O157:H7. For the estimated standard errors, 95% confidence intervals for the 5-log reduction time of each holding treatment were obtained using asymptotic theory (J. Osborne, NCSU, personal communication) for nonlinear regression models as described by Lu and others (2011) and the nonlinear data fitting procedure (NLIN) of the SAS statistical software package (SAS Inst., Inc. Cary, N.C., U.S.A.).

Results and Discussion

The survival of *E. coli* O157:H7 in commercial brines

Our study showed the ability of *E. coli* O157:H7 to survive more than 1 mo in commercial refrigerated pickle brines before a 5-log reduction was achieved (Figure 1). Prolonged survival of *E. coli* O157:H7 in low pH food at refrigerated temperatures has been demonstrated by several studies (Zhao and others 1993; Weagant and others 1994; Marques and others 2001; Bachrouri and others 2002; Breidt and others 2007). The inactivation rate of *E. coli* O157:H7 by weak acid treatment has been found to be temperature dependent, and can be substantially accelerated as temperature increases (Weagant and others 1994; Conner and Kotrola 1995). However, increasing the temperature of refrigerated pickles may result in undesirable "curing" (loss of white color in the mesocarp tissue), softening and other product quality defects, depending on the duration of the holding time at an elevated temperature. Therefore, thermal treatments should be minimized. We found cells survived significantly better in commercial brine A1 than in brine D (Table 2, Figure 1, $P < 0.01$), presumably due to the higher pH, lower acid and salt content in brine A1. The results indicated a potential safety risk if *E. coli* O157:H7 contamination occurs. The existing hurdles against cell survival in or on the fruit in current refrigerated pickle products include low pH (pH 3.7 to 4.0), salt (2%), cold temperatures (4 to 5 °C), and the presence of acetic and benzoic acids, which equilibrate throughout the product in the jars.

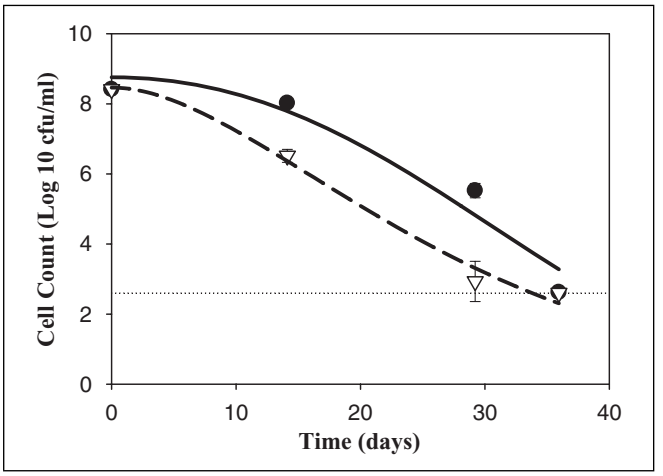

Figure 1–Survival of *Escherichia coli* O157:H7 in commercial refrigerated pickle brine A1 (circles, solid line) and brine D (triangles, dashed line), (as described in Table 2) at 4 °C. Experiments were done anaerobically. The horizontal dotted line indicates a 5-log reduction of *E. coli* O157:H7 compared to the initial value. Each data point represents the means of 3 replicates. Error bars indicate standard deviation. Data was fitted with a Weibull model.

Table 2–Composition of commercial refrigerated pickle brines.

Company	Acetic acid (mM)	Benzoic acid (mM)	pH	NaCl (%)
A1	72.8	5.3	3.83	2.42
A2	62.68	4.6	3.89	2.20
B1	90.19	6.7	3.79	2.00
B2	83.01	6	3.77	1.67
C1	68.34	6.6	3.86	1.83
C2	65.51	6.3	3.8	2.10
D	72.93	6.5	3.77	3.18
Mean	73.64	6.0	3.82	2.20

