*Journal of Food Protection, Vol. 56, No. 6, Pages 474-478, 484 (June 1993)*


# Differentiation of the Effects of pH and Lactic or Acetic Acid Concentration on the Kinetics of *Listeria Monocytogenes* Inactivation

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(Received for publication October 5, 1992)

ABSTRACT

The effects of pH and lactic acid or acetic acid concentration on *Listeria monocytogenes* inactivation were studied in brain heart infusion broth using a three strain mixture. Combinations of lactic acid/sodium lactate and acetic acid/sodium acetate were used to achieve concentrations of 0.1, 0.5, 1.0, and 2.0 M in conjunction with pH values of 4.0, 5.0, 6.0, and 7.0. Cultures adjusted with HCl to pH 3.0 to 7.0 in 0.5 pH unit intervals were used as 0.0 M controls. Each pH/concentration combination was inoculated to a level of $10^8$ CFU/ml and incubated at 28°C for up to 60 d. Bacterial populations were determined periodically by plate counts. Inactivation was exponential after an initial lag period. Survivor curves (log# versus time) were fitted using a linear model that incorporated a lag period. The model was subsequently used to calculate D values and "time to a 4-D (99.99%) inactivation" ($t_{4\text{-}D}$); $t_{4\text{-}D}$ values were directly related to pH and inversely related to acid concentration. At acid/pH combinations that supported growth, the level of the organism increased slightly (2- to 10-fold) before declining. In the HCl-adjusted controls with pH's ≤5.5, the rate of inactivation was linearly related to pH. In the presence of the monocarboxylic acids, the duration of the lag period and the rate of inactivation were dependent on the pH, as well as the identity and concentration of acid. 4-D inactivation times were related to the level of undissociated lactic and acetic acids. That relationship was described by the equations, $t_{4\text{-}D} = \exp(-0.1773*LA^{0.5} + 7.3482)$ and $t_{4\text{-}D} = \exp(-0.1468*AA^{0.5} + 7.3905)$ for lactic and acetic acids, respectively, where LA and AA are mM of undissociated acid. These relationships were used in conjunction with the Henderson-Hasselback equation to develop a model for predicting the rate of inactivation as a function of pH and total organic acid concentration.

A number of investigators have observed that when *Listeria monocytogenes* is placed in an acidic environment that does not support growth, the organism will be inactivated over time (*1,4,5,12*). It has also been observed that under nonideal pH conditions that still support growth, the organism will tend to decline after reaching stationary phase, particularly at elevated incubation temperatures (*12*). The inhibition or inactivation of *L. monocytogenes* is enhanced when organic acids are used as acidulants (*1,5-7,11,14,15*). Sufficiently high levels of organic acid salts such as sodium lactate and sodium acetate can inhibit or inactivate the pathogen, even at neutral pH levels (*13,16,17*). Various investigators have concluded that the rate of inactivation is dependent not only on the pH of the environment but also on the identity and concentration of the acidulant used to modify the pH. However, there have been no studies where the relative impact of the three variables have been separated and quantified. Accordingly, the objective of the current study was to quantitatively determine the inactivation kinetics for *L. monocytogenes* when exposed to lactic and acetic acids in a manner that permitted the effects of pH and acidulant concentration to be differentiated.

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

## MATERIALS AND METHODS

### *Microorganisms*

Three strains of *L. monocytogenes*, HO-VJ-S, V-7, and Scott A, were cultured separately in 250-ml Erlenmeyer flasks containing 25 ml of brain heart infusion broth (BHI; Difco, Detroit, MI) + 0.3% dextrose at 37°C for 24 h. The three cultures, each containing approximately $10^{10}$ CFU/ml, were then combined (total volume of 75 ml) for use as the inoculum.

