



Inactivation by lemon juice of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* in beef marinating for the ethnic food kelaguen

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ARTICLE INFO

Article history:

Received 28 February 2012

Received in revised form 10 October 2012

Accepted 8 November 2012

Available online 20 November 2012

Keywords:

Escherichia coli O157:H7

S. Enteritidis

L. monocytogenes

pH

Citric acid

D-value

ABSTRACT

Lemon juice, a major source of acidulant citric acid, is frequently used in the preparation of ethnic foods. Raw or partially cooked meats are marinated with lemon juice in the preparation of a popular Chamorro dish called kelaguen, which is, unfortunately, strongly associated with foodborne illness outbreaks in Guam. We investigated the efficacy of lemon juice in reducing numbers of *Escherichia coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* at stationary phase during marination. Beef inoculated with a three-strain mixture of *E. coli* O157:H7, *S. Enteritidis*, or *L. monocytogenes* at 10^6 CFU/mL was marinated with lemon juice from 0.2 to 10 mL/g for 48 h at 28 °C. The decline of the pathogens during marination exhibited various degrees of deviation from first-order kinetics. Based on calculations with both linear regression and Weibull models, the decimal reduction time (4-D values) over the range of lemon concentrations was 366–5.1 h for *E. coli* O157:H7, 282–2.4 h for *S. Enteritidis*, and 104–2.4 h for *L. monocytogenes*, indicating that *E. coli* O157:H7 was the most lemon-juice-resistant of the three. The pathogen reduction time (log 4-D values) plotted against undissociated titratable citric acid exhibited a biphasic pattern. The pathogen reduction time (log 4-D or δ values) was linearly correlated with the pH of the marinating beef ($R^2 = 0.92$ to 0.98). The Z_{pH} values (pH dependence of death rate) with beef marination were 1.03 for *E. coli* O157:H7, 0.92 for *S. Enteritidis*, and 1.29 for *L. monocytogenes*, indicating that *L. monocytogenes* was the most pH resistant of the three. *L. monocytogenes* exhibited less resistance to lemon juice than *S. Enteritidis* at pH of 3.5–4.4 but more resistance at pH of 2.6–2.8. In addition, at 4 °C, all three pathogens exhibited 4-D values 1.7–4.1 times greater than those at 24 °C at 5 mL lemon juice/g beef. In conclusion, the usual beef marinating practice for kelaguen preparation (<0.5 mL lemon juice/g beef for 1–12 h) did not sufficiently inactivate *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* to meet minimum food-safety requirements. To reduce the risk of kelaguen-associated foodborne illness, kelaguen preparation must include heat treatment in addition to marination with lemon juice.

Published by Elsevier B.V.

1. Introduction

Weak organic acids are one of the several primary agents used to control microorganisms in both fermented and nonfermented foods (Buchanan et al., 2002). For example, citric acid, acetic acid, and lactic acid are either naturally produced or added to food or marinades to achieve food safety and meet quality requirements. Weak acids exhibit antimicrobial activity mainly in their undissociated form, which penetrates the cell membrane, acidifies cytoplasm, and increases the toxic level of the dissociated acid anion (Lambert and Stratford, 1999; Mani-López et al., 2012; Young and Foegeding, 1993). In addition, the chelating properties of organic acids (such as citric and malic acid) can also destabilize the cell outer membrane (Mani-López et al., 2012).

The ethnic food “kelaguen” in Guam is a popular Chamorro meat dish with a pH range from 4.5 to 5.8 (Yang and Lee, 2009). In its preparation, lemon juice is either used to marinate raw meats (e.g., beef or seafood) or mixed with partially cooked meat (e.g., chicken) and other ingredients, such as peppers, onions, coconut, and salt (Yang and Lee, 2009). Unfortunately, kelaguen accounts for about 13% of food-borne-illness outbreaks in Guam and for about 16% of individual cases (Haddock, 2007). The pathogens known to be associated with kelaguen-related food-borne illnesses are *Salmonella* spp., *Staphylococcus aureus*, *Vibrio* spp., and *Shigella* spp. (Haddock, 2007). The use of raw or partially cooked meat is considered one of the major improper food-handling practices resulting in such illness.

Escherichia coli O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* are pathogens that commonly contaminate beef, raw chicken, and ready-to-eat meat products. *E. coli* O157:H7 can tolerate pH 2.0 and survives at pH 3–4; *S. Enteritidis* and *L. monocytogenes* survive at pH 4–5.5 (Conner and Kotrola, 1995; Gabriel and Nakano, 2010; Miller and Kaspar, 1994). Lemon juice (citric acid), the major

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acidulant for kelaguen, exerts bactericidal and bacteriostatic effects on all three (Buchanan and Golden, 1994; Sengun and Karpinar, 2004; Young and Foegeding, 1993). For example, lemon juice concentrate produces 5-log inactivation of all three at 0 °C, and single-strength lemon juice a 5-log inactivation of *E. coli* O157:H7 at 22 °C (Enache et al., 2009; Nogueira et al., 2003). Citric acid reduces *E. coli* O157:H7 and *L. monocytogenes* 4–5 logs in acidified media at 25–28 °C (Bjornsdottir et al., 2006; Buchanan and Golden, 1994; Conner and Kotrola, 1995).

The rate at which lemon juice or citric acid inactivates pathogens depends on acid concentration, pH, and temperature (Buchanan and Golden, 1994; Nogueira et al., 2003). The responses of food-borne pathogens to acid stress may or may not follow classical first-order kinetics (Buchanan et al., 2002). The high survival and the low infectious doses of *E. coli* O157:H7 and *S. Enteritidis* in acidic or acidified foods pose a great risk of foodborne illness. Recently, the Weibull model has been used to describe the acid-inactivation rate (δ and ρ) of pathogens for survival curves deviating from first-order kinetics (Virtó et al., 2006) and is considered more realistic than the linear regression model in the estimation of microbial inactivation time (van Boekel, 2002).