Survival of *E. coli* O157:H7 in modified refrigerated pickle brines

Commercial brines from companies A and D (Table 2) were inoculated with *E. coli* O157:H7 and showed a greater reduction in cell numbers as the concentration of fumaric acid increased, and/or temperature increased (Figure 2). Fumaric acid in refrigerated pickle brine at a concentration of 15 mM was insufficient to cause a 5-log reduction of *E. coli* O157:H7 within 10 d at 10 °C, and was therefore excluded for further testing. Regardless of holding temperatures, a 5-log reduction of *E. coli* O157:H7 was achieved in the brines containing 25 mM or more of fumaric acid. However, 35 mM fumaric acid wasn't significantly more effective than 25 mM of fumaric acid ($P < 0.01$). Based on these results and those shown in Table 2, an effective brine formulation that resulted in a 5-log reduction of *E. coli* O157:H7 cell numbers in less than 10 d in refrigerated pickle products was defined as: 25 mM fumaric acid (0.8 mM fully protonated acid), 70 mM acetic acid (63 mM protonated acid), 5 mM benzoic acid (3.6 mM protonated acid), and 2% salt at pH 3.8. The survival of *E. coli* O157:H7 in this brine at 10, 20, and 30 °C are shown in Figure 3. Linear models were chosen to fit the data, with an R^2 value of 0.97, 0.86, and 0.84, respectively. Increasing temperature shortened the 5-log reduction time from 8.83 ± 0.56 d at 10 °C to 3.12 ± 0.36 d at 20 °C, and to 1.52 ± 0.15 d at 30 °C. Similar 5-log reduction times were found for *Salmonella* strains (data not shown).

The antimicrobial effect of fumaric acid on *E. coli* O157:H7 has been demonstrated previously (Comes and Beelman 2002; Kondo and others 2006). A strong linear correlation between undissociated fumaric acid and log reduction of *E. coli* O157:H7 was observed in apple cider (Comes and Beelman 2002). In the same study, an effective non-thermal treatment of apple cider was developed at pH 3.3, with 0.15% fumaric acid (4 mM fully protonated acid) and 0.05% benzoic acid (3.5 mM protonated acid), and held

