

Interaction of Citric Acid Concentration and pH on the Kinetics of *Listeria monocytogenes* Inactivation

ROBERT L. BUCHANAN* and MARSHA H. GOLDEN

Microbial Food Safety Research Unit, U.S. Department of Agriculture, Eastern Regional Research Center, Agricultural Research Service, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118

(Received November 22, 1993/Accepted January 17, 1994)

ABSTRACT

The effects and interactions between pH and citric acid concentration on the inactivation of *Listeria monocytogenes* was determined using a three-strain mixture. Citric acid/sodium citrate combinations were added to brain heart infusion (BHI) broth to achieve concentrations of 0.1, 0.5, 1.0 and 2.0 M in conjunction with pH values of 4, 5, 6 and 7. The media were dispensed in 20-ml portions in dilution bottles, inoculated to approximately 10^8 CFU/ml, and incubated at 28°C. Survivor curves were generated using a linear model incorporating a lag term, and D-values and "time to 4-D inactivation" values were calculated. The results were compared against control cultures in which the pH was modified using hydrochloric acid (HCl). The rate of inactivation was dependent on both the pH and concentration of citric acid. Low levels of citric acid were protective, particularly at pH 5 and 6. At higher concentrations, a distinct anion effect was observed as compared to the HCl controls, with inactivation rates being correlated with the completely undissociated form of the acid. Comparison of the kinetic data with earlier results with lactic and acetic acids suggests that citric acid has both protective and bactericidal activity against *L. monocytogenes*, which involve different modes of action.

Key Words: Citric acid, pH, *Listeria monocytogenes*

When *L. monocytogenes* are placed in an acidic environment that does not support growth, pathogen levels decline with the rate of inactivation being a function of the severity of the conditions (1-5,10). Even when the microorganism does grow at a non-optimal pH, the population tends to decline soon after reaching the stationary phase (3,10). This is enhanced by elevating the incubation temperature.

The rate of inactivation is dependent on the pH as well as the identity and concentration of the acidulant (1-3, 5-7,11,12). Organic acids are generally more effective due to the combined pH and anion effects. Previous studies in our laboratory that quantified the times to a 4 log cycle inactivation (T_{4D}) of *L. monocytogenes* indicated that this measure of inactivation rates was linearly related to pH in

microbiological media adjusted with HCl (2). However, when moderate to high levels of lactic and acetic acids were used as acidulants, T_{4D} -values were also dependent on the concentration of the organic acid. It was observed further that an apparent linear relationship exists between the log of the T_{4D} -values and the square root of the molar concentration of the undissociated form of acetic or lactic acid. Since both acetic and lactic acids are monocarboxylic, the purpose of the present study was to determine if a similar relationship occurs when citric acid, a tricarboxylic acid, was used as an acidulant.

MATERIALS AND METHODS

Microorganisms

A three-strain mixture of *L. monocytogenes* (Scott A, HO-VJ-S and V-7) was used throughout the study. Stock cultures were maintained in BHI broth (Difco Laboratories, Detroit, MI) at 5°C, and transferred monthly. Cultures used as inocula were grown individually for 24 h in BHI + 0.3% dextrose incubated at 37°C on a rotary shaker (150 rpm). The cultures were then combined to obtain a mixed inoculum containing approximately equal numbers of the three strains.

Experimental design

A complete factorial design was used to assess the effects of pH (4, 5, 6 and 7) and citric acid concentration (0.1, 0.5, 1.0 and 2.0 M). This was accompanied with 0.0 M citric acid control cultures in which HCl was used to adjust the pH in 0.5 unit increments between pH 3.0 and 7.0. All variable combinations were tested at least twice.

Cultures techniques

The culture techniques were identical to those employed previously (2). Briefly, BHI was supplemented with citric acid and sodium citrate to achieve pH levels of 4, 5, 6 and 7 in conjunction with concentrations of 0.0, 0.1, 0.5, 1.0 and 2.0 M. Duplicate 20-ml portions of the 16 pH/concentration combinations were transferred to 150-ml screw-cap milk dilution bottles and autoclaved. The pH of the medium was verified after autoclaving. The bottles were then inoculated with the mixture of strains to a level of approximately 10^8 CFU/ml. The bottles were incubated without agitation on their side to maximize oxygen transfer at 28°C. Periodically, 0.1 ml samples were removed, diluted in sterile 0.1% peptone water, and viable counts determined on Tryptose Agar (Difco) using a Spiral Plater (Spiral

Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Biotech, Inc., Bethesda, MD). All plates were incubated for 24 h at 37°C and enumerated using a Laser Colony Counter (Model 500, Spiral Biotech). Sampling continued for 150 days or until the counts fell below the lower limit of detection ($\log_{10} \leq 1.03$ CFU/ml).