### *Preparation of test system*

BHI was supplemented with appropriate combinations of sodium lactate + lactic acid or sodium acetate + acetic acid to achieve pH levels of 7.0, 6.0, 5.0, and 4.0 in combination with concentrations of 0.1, 0.5, 1.0, and 2.0 M. These concentrations are roughly equivalent to 0.9, 4.5, 9.0, and 18.0% (wt/vol) for lactic acid and 0.6, 3.0, 6.0, and 12.0% for acetic acid, calculated on the basis of the acid. Duplicate 20-ml portions of the 16 pH/acid concentration combinations were transferred to milk dilution bottles and sterilized by autoclaving. Changes in observed pH after autoclaving were <0.2 pH units. A separate set of bottles containing BHI adjusted to pH levels of 3.0-7.0 in 0.5 pH unit increments using HCl was employed as a control.

### *Inactivation studies*

Each bottle was inoculated to a population density of approximately $10^8$ CFU/ml by adding 0.6 ml of the combined 24-h culture. The bottles were laid on their side to maximize oxygen transfer and incubated without agitation at 28°C. Periodically, samples were removed aseptically, diluted as needed in 0.1% peptone water, and surface plated on tryptose agar (Difco) using either a Spiral Plater (Spiral Systems, Inc., Cincinnati, OH) or spread plates (depending on the level of surviving cells anticipated). All plates were incubated for 24 h at 37°C and enumerated either by hand counting or using an automated colony counter (Model 500A, Spiral Systems, Inc.). Sampling was continued for 60 d or until the counts fell below the lower limit for detection (log No. <1.03 CFU/ml).

*Survivor curves*

Survivor curves were generated by fitting the data to the linear function that allows for the presence of a lag period before initiation of an exponential decline in population density.

$$Y = Y_0 \qquad [t < t_L] \qquad (1)$$
$$Y = Y_0 + s(t - t_L) \qquad [t \geq t_L]$$

where:

$Y$ = log count of bacteria at time t. Log(CFU/ml);
$Y_0$ = log count of bacteria at time t = 0. Log(CFU/ml);
$s$ = slope of the survivor curve. [Log(CFU/ml)]/h;
$t$ = time. (h);
$t_L$ = duration of lag period prior to initiation of inactivation. (h).

The curves were fitted using ABACUS, a nonlinear curve-fitting program developed by W. Damert (U.S. Department of Agriculture, Agricultural Research Center, Eastern Regional Research Center) that uses a Gauss-Newton iterative procedure. The D values were then calculated by taking the negative reciprocal of s. The time (h) to a 4-D (99.99%) inactivation was calculated using the equation,

$$t_{4\text{-}D} = t_L + (4^*D). \qquad (2)$$

## RESULTS AND DISCUSSION

After an initial lag period, *L. monocytogenes* populations declined exponentially. Examples of representative inactivation curves are presented in Fig. 1. The duration of the lag period ($t_L$) and the rate of inactivation were dependent on the severity of the conditions (Table 1). Some growth (2- to 10-fold increase) was observed with cultures that had a combination of a low acid concentration and a pH value that supported growth. Times to a 4-D inactivation ($t_{4\text{-}D}$) were calculated and compared to combine the effects on $t_L$ and D. The selection of a 4-D inactivation was arbitrary, and the model could be solved for alternate degrees of inactivation.

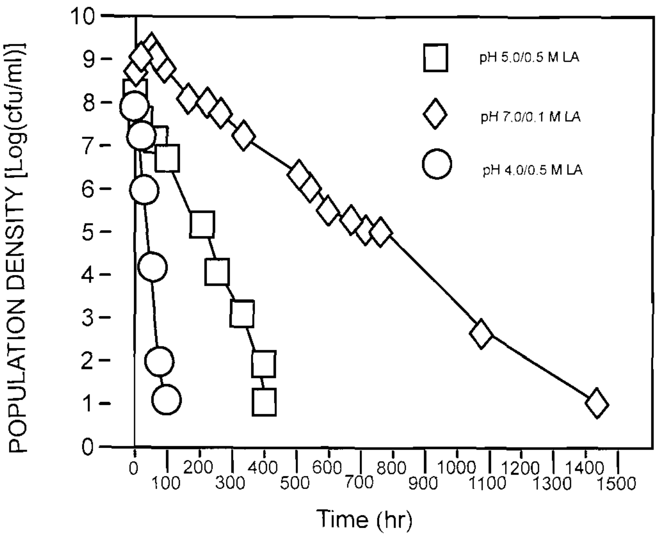


Figure 1. *Examples of inactivation curves observed with* L. monocytogenes *when exposed to various combinations of pH and lactic acid (LA) concentrations.*