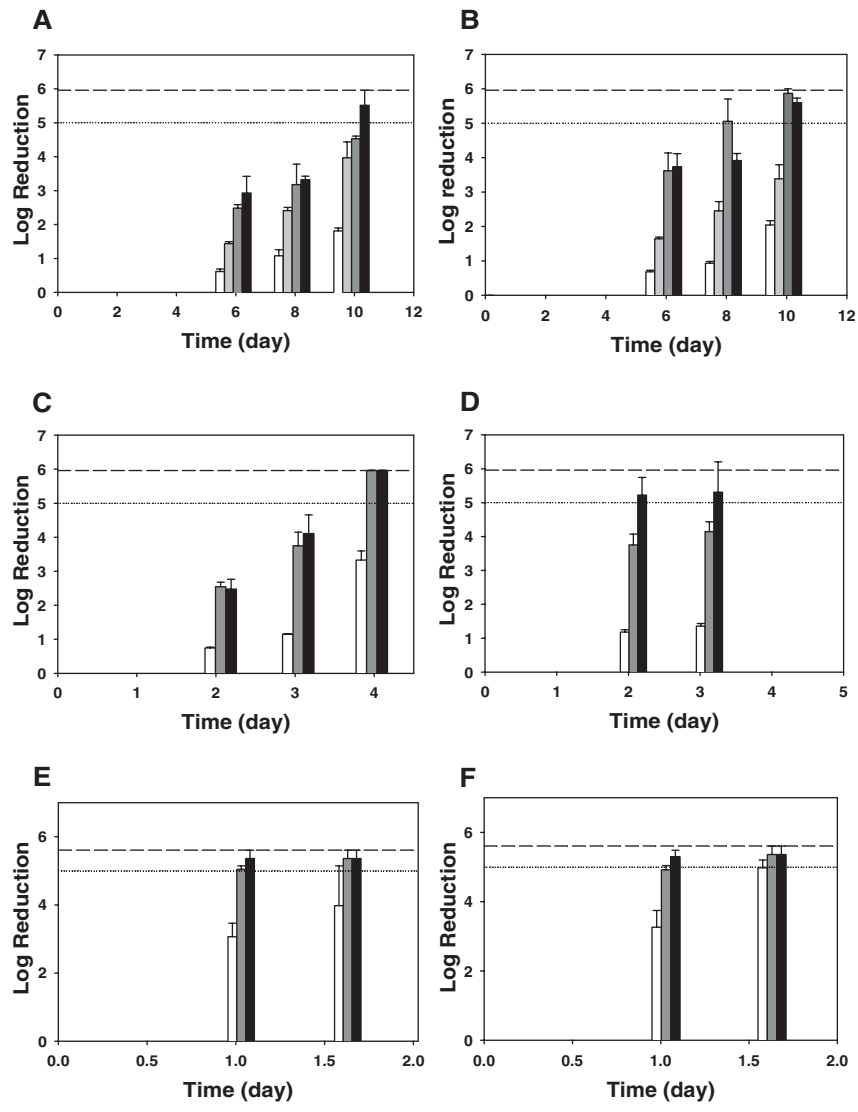

Figure 2–Antimicrobial effect of fumaric acid against *Escherichia coli* O157:H7 in Commercial Brines supplemented with 0 (white bars), 15(light gray bars), 25 (dark gray bars) and 35 (black bars) mM fumaric acid. Holding temperatures were 10 (A and B), 20 (C and D) and 30 °C (E and F). Dashed lines indicate detection limit, and the dotted lines indicate 5-log reduction. A, C and E were done with commercial brine A1, while B, D and F were done with commercial brine D. Each vertical bar represents means of 3 replicates. Error bars indicate standard deviation.

at 25 °C for 5 h (Comes and Beelman 2002). The organic acids (fumaric, benzoic and acetic acids), salts, and sugars from the brine and cucumber fruit will equilibrate in the pickle jars within 24 to 48 h. Therefore, *E. coli* O157:H7 and other vegetative bacteria that may be present in the product will be exposed to the organic acids even if they are embedded in the fruit. Although cucumbers were used in this study, the results may be valid for a variety of pickled vegetable products. Cucumber fruit has no known inhibitory compounds which may bias the study, and acetic and fumaric acids will equilibrate and penetrate into a variety of vegetables used for pickling.

Inhibition of lactic acid fermentation

Because refrigerated pickles are not heat processed, a spoilage fermentation by lactic acid bacteria may result in the product if it is temperature abused (10 °C or greater). The bacteriostatic effect of fumaric acid on lactic acid bacteria has previously been documented (Cofran and Meyer 1970; Ough and Kunkee 1974; Pérez-Díaz and McFeeters 2010; Pérez-Díaz 2011). We prepared a simulated pickle brine solution containing 50% cucumber juice, 15 to 35 mM fumaric acid, 5 mM benzoic acid and 2% NaCl (pH 4.0) to test the inhibitory effect of fumaric acid on fermentation. The time before the production of 15 mM lactic acid in these brines is shown in Table 3. Based on these observations, 15 and 25 mM fumaric acid was sufficient to retard lactic acid fermentation for 20 d at 10 °C. At higher temperatures, 25 and 35 mM fumaric acid (along with 5 mM benzoic acid) delayed fermentation for 3 to 8 d at 20 °C or 2 d at 30 °C. Since the bacteriostatic effect of fumaric acid can be significantly enhanced as pH decreases (data not shown), the same holding treatments remain valid for refrigerated products formulated with pH values lower than 4.0. These results indicate that spoilage by lactic acid bacteria will be inhibited if holding at 10 °C is used for refrigerated pickles containing 25 mM fumaric acid in addition to 5 mM benzoic acid.