Although we have previously reported controlling lemon concentration and pH to achieve safety of kelaguen without temperature control (Yang and Lee, 2009), how foodborne pathogens survive during beef marination with lemon for kelaguen is unclear. The objectives of the study reported here were to determine the inactivating effect of lemon juice during beef marination on *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* and to calculate the inactivation rate under both the linear regression and the Weibull models.

2. Materials and methods

2.1. Strains of microorganisms

The pathogens *E. coli* O157:H7 (ATCC# 35250, 43889, and 43890), *S. enterica* serovar Enteritidis (ATCC 19585, 13311, 14028), and *L. monocytogenes* (ATCC 7644, 19113, and 19114) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). They were maintained in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) containing 50% glycerol at –20 °C for less than 1 year before use.

2.2. Preparation of inoculum

Each strain of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* was thawed and transferred to 9 mL of TSB and incubated at 37 °C for 24 h. The TSB culture was streaked on a tryptic soy agar (TSA, Difco) plate and incubated overnight at 37 °C. Tryptic soy agar was stored at 4 °C for no more than 1 month before use. A single colony from a TSA plate was inoculated into 9 mL TSB and incubated at 37 °C for 24 h. A cocktail consisting of a mixture of the three strains of *E. coli* O157:H7, *S. Enteritidis*, or *L. monocytogenes* was prepared by combining equal volumes of the three strain's cultures in a centrifuge tube. The cocktail in the stationary phase was then centrifuged at 3600 rpm for 25 min with an IEC Centra CL2 centrifuge (Thermo Electronic Cooperation, Milford, MA, USA). The cell pellets were suspended in 27 mL of peptone water and used as the inoculum. We determined the initial number of bacteria in each inoculum suspension by plating 0.1 mL aliquots of appropriate dilutions with 1% peptone water on sorbitol-MacConkey agar (SMAC, Difco) for *E. coli* O157:H7, xylose lysine deoxycholate agar (XLD, Difco) for *S. Enteritidis*, and Oxford agar (OAB, Difco) for *L. monocytogenes* and incubating the plates at 37 °C for 24 h.

Raw beef (top round) was purchased from a local supermarket and stored at –18 °C for no more than 48 h. Before inoculation with the bacteria, the beef was thawed at 4 °C for 24 h and cut into slices (5×5×2.5 mm). The beef slices (100 g, about 100 slices) were placed

in an aseptic weighing boat (14×14 cm), inoculated with 1 mL of the three-strains inoculum (approximately 8.0 log CFU/mL) of *E. coli* O157:H7, *S. Enteritidis*, or *L. monocytogenes* by pipetting the inoculum drop by drop as evenly as possible across the slices, and further mixed with a sterile glass rod for even distribution of the inoculum. The inoculated beef slices were held for 10 min at 24 °C while the inoculum dried; the final bacterial concentration on the beef slices was approximately 6 log CFU/g. For analysis of three pathogens, the beef inoculation was prepared separately for testing *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes*.

2.3. Beef marination treatment

For marination treatment and sampling, five-g samples of the 100-g inoculated beef were transferred to sterile beakers. The number of sterile beakers depended on the number of sampling time in each marination treatment. For example, in testing *E. coli* O157:H7 during marination at a ratio of 0.2:1 (lemon juice:beef, mL/g), 1 mL of fresh-squeezed lemon juice – 7.2 g citric acid/100 mL – from locally grown lemons (*Citrus limon* L.) was added in each of 7 beakers; one beaker in this treatment was randomly selected and tested at times of 0, 4, 8, 12, 24, 36, and 48 h. The same marination treatment at the ratio of 0.2:1 and similar sampling were also used for testing *S. Enteritidis* and *L. monocytogenes*. The above procedures were used for the beef marination treatment at lemon ratios of 0.5:1, 1:1, 5:1, and 10:1. With the same procedures, the temperature effect was studied by marinating the beef samples with lemon juice at a ratio of 5:1 and at 4 °C; the citric acid effect was studied by marinating beef samples with citric acid solution (7.2%) at a ratio of 5:1 and 24 °C. All the above experiments were repeated from the inoculation to the beef marination treatment.

2.4. Microbiological analysis

For measurement of bacterial survival, the 5-g beef slices were taken from each treatment and combined with 45 mL of buffered peptone water in a Stomacher bag and pummeled with a Seward Stomacher® 80 (Norfolk, UK) for 2 min. 1 mL of sample from the Stomacher bag was serially diluted (1:10) with 9 mL of sterile peptone water (1%). Portions (0.1 mL) from appropriate dilutions were spread plated with a spiral plater (Model D; Spiral Biotech, Bethesda, MD) on SMAC, XLD, or OAB agar with modified Oxford antimicrobial supplement. The plates were then incubated at 37 °C for 24 h for *E. coli* O157:H7 and 48 h for *S. Enteritidis* and *L. monocytogenes*. Thereafter, bacterial colonies were counted and expressed as colony forming units (log CFU/g). The mean of log CFU/g calculated from two replications of each sampling point were expressed as bacterial survival counts.

2.5. pH, titratable acidity, and undissociated citric acid

The pH of the mixtures of beef and lemon juice was measured with a pH meter (Corning Scholar 425, Corning, N.Y.). The titratable acidity of the marination mixture was titrated with NaOH solution and expressed as citric acid g/100 g based on citric acid standard curve. The concentration of undissociated citric acid of the marination mixture was calculated with Henderson–Hasselbalch equation based on the titratable acidity, pH, and pK_3 value (3.128) of citric acid (H_3B).

2.6. Calculation of D-values and Z-values

The survival curves of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* were the log bacterial survival counts plotted against the marination time. With the linear model, the decimal reduction time (D-value) – the marination time necessary to inactivate 90% of the bacterial population – was calculated from the slope of the survival curve. With the Weibull model, the survival parameters of the bacteria were described by $\log_{10}S(t) = (t/\delta)^\rho$ (Mafart et al., 2002), where $\log_{10}$

$S(t)$ represents the decimal reduction ratio at time t , parameter δ represents the time of the first decimal reduction, and parameter ρ represents the curve shape parameter. The D-value, the shape parameter (ρ), the decimal reduction parameter (δ), and 4-D values were evaluated with the linear model and the Weibull model by GInaFIT software (Geeraerd et al., 2005). The Z_{pH} -value, which is defined as a pH value resulting in a 90% change of the D-values, was determined by computation of the reciprocal of the slope of the linear regression curve between the $\log_{10}$ 4-D values and their corresponding marination pH values.