TABLE 1. *Effect of pH, acidulant identity, and acidulant concentration on the lag period prior to inactivation ($t_L$), D value, and time to 4-D inactivation ($t_{4\text{-}D}$) for* L. monocytogenes.

| | | Lactic acid | | | Acetic acid | | |
|---|---|---|---|---|---|---|---|
| *pH* | Conc. (*M*) | $t_L$ (h) | D Value (h) | $t_{4\text{-}D}$ (h) | $t_L$ (h) | D Value (h) | $t_{4\text{-}D}$ (h) |
| 7.0 | 0.1 | 263.0 | 163.2 | 915.8* | NI | NI | >1440.0* |
| | 0.5 | 136.5 | 169.4 | 813.9* | 200.0 | 115.4 | 661.7* |
| | 1.0 | 76.5 | 127.0 | 584.3 | 154.5 | 108.5 | 588.4 |
| | 2.0 | 0.0 | 100.3 | 401.2 | 88.0 | 57.3 | 317.0 |
| 6.0 | 0.1 | 78.0 | 184.1 | 814.4 | 96.0 | 194.7 | 874.8* |
| | 0.5 | 53.0 | 128.5 | 567.0 | 169.5 | 86.1 | 513.8 |
| | 1.0 | 10.5 | 92.3 | 379.7 | 136.5 | 44.1 | 313.0 |
| | 2.0 | 0.0 | 56.7 | 226.6 | 43.5 | 46.5 | 229.5 |
| 5.0 | 0.1 | 44.0 | 82.2 | 372.6 | 127.0 | 42.3 | 296.1 |
| | 0.5 | 0.2 | 50.4 | 201.8 | 60.0 | 28.0 | 171.9 |
| | 1.0 | 3.0 | 33.4 | 136.4 | 24.0 | 25.9 | 127.6 |
| | 2.0 | 0.0 | 15.8 | 63.2 | 16.5 | 11.0 | 60.3 |
| 4.0 | 0.1 | 6.0 | 42.4 | 175.4 | 2.0 | 26.9 | 109.5 |
| | 0.5 | 4.0 | 11.6 | 50.2 | 0.0 | 12.6 | 50.2 |
| | 1.0 | 0.0 | 8.8 | 35.2 | 0.0 | 1.1 | 4.5 |
| | 2.0 | 0.0 | 1.1 | 4.2 | 0.0 | 0.9 | 3.5 |

| | HCl-Adjusted controls | | |
|---|---|---|---|
| pH | $t_L$ (h) | D Value (h) | $t_{4\text{-}D}$ (h) |
| 7.0 | NI | NI | >1440.0* |
| 6.5 | NI | NI | >1440.0* |
| 6.0 | 72.0 | 195.7 | 854.6* |
| 5.5 | 40.0 | 125.0 | 539.8* |
| 5.0 | 84.0 | 102.3 | 493.0* |
| 4.5 | 109.5 | 60.9 | 353.0 |
| 4.0 | 6.0 | 67.6 | 276.2 |
| 3.5 | 10.0 | 29.1 | 126.2 |
| 3.0 | 4.0 | 20.2 | 84.2 |

NI = Less than 1 log cycle of decline over the course of the experiment.
* = 2- to 10-fold increase in population density during initial phase of experiment.
All values are the means of two independent determinations.