Survivors curves

Survivor curves were generated by fitting the \log_{10} counts to a linear primary model (2,3).

$$Y = Y_0 \quad \text{for } T \leq T_L$$

$$Y = Y_0 + m(T - T_L) \quad \text{for } T \geq T_L$$

Where:

$Y = \text{Log}_{10}$ count of bacteria at time T . [Log_{10} (CFU/ml)]

$Y_0 = \text{Log}_{10}$ count of bacteria at time $T = 0$. [Log_{10} (CFU/ml)]

$m = \text{Slope of the survivor curve. } [(\text{Log}_{10}(\text{CFU/ml}))/\text{h}]$

$T = \text{Time. [h]}$

$T_L = \text{Duration of lag period prior to initiation of inactivation. [h]}$

The " T_L " and " m " terms were fitted using ABACUS, a curve-fitting program developed by W. Damert (U.S. Department of Agriculture [USDA], ARS Eastern Regional Research Center). The Y_0 -value was fixed at that observed for the 0-h sample. In those instances where there was some growth before the initiation of inactivation, T_L -values were calculated based on fixing the Y_0 -value. D-values were calculated as the negative reciprocal of m , and the " T_{4D} " were calculated using the equation:

$$T_{4D} = T_L + 4 \times D$$

RESULTS

The general pattern of *L. monocytogenes* survival was similar to that observed previously (2), with exponential inactivations beginning after an initial lag period. More severe conditions increased the rate of inactivation and decreased the duration of the lag period. Some of the less severe conditions initially supported increases in population densities of *L. monocytogenes* by 2- to 10-fold, followed by exponential declines.

The time to achieve a 10,000-fold decrease (T_{4D}) in *L. monocytogenes* in BHI adjusted with HCl was linearly related to pH at values ≤ 5.0 (Fig. 1). The average response for pH 5.5 and 6.0 cultures approximated this linear relationship; however, the variability in observed T_{4D} -values was high. At pH 6.5 and 7.0, inactivation was slowed substantially, and again had a high degree of variability. Overall, these results are consistent with those reported by Buchanan et al. (2).

The rate of *L. monocytogenes* inactivation in the presence of moderate to high levels of citric acid was dependent on both pH and concentration (Table 1). When compared against the HCl controls (Fig. 2), a substantial protective effect was indicated for the lower concentrations of citric acid, particularly at pH values of 5.0 and 6.0. Higher concentrations of the organic acid enhanced inactivation.

The concentrations of each of the ion forms of citric acid were calculated using the Henderson-Hasselbalch equation and compared against the T_{4D} -values (not shown). When the $\text{Ln}(T_{4D})$ versus $[\text{H}_3\text{A}]^{0.5}$ relationship observed previously with lactic and acetic acids was evaluated, there

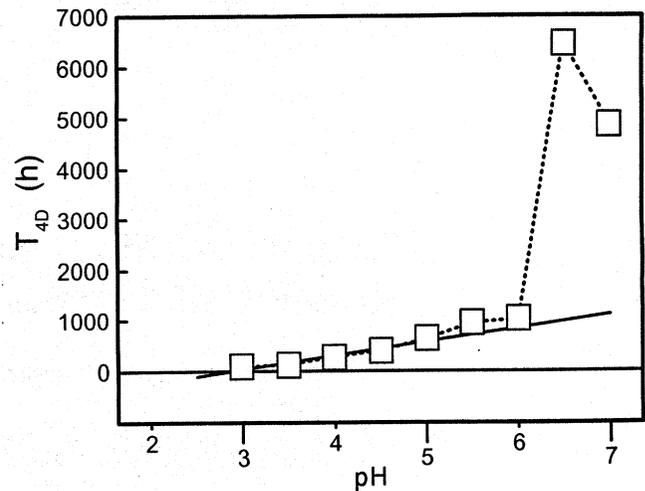


Figure 1. The time for *L. monocytogenes* populations to decline 10,000-fold (T_{4D}) in BHI broth (0.5% NaCl, 28°C) adjusted to different initial pH levels using HCl. Straight line indicates linear regression based on values between pH 3.0 and 5.0