2.7. Statistical analysis

Based on two replications of the inoculation and marination experiment, the statistically significant differences between D values obtained at different concentrations were determined using a one-way analysis of variance; the LSD test at $p < 0.05$ was performed with PASW Statistics 18 (SPSS, 2009). These analyses were separately conducted for each organism and for each of the two models fitted to the data.

3. Results and discussion

3.1. Lemon juice concentration in beef marination

The concentration of lemon juice used in kelaguen recipes ranges from 3 to 24%. Based on the lemon concentration, the dish is classified as low-acid (0–15% lemon juice; pH above 4.6) or high-acid kelaguen (16–25% lemon juice; pH below 4.6) (Yang and Lee, 2009).

The range of lemon-juice concentrations used in this study ranged from 16.6 to 90.9% and covered the range of concentrations used high-acid kelaguen. The corresponding range of pH values was 4.40–2.61, titratable acidity 1.43–4.50 g citric acid/100 g (or 74.3–234 mM), and undissociated acid concentration 2.60–180 mM (Table 1). Because *E. coli* O157:H7 is inactivated by 4–5 logs at a citric-acid concentration of 50 mM (undissociated form) or in media at a pH of 3.2 without weak acid after exposure for 6 h (Bjornsdottir et al., 2006), the range of lemon concentrations and pH used in our study should exert antimicrobial activity against *E. coli* O157:H7.

3.2. Microbial survival curves on beef marinating in lemon juice

The *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* cells used in the survival study were in the stationary phase. In addition to contamination, one of reasons to select *S. Enteritidis* rather than acid-resistant *Salmonella* serovars in this study is that the survival of *S. Enteritidis* in regular practice of beef marination also indicates the survival of the acid-resistant *Salmonella* serovars.

When the beef was marinated at 24 °C, the range of lemon-juice concentrations produced immediate inactivation of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* without a lag phase in the survival

curves (Fig. 1). Their declines with time of marination followed first-order kinetics in some cases and non-first-order kinetics in others. Buchanan et al. (2002) reported that the acid inactivation of pathogens typically follows first-order kinetics in a strongly acidic environment (pH 1.5–3.5) and non-first-order kinetics, often with shoulders or tails, under less acidic conditions. The acid-inactivation kinetics of foodborne pathogens is complex and affected by physiological processes as well as intrinsic and extrinsic factors (such as pH, acid identity, acid concentration, temperature, water activity, and “age” of cells) (Buchanan et al., 2002). The response of cells to treatments at various concentrations in this study affected the kinetics of the survival curves.

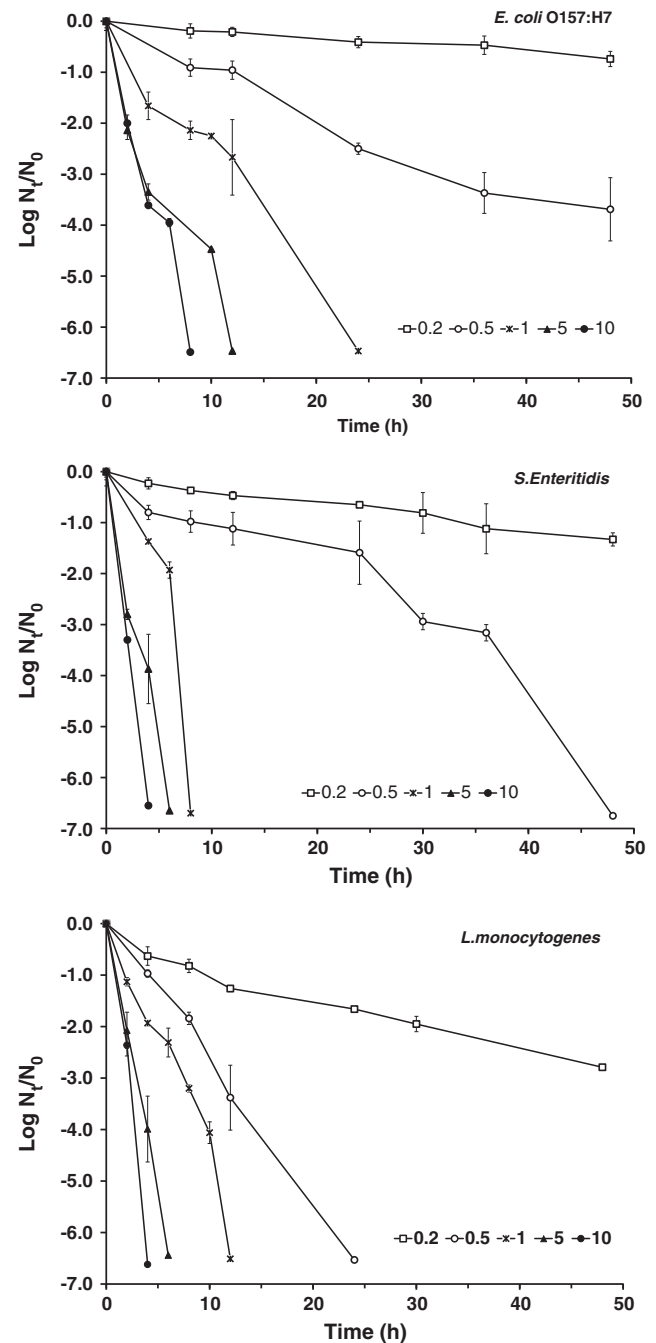


Fig. 1. Survival curves of *E. coli* O157:H7 (top), *S. Enteritidis* (middle), and *L. monocytogenes* (bottom) on beef marinated in lemon juice. Point shapes represent ratios of lemon juice to beef (mL/g).