Cultures adjusted to various pH levels using HCl were employed as controls to estimate the effect of pH alone, on the basis that this mineral acid is completely dissociated, and the lowest pH values that have been reported to support *L. monocytogenes* growth have been in conjunction with microbiological media adjusted with HCl (*1-3,5,7,8,15*). At pH levels ≤5.5, the $t_{4\text{-}D}$ for HCl-adjusted cultures was linearly related to pH (Fig. 2) and could be described by the regression equation:

$$t_{4\text{-}D} = 197.3^*\text{pH} - 526.5. \qquad (3)$$

These results are similar to those of Parish and Higgins (*12*) who reported that the duration of the lag period before

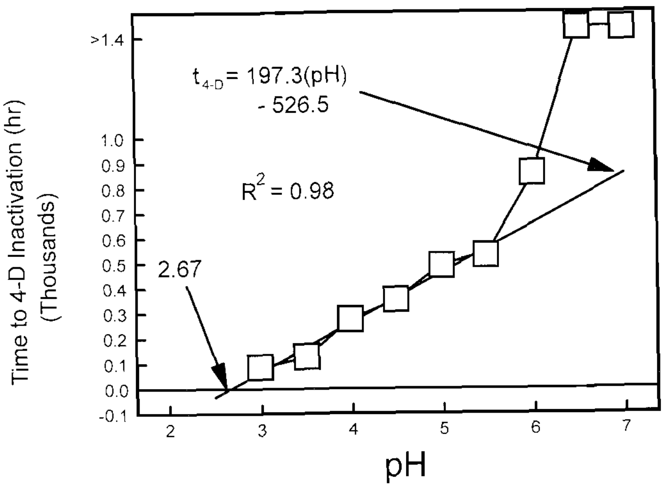


Figure 2. *Effect of pH on the inactivation of* L. monocytogenes *in control cultures adjusted to various pH levels using HCl.*

initiation of inactivation of *L. monocytogenes* in orange serum adjusted to pH 3.6-4.8 with HCl and NaOH was linear with respect to pH.

The relationship between pH and $t_{4\text{-}D}$ could also be described using the equation:

$$t_{4\text{-}D} = 197.3(\mathrm{pH} - \mathrm{pH}_0), \tag{4}$$

where $\mathrm{pH}_0$ = 2.67 (Fig. 3). The term, $\mathrm{pH}_0$, is the point where the extrapolated regression line crosses the X-axis. It can be considered a hypothetical value indicating the pH at which a 4-D inactivation is instantaneous.

The results of adding various concentrations of lactic acid (Fig. 4) and acetic acid (Fig. 5) at set pH values establishes unequivocally that the rate at which lactic and acetic acids inactivate *L. monocytogenes* is dependent on the identity of the acid, the concentration of the acid, and the pH of the system. Lactic acid was more effective than acetic acid at the higher pH levels, whereas this relationship was reversed at lower pH values. While the bacterium is inactivated by pH alone, there was a clear enhancement attributable to

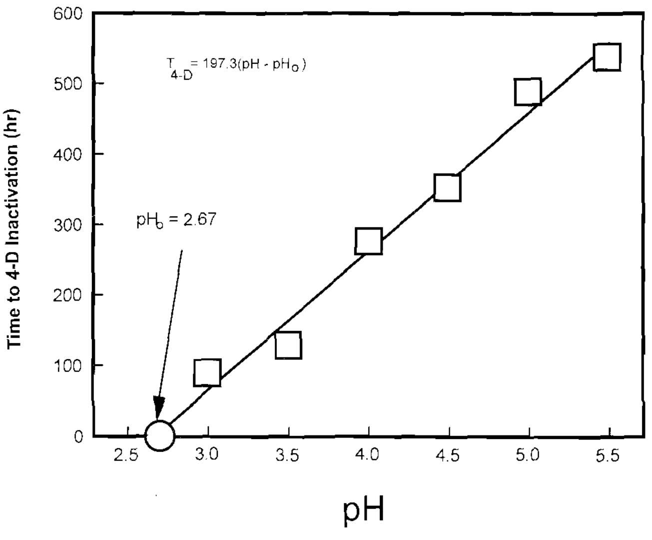


Figure 3. *Depiction of the HCl-adjusted control data with pH ≤5.5 using the relationship,* $t_{4\text{-}D}$ = *193.3(pH -* $pH_0$*), where* $pH_0$ *is notational pH depicting value when regression line crosses X-axis (i.e., time when 4-D inactivation is instantaneous).*

