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

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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 10^6 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_{40}) 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_{40}-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_{40}-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-5 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 where 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 10^6 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...
Survivors curves

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

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

Where:
- \( Y = \log_{10} \) count of bacteria at time \( T \). \([\log_{10} \text{(CFU/ml)}]\)
- \( Y_0 = \log_{10} \) count of bacteria at time \( T = 0 \). \([\log_{10} \text{(CFU/ml)}]\)
- \( m \) = Slope of the survivor curve. \([\log_{10} \text{(CFU/ml)}]/h]\)
- \( T \) = Time, [h]
- \( T_L \) = Duration of lag period prior to initiation of inactivation.

The "TL" 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_{40}" were calculated using the equation:

\[ T_{40} = T_L + 4 \times D \]

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 (\( T_{40} \)) in \( L.\) monocytogenes in BHI broth was linearly related to \( pH \) at values \( \leq 5.0 \) (Fig. 1). The average response for \( pH \) 5.5 and 6.0 cultures approximated this linear relationship; however, the variability in observed \( T_{40} \)-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-Hassalbach equation and compared against the \( T_{40} \)-values (not shown). When the \( \ln(T_{40}) \) versus \( [\text{H}^+]A^0.5 \) relationship observed previously with lactic and acetic acids was evaluated, there 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 \( T_{40} \)-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.
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**DISCUSSION**

where:

- 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 (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 ≥lactic ≥citric. Young and Foegeding (13) reported that the bacteriostatic activity against L. monocytogenes on an equimolar basis for total acid was acetic ≥lactic ≥citric, while Sorrells et al. (12) reported the opposite, citric ≥lactic ≥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 ≥lactic ≥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

**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.)**

This relationship is described by the equation:

\[
\text{Ln}(T_{4D}) = -0.16646 \times [H_3A]^{0.5} + 6.2456
\]

where:

- \([H_3A] = \text{mM of completely undissociated citric acid.}\)

**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).

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molar concentration of undissociated acid was citric ≥ lactic ≥ 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 ≥ lactic ≥ 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.

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 effect, 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