TABLE 1. Inactivation of *L. monocytogenes* BHI broth adjusted to have various combinations of citric acid concentration and initial pH.

pH	Citric acid (M)	n	T_L (h)	D-Value (h)	T_{4D} (h)
7.0	0.1	2	240	770.9	3323.4 ^a
	0.5	2	240	742.3	3209.2 ^a
	1.0	2	2	324.2	1298.8
	2.0	2	0	106.1	424.2
6.0	0.1	4	230	891.7	3796.9 ^a
	0.5	4	252	692.2	3030.9
	1.0	2	149	90.5	510.8
	2.0	2	157	72.8	447.7
5.0	0.1	2	24	585.3	2365.0
	0.5	2	279	34.8	418.4
	1.0	2	242	38.8	396.4
	2.0	2	201	43.8	376.4
4.0	0.1	2	268	24.5	365.6
	0.5	2	6	25.1	106.4
	1.0	2	0	15.4	61.4
	2.0	2	0	13.5	54.0

^a Cultures for which there was a 2- to 10-fold increase in population densities before initiation of inactivation.

was an apparent two-phase response (Fig. 3). At low undissociated acid concentrations there was a cluster of responses with elevated survival times, whereas at higher concentrations there was a linear relationship. A strong linear relationship ($R^2 = 0.95$) was indicated (Fig. 3, insert) after excluding the data for pH 7.0 cultures and those citric acid containing cultures, which had T_{4D} -values greater than the corresponding HCl controls. These were excluded on the basis of large variability associated with the pH 7.0 controls and the apparent protective effect associated with specific citric concentrations, respectively.

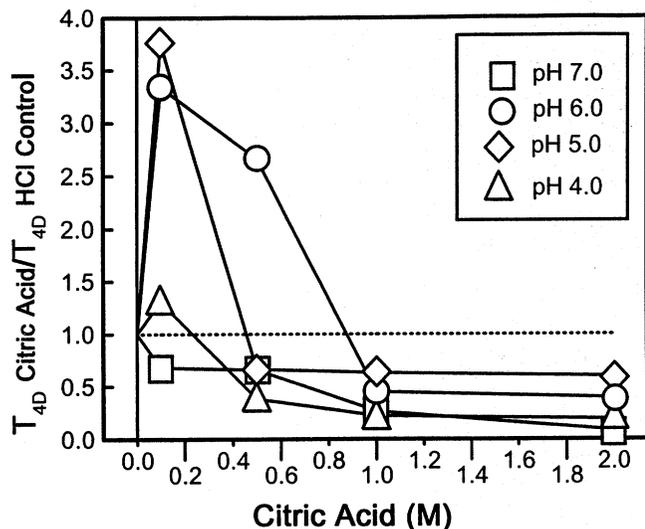


Figure 2. Effects of pH and citric acid/sodium citrate concentration on the inactivation of *L. monocytogenes*. (The 0.0 M-values are those from Fig. 1.)

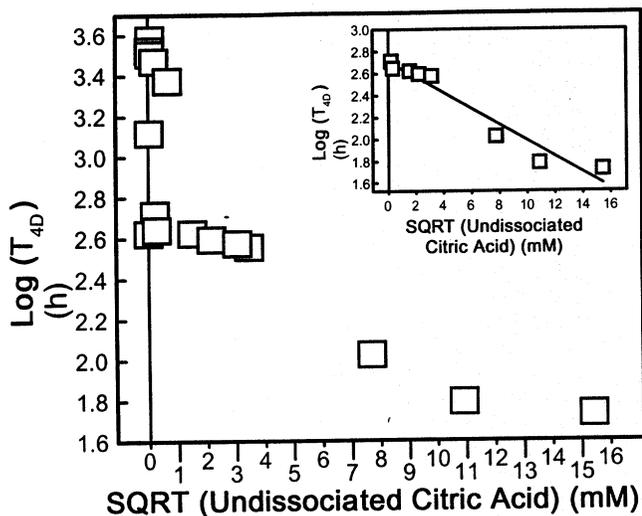


Figure 3. The relationship between the $\text{Log}(T_{4D})$ and the square root of the calculated concentrations of undissociated citric acid. The insert is the same data where the T_{4D} -values for selected variable combinations are excluded. (See text.)