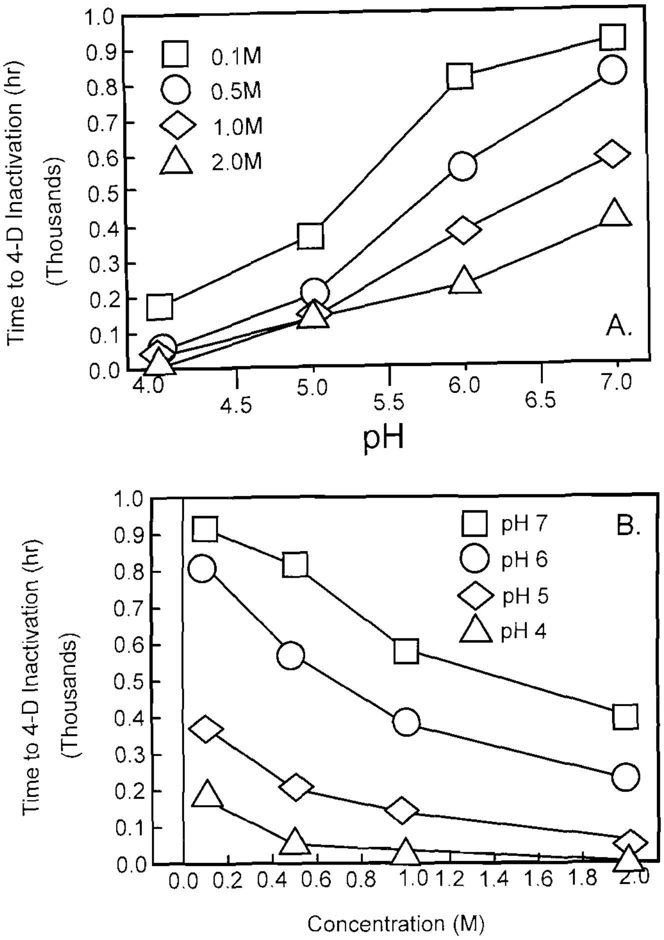


Figure 4a. *Effect of pH at various lactic acid/sodium lactate concentrations (A) and the effect of lactic acid/sodium lactate concentrations at various pH levels (B) on the inactivation of* L. monocytogenes.

the anion. The response to pH within an acid concentration could be approximated by a linear relationship, particularly at pH levels ≤6.0. However, the response to concentration within a pH level was nonlinear.

The antimicrobial activity of monocarboxylic organic acids has been hypothesized to require the molecules to be in their undissociated form (*9,10*). This prompted an assessment of the relationship between the calculated concentrations of undissociated lactic (Fig. 6) and acetic (Fig. 7) acids and the observed $t_{4\text{-}D}$ values. A distinct hyperbolic relationship was apparent for both acids. A further evaluation of the data was conducted using various mathematical transformations. When the logarithms of the $t_{4\text{-}D}$ values were plotted against the square roots of the concentrations of undissociated acid, a linear relationship was evident for both lactic (Fig. 8) and acetic (Fig. 9) acids. This allowed the inactivation of *L. monocytogenes* to be modeled based on the general regression-derived equation:

$$\mathrm{Log}(t_{4\text{-}D}) = m[\mathrm{HA}]^{0.5} + b, \tag{5}$$

where [HA] = mM of undissociated acid,
m = slope of the regression line (Fig. 8 and 9),
b = y - intercept of the regression line (Fig. 8 and 9).