Table 1

Experimental concentrations of lemon juice, pH, and acidity (mean $\pm$ SD) used in marination of beef as for preparation of kelaguen.

Ratio of lemon juice to beef (mL/g)	pH	Titratable acidity		Undissociated citric acid (mM) ^b
		Citric acid (g/100 g)	Citric acid (mM)	
0.2 (16.6%) ^a	4.40 $\pm$ 0.01	1.43 $\pm$ 0.01	74.3 $\pm$ 0.73	2.60 $\pm$ 0.03
0.5 (33.3%)	3.90 $\pm$ 0.00	2.12 $\pm$ 0.03	110 $\pm$ 1.45	14.3 $\pm$ 0.19
1.0 (50.0%)	3.47 $\pm$ 0.00	2.81 $\pm$ 0.06	146 $\pm$ 3.27	44.3 $\pm$ 0.99
5.0 (83.3%)	2.75 $\pm$ 0.02	4.18 $\pm$ 0.15	217 $\pm$ 8.05	146 $\pm$ 5.41
10.0 (90.9%)	2.61 $\pm$ 0.01	4.50 $\pm$ 0.11	234 $\pm$ 5.87	180 $\pm$ 4.52

^a Calculated concentration (%) of lemon juice during beef marination.

^b Calculated concentration of citric-acid undissociated form based on titratable acidity with pK_3 of citric acid at 3.128.

For foodborne pathogens with non-first-order kinetics in acid-inactivation, the Weibull model is valuable for describing acid-inactivation parameters – the shape value ρ and the first-decimal-reduction-time δ value (Virtto et al., 2006). If $\rho < 1$, $\rho = 1$, or $\rho > 1$, the shape of a survival curve is concave upward, linear, or concave downward, respectively. The linear shape ($\rho = 1$) represents the first order kinetics. With regard to physiological effects, the upwardly concave curves are associated with the adaptation of the remaining cells to the applied stress and the downwardly concave with increased damage of the remaining cells in applied stress (van Boekel, 2002). With beef marination, the shape values ρ of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* varied from 0.57 to 4.38 (Table 2), indicating that three pathogens responded differently to the stress of lemon juice.

3.3. Pathogen decimal reduction time with beef marination

Because the three pathogens exhibited both linear and nonlinear survival curves (Fig. 1), both the linear-regression model and the Weibull model were used to determine the pathogen decimal reduction time (D- and δ -value). Table 2 shows the D- and δ -values for the three pathogens at the five lemon-juice concentrations we used. At the two lowest concentrations (0.2, 0.5 mL/g), the order of pathogen resistance to lemon juice was *E. coli* O157:H7 > *S. Enteritidis* > *L. monocytogenes*; at the intermediate concentration (1 mL/g), the order was *E. coli*

O157:H7 > *L. monocytogenes* > *S. Enteritidis*; at the two highest concentrations (5, 10 mL/g), the order was *E. coli* O157:H7 > *S. Enteritidis* = *L. monocytogenes*. At all concentrations, *E. coli* O157:H7 was the most resistant of the three to lemon juice. At the two highest concentrations, the differences in resistance to lemon juice between *S. Enteritidis* and *L. monocytogenes* became nonsignificant. The resistance of the pathogens to lemon juice therefore changed with acidity during beef marination and depended on the physiological characteristics of the individual pathogens.

Bingol et al. (2011) reported that a treatment of lemon juice to cig kofte (Turkish raw meatball) at 0.6 mL/g for 1 h inactivates *S. Enteritidis* by 1.5 logs and *E. coli* by 1.9 logs. The extrapolated D-values are 0.67 h for *S. Enteritidis* and 0.48 h for *E. coli*, much lower than the D-values we observed at 0.5 mL/g (Table 2). The difference in D-values may be attributable to the synergistic antimicrobial effect of lemon juice with other ingredients, such as paprika and black pepper, in cig kofte (Bingol et al., 2011). Enache et al. (2009) observed that a single-strength lemon juice at 22 °C reduces *E. coli* O157:H7 by 5.70 logs at the treatment of 48 h. The extrapolated D-value is 8.42 h, about 6.3 times greater than the D-value we observed in our highest-concentration treatment (Table 2). Since both treatments has a pH value of 2.5–2.6 and titratable acidity of 4.5%, the difference may be attributable to the difference of other experiment conditions as well as the temperature difference between two studies. Sengun and Karpinar (2004) noted that a treatment with fresh lemon juice (4.5% citric acid) at room temperature for 1 h reduced

Table 2
Decimal reduction time (mean $\pm$ SD) of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* on beef marinated in lemon juice, calculated by linear regression and Weibull models. "Lemon ratio" is defined as in Table 1.