This relationship is described by the equation:

$$\text{Ln}(T_{4D}) = -0.16646 \times [\text{H}_3\text{A}]^{0.5} + 6.2456$$

where:

$[\text{H}_3\text{A}]$ = mM of completely undissociated citric acid.

DISCUSSION

The current study and our previous work (2) has demonstrated that the rate of inactivation of *L. monocytogenes* in different pH environments is dependent on three factors: The pH, the acidulant and the acidulant's concentration. For lactic and acetic acids, pH was the predominant

factor at low acid concentrations; anion effects being more important as concentrations were increased. Citric acid also displayed significant anion effects at high (≥ 1 M) concentrations; however, at lower concentrations the acid appears to enhance the survival of the microorganism. This effect was most evident under mildly acidic conditions (pH 5 to 6). Young and Foegeding (13) reported that for certain combinations of pH and concentration, citric acid stimulated the growth of *L. monocytogenes* as compared to HCl-adjusted control cultures. They hypothesized that the effect could be due to citrate serving as a carbon source or through the chelation of metal ions. Conner et al. (5) reported that low levels of citric acid (0.029 M) did not affect the minimum pH that supported *L. monocytogenes* growth as compared to HCl. However, they did observe some depression of inactivation rates at pH 4.0 in the citric acid containing cultures. Little et al. (8) did not observe a citric acid associated protective effect during inactivation studies with *Yersinia enterocolitica*; however, this could have been due to either species differences or the low pH range (3.0 to 4.0). Minimal protective effect was observed at pH 4.0 in the current study.

The pH dependent nature of the competing protective and toxic effects associated with citric acid suggest that they may involve different ionic forms of the molecule. The toxic effect correlated best with the concentration of the undissociated form of the molecule, whereas, the protective effect appeared related to the calculated levels of the mono- or dihydroxy form. This suggests that the protective effect may be related to the chelating capacity of the partially ionized acid. The apparent linear relationship (Fig. 3) between the natural log of the inactivation rate ($\text{Ln}[T_{4D}]$) and the square root of the concentration of the undissociated acid is similar to that observed earlier with lactic and acetic acids (2). This suggests that there is some underlying physiological/biochemical factor associated with the bactericidal activity of organic acids that is responsible for these kinetics. However, the identification of this locus will require further research.

The relative bacteriostatic and bactericidal activities associated with citric, lactic and acetic acids have been assessed by a number of investigators. However, it is difficult to compare the results due to differences in species investigated and the methods for evaluating and reporting acidulant concentrations and pH, as well as other differences in environmental parameters such as incubation temperatures. Ahamad and Marth (1) concluded that on an equal weight basis, the relative bactericidal activity against *L. monocytogenes* was acetic \geq lactic \geq citric. Young and Foegeding (13) reported that the bacteriostatic activity against *L. monocytogenes* on a equimolar basis for total acid was acetic \geq lactic \geq citric, while Sorrells et al. (12) reported the opposite, citric \geq lactic \geq acetic. Based on the achievement of equivalent pH-values in media systems, a number of investigators have concluded that relative bacteriostatic and bactericidal activities are acetic \geq lactic \geq citric for *L. monocytogenes* (7,12,13), *Aeromonas hydrophila* (9) and *Y. enterocolitica* (8). Alternatively, Young and Foegeding (13) reported that the relative bacteriostatic activity of the three acids when expressed on the basis of

molar concentration of undissociated acid was citric \geq lactic \geq acetic. The models developed in the current study and our earlier work (2) allow a more detailed evaluation of relative bactericidal activity. While citric acid was substantially less effective than lactic and acetic acid on either a weight or total acid concentration basis, the order of activities based on undissociated acid concentrations was citric \geq lactic \geq acetic (Fig. 4). However, the extent of this differential was dependent on the concentration of the undissociated acid, with citric acid being substantially more effective at lower concentrations.