The simplified forms for the equations for lactic acid (Fig. 8) and acetic acid (Fig. 9) are

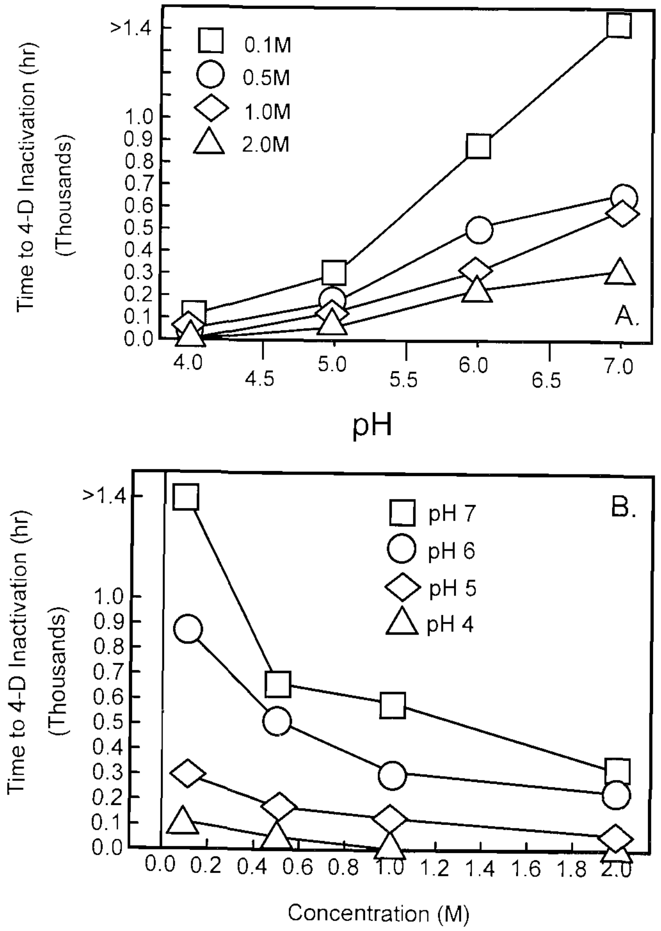


Figure 5. *Effect of pH at various acetic acid/sodium acetate concentrations (A) and the effect of acetic acid/sodium acetate concentrations at various pH levels (B) on the inactivation of* L. monocytogenes.

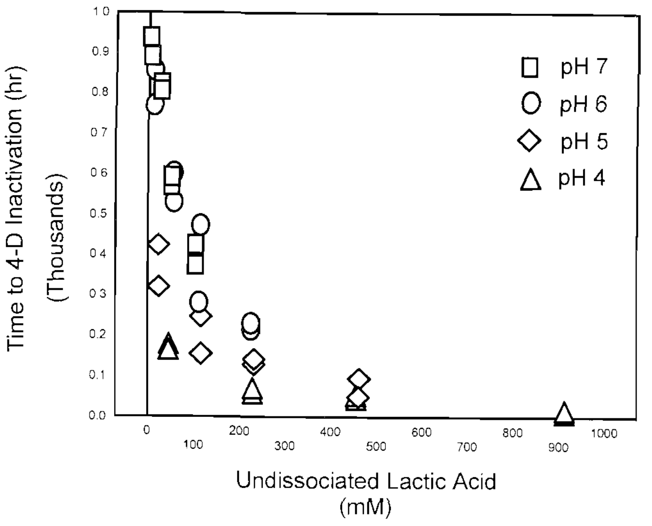


Figure 6. *Comparison of 4-D inactivation times versus calculated concentrations of undissociated lactic acid for the various pH/ concentration combinations.*

Lactic acid

$$t_{4\text{-}D} = \exp(-0.1773[HA]^{0.5} + 7.3482) \quad (6)$$

Acetic acid

$$t_{4\text{-}D} = \exp(-0.1468[HA]^{0.5} + 7.3905). \quad (7)$$

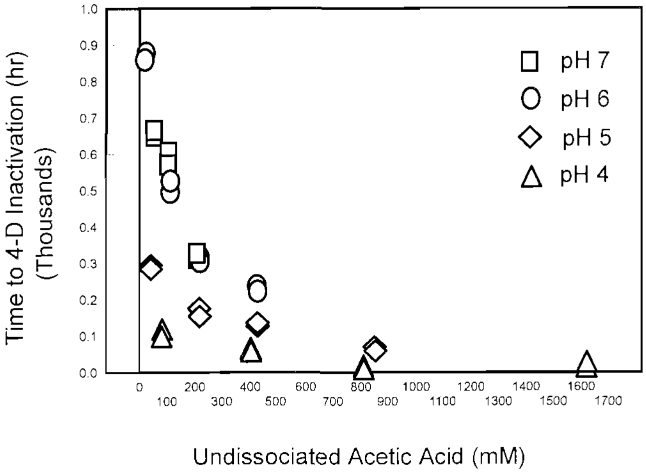


Figure 7. *Comparison of 4-D inactivation times versus calculated concentrations of undissociated acetic acid for the various pH/ concentration combinations.*