Lemon ratio	Linear model		Weibull model				4-D value (h)	
	D value (h)	r ²	δ (h)	ρ	r ²	RMSE	Linear model	Weibull model
<i>E. coli</i> O157:H7								
0.2	70.0 $\pm$ 5.60 (a ^a A ^b)	0.96	74.4 $\pm$ 5.73 (a A)	0.87	0.95	0.06	281 $\pm$ 22.4 (a A)	366 $\pm$ 28.2 (a A)
0.5	12.1 $\pm$ 1.43 (b A)	0.95	8.14 $\pm$ 1.09 (b A)	0.78	0.96	0.31	48.5 $\pm$ 5.73 (b A)	48.1 $\pm$ 6.32 (b A)
1.0	3.96 $\pm$ 0.13 (c A)	0.96	5.07 $\pm$ 0.12 (bc A)	1.16	0.95	0.47	15.8 $\pm$ 0.51 (c A)	16.8 $\pm$ 0.51 (bc C)
5.0	2.25 $\pm$ 0.09 (d A)	0.89	0.55 $\pm$ 0.04 (c A)	0.57	0.91	0.73	9.00 $\pm$ 0.34 (d A)	6.24 $\pm$ 0.34 (c A)
10.0	1.34 $\pm$ 0.02 (d A)	0.95	1.06 $\pm$ 0.12 (a A)	0.89	0.92	0.66	5.36 $\pm$ 0.06 (d A)	5.12 $\pm$ 0.06 (c A)
<i>S. Enteritidis</i>								
0.2	46.9 $\pm$ 3.57 (a B)	0.94	35.6 $\pm$ 1.32 (a B)	0.67	0.97	0.09	187 $\pm$ 14.3 (a B)	282 $\pm$ 10.4 (a B)
0.5	8.52 $\pm$ 0.26 (b B)	0.85	23.1 $\pm$ 0.57 (b B)	2.46	0.96	0.44	34.1 $\pm$ 1.02 (b B)	40.8 $\pm$ 0.00 (b A)
1.0	1.38 $\pm$ 0.02 (c B)	0.61	5.27 $\pm$ 0.27 (c A)	4.38	0.92	0.82	5.52 $\pm$ 0.06 (c B)	7.28 $\pm$ 0.06 (a B)
5.0	0.96 $\pm$ 0.04 (c A)	0.96	0.79 $\pm$ 0.04 (d B)	0.91	0.93	0.74	3.84 $\pm$ 0.17 (c A)	3.66 $\pm$ 0.17 (c B)
10.0	0.62 $\pm$ 0.02 (c B)	1.00	0.60 $\pm$ 0.06 (d A)	0.98	– ^c	–	2.48 $\pm$ 0.06 (c A)	2.44 $\pm$ 0.11 (c B)
<i>L. monocytogenes</i>								
0.2	21.4 $\pm$ 0.47 (a C)	0.91	10.7 $\pm$ 0.54 (a C)	0.61	0.93	0.23	85.8 $\pm$ 1.86 (a C)	104 $\pm$ 5.30 (a C)
0.5	3.66 $\pm$ 0.04 (b C)	0.99	3.97 $\pm$ 0.04 (b C)	1.05	0.99	0.22	14.6 $\pm$ 0.17 (b C)	15.1 $\pm$ 0.17 (b B)
1.0	2.13 $\pm$ 0.02 (c C)	0.92	4.04 $\pm$ 0.01 (b B)	1.58	0.94	0.54	8.52 $\pm$ 0.08 (c C)	9.72 $\pm$ 0.16B (c B)
5.0	0.95 $\pm$ 0.01 (d A)	0.99	1.09 $\pm$ 0.00 (c C)	1.08	0.99	0.18	3.78 $\pm$ 0.04 (d D)	3.96 $\pm$ 0.04 (cd B)
10.0	0.61 $\pm$ 0.01 (e B)	0.97	1.12 $\pm$ 0.01 (c B)	1.49	–	–	2.44 $\pm$ 0.03 (e B)	2.88 $\pm$ 0.00 (d C)

^a Entries with different lower-case letters in the same column under the same pathogen are significantly different ($p < 0.05$).

^b Entries with different capital letters in the same column at the same lemon ratio are significantly different ($p < 0.05$).

^c The value were not reported because of the low observation number ($n = 3$) in the survival curve.

Salmonella Typhimurium on carrots by 2.64–3.95 logs. The extrapolated D-values (0.25–0.38 h) are about 5.4–3.5 times lower than the D-value we observed for *S. Enteritidis* at our highest experimental concentration (Table 2); the different may be resulted from using different pathogen species and experiment conditions.

Under our experimental conditions, the 4-D values extrapolated with the Weibull model were 0.7–53.1% (average 15.8%) greater than those with the linear-regression model (Table 2). The shape ρ value affected the difference of the extrapolated 4-D values between the two models. For example, at ρ value between 0.75 and 1.5, the average difference in 4-D values between the two models was 6.0%. Above or below that range, the average difference reached 19.7%. Therefore, for a survival curve that greatly deviates from log-linear kinetics, the Weibull model should be used to predict the pathogen reduction time (such as 4-D values) needed to meet food safety requirements. The Weibull model is considered more realistic than the linear regression model in the estimation of microbial inactivation time (van Boekel, 2002). Besides, the difference of extrapolated 4-D values between the two models was greatest at our lowest lemon-juice concentration (0.2 mL/g), reaching 32%. The main reason was that observed data points did not cover the range of pathogen 4-log reductions – for accuracy in predicting pathogen reduction times like 4-D values, the observations must cover the objective of the food safety requirement, such as a 4-log reduction of a pathogen.

3.4. Change in pathogen susceptibility to lemon juice with D-values and δ -value

The D- and δ -values of the three pathogens decreased greatly with increasing lemon concentration in the beef marination (Table 2). Over the range of concentrations from 0.2 to 10 mL/g, the 4-D (or D) values from the linear model decreased by factors of 52, 75, and 35 times for *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes*, respectively. The 4-D value (or δ -value) from the Weibull model decreased by factors of 71 (70), 116 (59), and 36 (9.6) times for *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes*, respectively. The relative ratios of changes in 4-D values for the three pathogens were 1.5:2.1:1 (linear model) and 2.0:3.2:1 (Weibull model); the relative ratios of changes in D- and δ -values were 1.5:2.1:1 and 7.3:6.1:1, respectively. Except δ -value, the ratios of 4-D value changes from the linear and the Weibull models exhibited the same order of the pathogens' sensitivity to the changes in lemon juice concentration: *S. Enteritidis* > *E. coli* O157:H7 > *L. monocytogenes*. The results indicate that pathogen reduction time (4-D values) is a better parameter than δ -value for evaluation or comparison of the susceptibility of pathogens to environmental stress (Fig. 2).