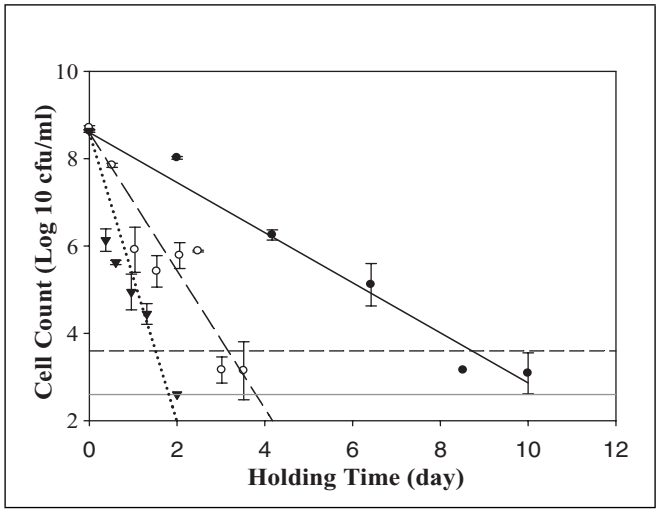

Figure 3–The die-off of *E. coli* O157:H7 in cucumber jars filled with simulated brine solutions prepared with the effective formulation (25 mM fumaric acid, 70 mM acetic acid, 5 mM benzoic acid, 2% salt, and pH 3.8) at a holding temperature of 10 °C (filled circle), 20 °C (open circle) and 30 °C (filled triangle). Linear models were used to fit the data of 10 °C (solid line), 20 °C (long dash line) and 30 °C (dotted line), with an R^2 of 0.97, 0.86 and 0.84, respectively. Horizontal short dash line indicates a 5-log reduction; and solid line indicates the detection limit.

Table 3–Inhibitive effect of fumaric acid on lactic acid fermentation.

Temperature (°C)	Total fumaric acid conc. (mM)	Time before [lactic acid] reach 15 mM (d)
10	15	>20
	25	>20
	35	>20
20	15	2
	25	3
	35	>3, <8
30	15	1
	25	2
	35	2

Table 4–Sensory analysis of refrigerated pickles.

	Overall	Flavor	Texture
Control	6.3[a*]	6.2[b]	7.2[c]
Fumaric	6.4[a]	6.2[b]	7.3[c]

*Mean score from the 9-point hedonic scale for overall, flavor, and texture liking. Similar letters within a column indicate no significant difference ($P > 0.01$).

Sensory characteristics

The flavor of fumaric acid has been described as tartness and grape like (Gardner 1977). In pickle products with acetic acid, the flavor contribution by fumaric acid was not known. We were, however, able to predict the impact of the acid species in solution on sour taste. The acid species in solution (anion and protonated) that have one or more protonated carboxyl groups (protonated acetic acid or mono- or di-protonated fumaric acid) should all equally contribute to sour taste, but acid anions without protonated carboxyl groups do not (Da Conceicao Neta 2007). The concentration of the protonated form(s) of the acetic and fumaric acids were determined at pH 3.8. The acetic acid (total acid concentration) was, therefore, reduced from 90 to 74 mM in brines containing 25 mM fumaric acid to result in equivalent sour taste in the fumaric acid treated and control brines. The brine pH for the control and fumaric acid samples was determined to be pH 3.6 and pH 3.3, respectively. Although the target pH (pH 3.7) was not achieved in either sample, results from the consumer acceptance testing showed that there were no significant differences ($P < 0.01$) in the overall, flavor or texture liking between the control and fumaric acid pickles (Table 4).

Conclusions

We have developed a modified formulation for refrigerated pickles that results in a 5-log reduction in *E. coli* O157:H7 and *Salmonella*. In current product brines, which contain acetic acid and benzoic acid, we found that *E. coli* O157:H7 can survive for up to 1 mo. To improve the safety of refrigerated pickle products, a brine formulation with added fumaric acid and reduced acetic acid was developed. Fumaric acid is generally regarded as safe, and is used in a variety of food products. The modified formulation did not alter the characteristic sensory properties of refrigerated pickles, and was found to assure a 5-log reduction in cell numbers of *E. coli* O157:H7 without a heat process.

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