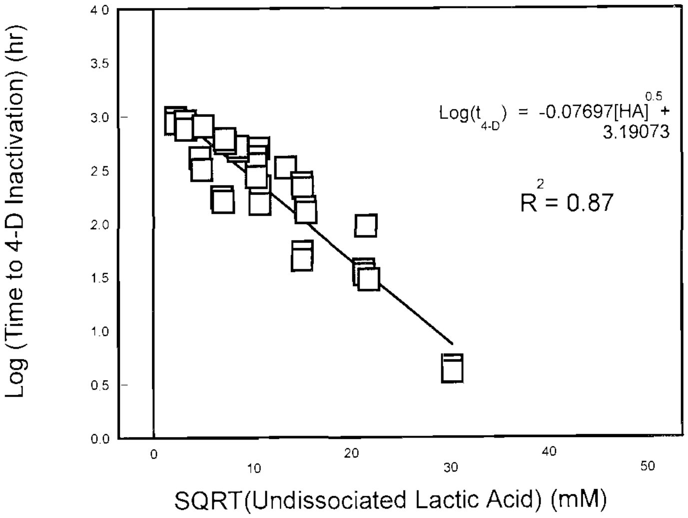


Figure 8. *Linear relationship observed by comparing logarithm of 4-D inactivation times versus the square root of the concentration of undissociated lactic acid.*

The agreement between observed and predicted values (Fig. 10 and 11) was reasonable, and indicated that the models could be used to provide initial estimates for the inactivation of *L. monocytogenes* within the experimental range of concentrations and pH values. The $R^2$ values suggest that the models could benefit from additional data encompassing other pH/concentration combinations.

These models indicated that lactic acid was more effective than acetic acid for inactivating *L. monocytogenes* when expressed in terms of concentration of undissociated acid. The strong correlation of inactivation activity with the concentration of undissociated organic acid supports the concept that this is the active form for both acids. The results observed at neutral pH values indicate further that the reported antilisterial activity of sodium lactate and sodium acetate (*13,16,17*) remains attributable to the undissociated acid and argues against alternative hypotheses of separate inhibitory mechanisms at higher pH values. This implies that increases in antimicrobial activity can be expected if the pH of an acetate- or lactate-containing system was decreased even to a small extent such that it was closer to the acid's $pK_a$.

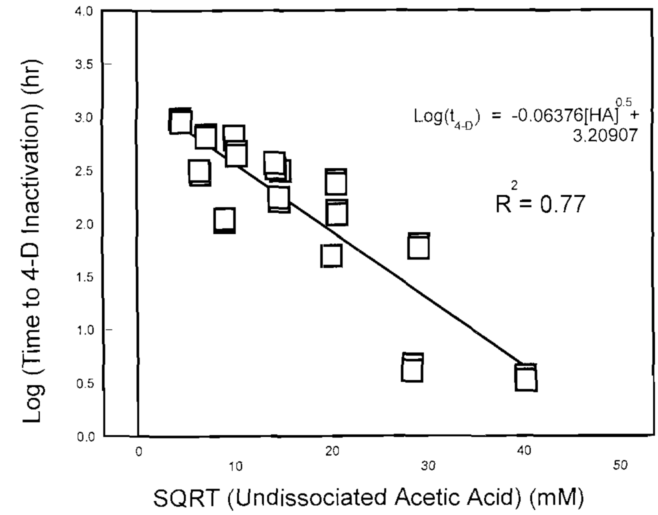


Figure 9. *Linear relationship observed by comparing logarithm of 4-D inactivation times versus the square root of the concentration of undissociated acetic acid.*