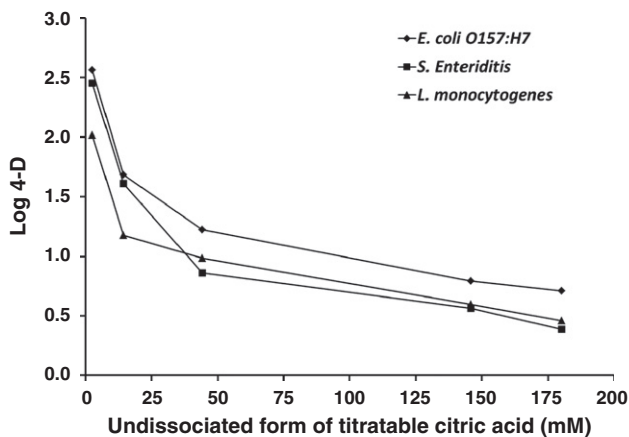


Fig. 2. Effect of undissociated citric acid on the decimal reduction time (log 4-D values based on the Weibull model) of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* on marinated beef.

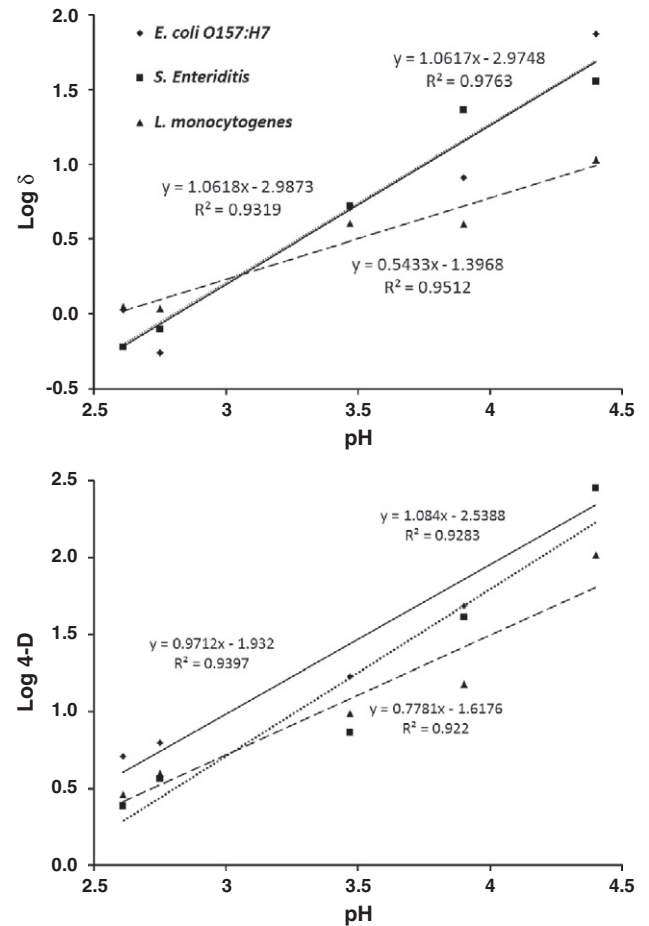


Fig. 3. Effect of pH on the decimal reduction time (log δ and log 4-D values based on the Weibull model) of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* on marinated beef.

3.5. Patterns of pathogen reduction time (4-D values)

The undissociated form of citric acid and pH are two critical factors in the acid-inactivation of foodborne pathogens (Buchanan et al., 2002). When log 4-D values were plotted against undissociated citric acid concentration, the decimal reduction time of pathogens with beef marination exhibited a biphasic pattern – a fast reduction and a slow reduction. The turning points between the fast and slow phases were at lemon-juice concentrations between 0.5 and 1.0 mL/g beef, at concentrations between 14.3 and 44.3 mM of undissociated citric acid, and at pH's between 3.5 and 3.9 (Fig. 3, Table 1).

The fast phase accounted for about 90–95% of reduction in 4-D values and covered concentrations of undissociated citric acid from 2.6 to 44.3 mM and pH's from 4.4 to 3.5, suggesting that *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* were very susceptible to increases in concentration of undissociated citric acid and decrease in pH in these ranges. The slow phases accounted for about a 5–10% and covered concentrations of undissociated citric acid from 44.3 to 180 mM and pH's from 3.5 to 2.6, suggesting that further increases in concentration of undissociated citric acid and decreases in pH produced little increase in inactivation of the pathogens and therefore approached the maximum pathogen inactivation rate.

Buchanan et al. (1993, 1994) observed a good linear relationship between the log 4-D value of *L. monocytogenes* and the square root of the calculated undissociated form of citric, lactic, or acetic acid in brain heat-infusion broth at pH of 4–7 and concentrations of 0.1–2.0 M. Virto et al. (2005, 2006) reported a linear relationship between the 3-D values of *E. coli*, *L. monocytogenes*, and *Yersinia enterocolitica* and citric acid concentrations ranging from 1 to 20%, but we did not observe a linear

relationship using their models and only observed biphasic patterns between the pathogen decimal reduction time and citric-acid concentrations (the calculated undissociated form of citric acid). The difference may be attributable to the differences in experimental conditions between studies, including pH, acid concentrations, types of media, temperature, type of acidulant used, and pathogen species.

In addition, Buchanan et al. (1993) observed a linear relationship between the 4-D values of *L. monocytogenes* in HCl-adjusted cultures and pH values (≤ 5.5); by extrapolation, they formed the hypothesis that a pH of 2.67 could instantly produce 4-D inactivation of *L. monocytogenes*. Bjornsdottir et al. (2006) noted that a condition at pH 3.2 (a control without citric acid) produced 4-D inactivation of *E. coli* O157:H7 within 6 h; at pH 3.2, higher concentrations (20–40 mM) of undissociated citric-acid increased inactivation by only 1–1.5 D. Therefore, in addition to the increase in concentration of undissociated citric acid from 2.60 to 44.3 mM, the decrease in pH (from 4.4 to 3.5) in the fast phase might also significantly decrease 4-D values.