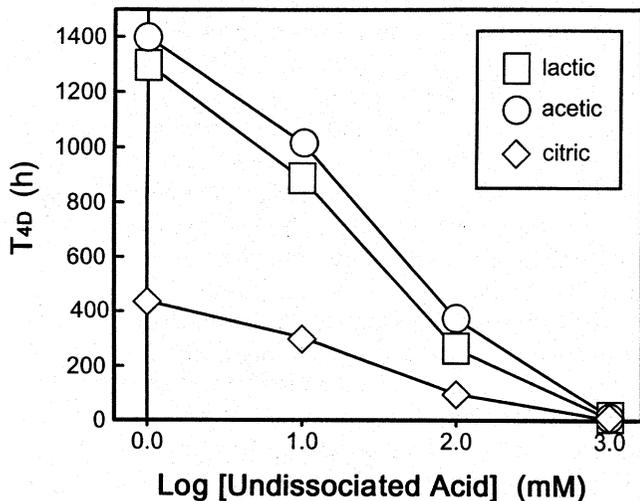


Figure 4. Comparison of the relative bactericidal activities of citric, lactic and acetic acids against *L. monocytogenes* based on concentrations of undissociated acid. Values calculated using model from current study and those from Buchanan et al. (2).

In summary, the current research has determined that the effects of citric acid on the inactivation of *L. monocytogenes* is dependent both on concentration and pH, and appears to involve two competing effects, protection versus toxicity. The observation that the bactericidal effects at high concentrations fit the previously identified relationship between inactivation rate and concentration

of undissociated acid suggests a general response, and warrants additional research. Likewise, the mechanism underlying the observed protective effect at concentrations commonly used in food products needs to be characterized further. Additional knowledge of this type should permit a more systematic means for selecting acidulants to optimize the inhibition or inactivation of foodborne pathogens.

REFERENCES

- Ahamad, N. and E. H. Marth. 1989. Behavior of *Listeria monocytogenes* at 7, 13, 21 and 35°C in tryptose broth acidified with acetic, citric or lactic acid. *J. Food Prot.* 52:688-695.
- Buchanan, R. L., M. H. Golden and R. C. Whiting. 1993. Differentiation of the effects of pH and lactic or acetic acid concentration on the kinetics of *Listeria monocytogenes* inactivation. *J. Food Prot.* 56:474-478.
- Buchanan, R. L., M. H. Golden, R. C. Whiting, J. Phillips and J. L. Smith. 1994. Model for the non-thermal inactivation of *Listeria monocytogenes*. *J. Food Sci.* 59:179-188.
- Cole, M. B., M. V. Jones and C. Holyoak. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *J. Appl. Bacteriol.* 69:63-72.
- Conner, D. E., V. N. Scott and D. T. Bernard. 1990. Growth, inhibition and survival of *Listeria monocytogenes* as affected by acidic conditions. *J. Food Prot.* 53:652-655.
- El-Shenawy, M. A. and E. H. Marth. 1989. Inhibition or inactivation of *Listeria monocytogenes* by sodium benzoate together with some organic acids. *J. Food Prot.* 52:771-776.
- Ita, P. S. and R. W. Hutkins. 1991. Intracellular pH and survival of *Listeria monocytogenes* Scott A in tryptic soy broth containing acetic, lactic and hydrochloric acids. *J. Food Prot.* 54:15-19.
- Little, C. L., M. R. Adams and M. C. Easter. 1992. The effect of pH, acidulant and temperature on the survival of *Yersinia enterocolitica*. *Lett. Appl. Microbiol.* 14:148-152.
- Palumbo, S. A. and A. C. Williams. 1992. Growth of *Aeromonas hydrophila* K144 as affected by organic acids. *J. Food Sci.* 57:233-235.
- Parish, M. E. and D. P. Higgins. 1989. Survival of *Listeria monocytogenes* in low pH model broth systems. *J. Food Prot.* 52:144-147.
- Sorrells, K. M. and D. C. Enigl. 1990. Effect of pH, acidulant, sodium chloride and temperature on the growth of *Listeria monocytogenes*. *J. Food Safety* 11:31-37.
- Sorrells, K. M., D. C. Enigl and J. R. Hatfield. 1989. Effect of pH, acidulant, time and temperature on the growth and survival of *Listeria monocytogenes*. *J. Food Prot.* 52:571-573.
- Young, K. M. and P. M. Foegeding. 1993. Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* Scott A and the effect on intracellular pH. *J. Appl. Bacteriol.* 74:515-520.