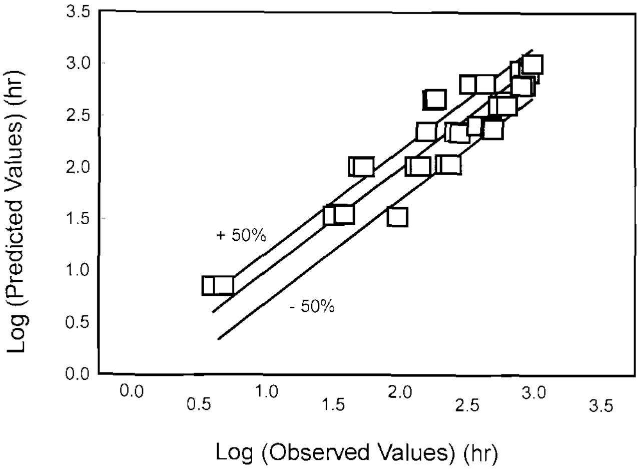


Figure 10. *Comparison of observed $t_{4\text{-}D}$ values for the inactivation of* L. monocytogenes *by lactic acid versus values predicted using the regression equation from Fig. 8. The center line is the line of identity, and the two exterior lines represent ±50% of the observed values.*

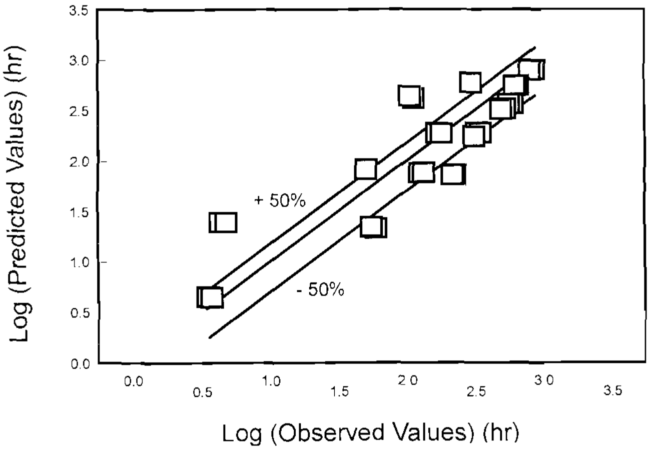


Figure 11. *Comparison of observed $t_{4\text{-}D}$ values for the inactivation of* L. monocytogenes *by acetic acid versus values predicted using the regression equation from Fig. 9. The center line is the line of identity, and the two exterior lines represent ±50% of the observed values.*

The mathematical relationships observed provide a promising new approach for the development of models to describe the pH/acidulant inactivation of *L. monocytogenes* and other foodborne pathogens. If the relationship between the logarithm of $t_{4\text{-}D}$ and the square root of the concentration of undissociated acid holds true for other organic acids, it could serve as the basis of a semimechanistic model for describing the combined effects of foodgrade organic acids. Using the Henderson-Hasselback equation, equations 6 and 7 can be modified so $t_{4\text{-}D}$ values can be predicted using the pH and the total organic acid concentration of the system accordingly to the relationship:

$$t_{4\text{-}D} = \exp(2.303\{m[(T/\exp((pH-pK)/2.303))/(1 + \exp((pH-pK)/2.303))]^{0.5} + b\}), \quad (8)$$

where

T = total concentration (mM) of organic acid,
m = slope of regression line from Fig. 8 or 9,
b = y-intercept of regression line from Fig. 8 or 9,
$t_{4\text{-}D}$ = time (h) to 10,000-fold decrease in population.

The current study identifies a new approach for modeling the inactivation of foodborne pathogens exposed to organic acids. Validation studies are needed to determine how well the models predict *L. monocytogenes* inactivation in both new pH/concentration combinations and representative foods. Likewise, future investigations are needed to determine how incubation temperature and water activity affect the relationship. Work is currently underway to determine if the relationship observed with these two monocarboxylic acids also occurs with other mono-, di-, and tricarboxylic acids.

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