3.6. The pH dependence of death rate (Z_{pH} -values)

The acid-death pH curves show the relationship of pathogen reduction times (δ and 4-D values) and pH during beef marination (Fig. 3). In the plots with a linear regression model, the acid-death pH curves of three pathogens exhibited the correlation coefficients (R) of 0.96–0.99 for the δ value and 0.96–0.97 for the 4-D value. The Z_{pH} value is the pH dependence of death rate, which is defined as the pH change that results in a 10-fold change in the decimal reduction time (δ or D value). From pH 2.6 to 4.4, the $Z_{pH-\delta}$ and Z_{pH-D} value was 0.94 and 1.03 for *E. coli* O157:H7, 0.94 and 0.92 for *S. Enteritidis*, and 1.84 and 1.29 *L. monocytogenes*, respectively. Based on both $Z_{pH-\delta}$ and Z_{pH-D} values, *L. monocytogenes* was the most low-pH-resistant pathogen of the three. Based on the Z_{pH-D} value, the order of resistance to low pH was: *L. monocytogenes* > *E. coli* O157:H7 > *S. Enteritidis*, the same order reported by Gabriel and Nakano (2010) for resistance to physico-chemical stresses (pH, a_w , temperature). Based on the $Z_{pH-\delta}$ value, *E. coli* O157:H7 and *S. Enteritidis* exhibited the same resistance to the pH changes; but for comparisons of pathogen resistance to the low-pH, the Z_{pH-D} value should be more reliable than the $Z_{pH-\delta}$ value because the total pathogen reduction time (e.g., 4-D value) in a stressful environment depends on both δ and ρ .

Based on the predicted acid-death pH curves (Fig. 3), the relative resistance of the three pathogens to inactivation by lemon juice changed with pH. As shown by Fig. 3, above pH 3.0, *S. Enteritidis* was more resistant to lemon juice than *L. monocytogenes*, but below pH 3.0, *L. monocytogenes* was more resistant than *S. Enteritidis*. Therefore, pH must be considered during attempts to inactivate pathogens to meet food safety requirements.

Table 3

Decimal reduction time (mean $\pm$ SD) of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* on beef marinating in lemon juice and citric-acid solution (7.2%) at a ratio of 5 mL/g, calculated by linear regression model.

Treatment	Pathogen	D value (h)	r^2	4-D value (h)
Lemon juice (4 °C)	<i>E. coli</i> O157:H7	5.55 $\pm$ 0.11a ^a	0.90	22.2 $\pm$ 0.44a
	<i>S. Enteritidis</i>	1.70 $\pm$ 0.05b	0.93	6.80 $\pm$ 0.20b
	<i>L. monocytogenes</i>	3.90 $\pm$ 0.21c	0.98	15.6 $\pm$ 0.84c
Citric acid (24 °C)	<i>E. coli</i> O157:H7	0.99 $\pm$ 0.01a	0.96	3.96 $\pm$ 0.01a
	<i>S. Enteritidis</i>	0.89 $\pm$ 0.10a	0.97	3.56 $\pm$ 0.40a
	<i>L. monocytogenes</i>	0.65 $\pm$ 0.01b	0.80	2.60 $\pm$ 0.04b

^a Entries with different lower-case letters in the same column within each treatment are significantly different ($p < 0.05$).

3.7. Temperature effect

The 4-D inactivation values of all three pathogens were 2–5 times greater at 4 °C than at 24 °C (Table 2 and Table 3). Conner et al. (1990) and Conner and Kotrola (1995) also noted that the bacteriostatic and bactericidal effects of citric acid on *L. monocytogenes* decline with decreasing temperature. Miller and Kaspar (1994) observed that survival of *E. coli* O157:H7 and *E. coli* at pH 2–12 was higher at 4 °C than at 25 °C. We found that marinating beef at 4 °C and a ratio of lemon juice to beef of 5 required 22 and 16 h to achieve a 4-log reduction of *E. coli* O157:H7 and *L. monocytogenes*, respectively (Table 3), much longer than the marination regularly used for kelaguen preparation (1–12 h). Therefore, refrigerating the mixture during marination does not make the kelaguen safer and may have the opposite effect.

At 4 °C, the order of pathogen resistance at a ratio of lemon juice to beef of 5 (pH 2.8) was: *E. coli* O157:H7 > *L. monocytogenes* > *S. Enteritidis*. Just as at 24 °C, *E. coli* O157:H7 was the most resistant (Fig. 3). Under these conditions, *L. monocytogenes* was also significantly more resistant to lemon juice than *S. Enteritidis*, perhaps because of its greater Z_{pH-D} value (Fig. 3). Furthermore, the physiological characteristic of *L. monocytogenes* – growing slowly at refrigerator temperatures – may contribute to its resistance to lemon juice.

3.8. Effects of citric acid

A citric acid solution with the same titratable acidity (7.2%) as the lemon juice was used in beef marination at 24 °C and a concentration of 5 mL/g. The marination with citric acid solution had a pH of 2.6, lower than that with lemon juice, and a titratable acidity of 5.82, higher than that with lemon juice (Table 1). The citric acid marinade at 24 °C inactivated all three pathogens more effectively than lemon juice at 24 °C, as revealed by D-values lower than those of the lemon juice marinade (Tables 2 and 3). The D-values of *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* were 0.99, 0.89, and 0.65 h in the citric acid marinade and 2.25, 0.96, and 0.95 h in the lemon juice marinade, respectively (Tables 2 and 3). This result is attributable to the lower pH value and the greater concentration of undissociated citric acid (232.5 mM) in the citric acid marinade than in the lemon juice marinade. Park et al. (2011) observed that a treatment with 2% citric acid at pH 2.09 for 10 min inactivates *E. coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* on apple and fresh lettuce by from 2.32 to >3.42 logs. The extrapolated D-values in their study are much lower than those obtained in our study; the low pH value in their study may contribute to the difference.

3.9. Kelaguen safety during beef marination

Our treatment at a ratio of lemon juice to beef of 0.5, which was above the ratio normally used to prepare high-acid kelaguen, took 48 h to reduce all three pathogens by 4 logs (Table 2). In regular kelaguen preparation, marination time for beef ranges from 1 to 12 h. Therefore, marinating beef at a ratio of lemon juice to beef below 0.5 for 12 h cannot kill *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* by a 4-log reduction. Although marination at a ratio of 5–10 for 12 h successfully reduced all three pathogens by 4 logs, these ratios are too high for use in regular kelaguen preparation by consumers. Preparation of kelaguen by marination of raw beef with lemon juice will be unsafe if raw beef is contaminated; therefore, a heat treatment must be included in preparation to make kelaguen safe for consumption.

4. Conclusion

Marinating beef with lemon juice is an essential step for kelaguen preparation, but we have shown that this marination alone, at the lemon-juice ratios and for the durations used by consumers, is

insufficient to inactivate high levels of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes* to make the dish safe. *E. coli* O157:H7 proved much more resistant than the other two, but in resistance to pH change alone, *L. monocytogenes* ranked first. Generally, the decimal reduction times (4-D values) calculated from the Weibull model were more conservative than those from the linear regression model, suggesting that the Weibull model is more suitable for formulating guidelines for food preparation or processing for food safety criteria.

Marinating beef for kelaguen at a ratio of lemon juice to beef <0.5 for 1–12 h did not produce a 4-log reduction of *E. coli* O157:H7, *S. Enteritidis*, and *L. monocytogenes*. The regular practice of marinating beef at ambient or refrigerator temperatures is therefore unsafe. To reduce the risk of kelaguen-associated food-borne illness, heat treatment must be applied in addition to marination of beef with lemon juice.

Acknowledgments

This project was supported by the National Integrated Food Safety Initiative (NIFSI), National Institute of Food and Agriculture, United States Department of Agriculture, grant number 2004-51110-02158.

References

- Bingol, E.B., Cetin, O., Muratoglu, K., 2011. Effect of lemon juice on the survival of *Salmonella* Enteritidis and *Escherichia coli* in cig kofte (raw meatball). *British Food Journal* 113, 1183–1194.
- Bjornsdottir, K., Breidt Jr., F., McFeeters, R.F., 2006. Protective effects of organic acids on survival of *Escherichia coli* O157:H7 in acidic environments. *Applied and Environmental Microbiology* 72, 660–664.
- Buchanan, R.L., Golden, M.H., 1994. Interaction of citric acid concentration and pH on the kinetics of *Listeria monocytogenes* inactivation. *Journal of Food Protection* 57, 567–570.
- Buchanan, R.L., Golden, M.H., Whiting, R.C., 1993. Differentiation of the effects of pH and lactic or acetic acid concentration on the kinetics of *Listeria monocytogenes* inactivation. *Journal of Food Protection* 56, 474–478.
- Buchanan, R.L., Whiting, R.C., Golden, M.H., 2002. Modeling acid inactivation of foodborne microorganisms. In: Juneja, V.K., Sofos, J.N. (Eds.), *Control of Foodborne Microorganisms*. Marcel Dekker, New York, pp. 461–478.
- Conner, D.E., Kotrola, J., 1995. Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Applied and Environmental Microbiology* 61, 382–385.
- Conner, D.E., Scott, V.N., Bernard, D.T., 1990. Growth, inhibition, and survival of *Listeria monocytogenes* as affected by acidic conditions. *Journal of Food Protection* 53, 652–655.
- Enache, E., Chen, Y., Elliott, P., 2009. Inactivation of *Escherichia coli* O157:H7 in single-strength lemon and lime juices. *Journal of Food Protection* 72, 235–240.
- Gabriel, A.A., Nakano, H., 2010. Responses of *E. coli* O157:H7, *L. monocytogenes* 1/2 c and *Salmonella enteritidis* to pH, a_w and temperature stress combinations. *Food Control* 21, 644–650.
- Geeraerd, A.H., Valdramidis, V.P., van Impe, J.F., 2005. GlnaFIT, a freeware tool to assess non-log-linear microbial survivor curves. *International Journal of Food Microbiology* 95–105 (2005).
- Haddock, R.L., 2007. Foodborne illness associated with kelaguen. Office of Epidemiology and Research Department of Public Health and Social Service, Government of Guam.
- Lambert, R.J., Stratford, M., 1999. Weak-acid preservatives: modelling microbial inhibition and response. *Journal of Applied Microbiology* 86, 157–164.
- Mafart, P., Couvert, O., Gaillard, S., Leguerinel, I., 2002. On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology* 72, 107–113.
- Mani-López, E., García, H.S., López-Malo, A., 2012. Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Research International* 45, 713–721.
- Miller, L.G., Kaspar, C.W., 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. *Journal of Food Protection* 57, 460–464.
- Nogueira, M.C.L., Oyarzabal, O.A., Gombas, D.E., 2003. Inactivation of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in cranberry, lemon, and lime juice concentration. *Journal of Food Protection* 2003, 1637–1641.
- Park, S.H., Choi, M.R., Park, J.W., Park, K.H., Chung, M.S., Ryu, S., Kang, D.H., 2011. Use of organic acids to inactivate *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on organic fresh apples and lettuce. *Journal of Food Science* 76, M293–M297.
- Sengun, I.Y., Karpinar, M., 2004. Effectiveness of lemon juice, vinegar and their mixture in the elimination of *Salmonella typhimurium* on carrots (*Daucus carota* L.). *International Journal of Microbiology* 96, 301–305.
- SPSS, 2009. PASW Advanced Statistics. Version 18.0. IBM, Chicago, IL.
- van Boekel, M.A.J.S., 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *International Journal of Food Microbiology* 74, 139–159.
- Virto, R., Sanz, D., Alvarez, I., Raso, J., 2005. Inactivation kinetics of *Yersinia enterocolitica* by citric and lactic acid at different temperature. *International Journal of Food Microbiology* 103, 251–257.
- Virto, R., Sanz, D., Alvarez, I., Condon, S., Raso, J., 2006. Application of the Weibull model to describe inactivation of *Listeria monocytogenes* and *Escherichia coli* by citric and lactic acid at different temperature. *Journal of the Science of Food and Agriculture* 86, 865–870.
- Yang, J., Lee, D., 2009. Lemon, pH and citric acid for kelaguen safety without temperature control. *Micronesia* 41, 19–31.
- Young, K.M., Foegeding, P.M., 1993. Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* Scott A and the effect on intracellular pH. *Journal of Applied Microbiology* 74, 515–520.