Growth, Inhibition, and Survival of *Listeria monocytogenes* as Affected by Acidic Conditions

DONALD E. CONNER1, VIRGINIA N. SCOTT*, and DANE T. BERNARD

National Food Processors Association, Microbiology Division, 1401 New York Ave., N.W., Washington, DC 20005

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**ABSTRACT**

Growth and survival of four strains of *Listeria monocytogenes* under acidic conditions were investigated. Tryptic soy broth with yeast extract (TSBYE) was acidified with acetic, citric, hydrochloric, lactic, or propionic acid to pH 4.0-6.0, inoculated with *L. monocytogenes* and incubated at 30 or 4°C. The minimum test pH at which *L. monocytogenes* did not grow (inhibitory pH) was determined for each acid. In the pH range tested, this inhibitory pH was 5.0 for propionic acid, 4.5 for acetic and lactic acids, and 4.0 for citric and hydrochloric acids. All four strains gave similar results. Subsequent studies were conducted at 10 and 30°C to determine changes in cell populations in TSBYE adjusted to each inhibitory pH. Initial populations of viable cells (10⁴ CFU/ml) were reduced to <10 CFU/ml within 1-3 weeks at 30°C, whereas at 10°C, *L. monocytogenes* survived for 11-12 weeks in acetic, citric, or propionic acid-adjusted media and for 6 weeks in media adjusted with hydrochloric or lactic acid. The concentration of undissociated lactic acid was 0.002 M at pH 4.5.

It is now recognized that the human pathogen *Listeria monocytogenes* is widely distributed in nature and can be isolated from a variety of foods and food ingredients (2,6,9,11,13). Recent outbreaks of human listeriosis as well as epidemiological studies of sporadic cases of human listeriosis have demonstrated that food is a possible vehicle for transmitting *L. monocytogenes* to humans (2,5,6,8,9,11,13).

Although *L. monocytogenes* may be isolated from many points in the food chain, there is little information concerning the behavior of this bacterium in food environments other than in dairy products. Conner et al. (4) indicated that *L. monocytogenes* is more tolerant to acidic conditions than previously believed and demonstrated that *L. monocytogenes* grew in cabbage juice at pH 5.0 but died off at pH ≤4.6. It has also been demonstrated that the effect of acids on the growth of *L. monocytogenes* may be influenced not only by pH but by other factors such as salt levels and temperature (4,12,14).

To better assess the role of *L. monocytogenes* in foodborne disease as well as identify potential means of controlling this bacterium in foods, it is important to identify and quantify the effect of various environmental factors on the behavior of *L. monocytogenes*. Therefore, this study was undertaken to investigate the fate of several strains of *L. monocytogenes* in the presence of selected organic acids and hydrochloric acid in laboratory medium.

**MATERIALS AND METHODS**

**Cultures**

Four isolates of *L. monocytogenes* were used: V-37, serotype 4b, isolated from bulk milk; N7045, serotype 4b, isolated from orange sherbet; N7095, serotype 1/2a, isolated from bacon brine; and N7183, serotype 1/2a, isolated from salami. Cultures were grown at 30°C in tryptic soy broth containing 0.6% yeast extract (TSBYE, Difco, Detroit, MI) and maintained in TSBYE-glycerol (50:50, v/v) at -20°C. Before preparing inoculum for the test media, cultures were activated by two successive transfers in TSBYE at 30°C.

**Acids**

Stock solutions of known concentrations of reagent grade acetic, citric, lactic, propionic, and hydrochloric acids were prepared, filter-sterilized, and used to aseptically acidify batches of TSBYE to pH 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5. The volume of acid added to achieve the desired pH was noted and used to calculate the concentration of each acid in TSBYE at each test pH. Concentrations of the undissociated forms of the organic acids at each pH were calculated using the Henderson-Hasselbalch equation (3):

\[
\text{pH} = \text{pK}_a + \log \left[ \frac{[A^-]}{[HA]} \right]
\]

where \(\text{pK}_a\) is the dissociation constant, \([A^-]\) is the concentration of the dissociated (unprotonated) acid, and \([HA]\) is the concentration of the undissociated (protonated) acid. Citric acid concentration was calculated using only \(\text{pK}_a\).

**Screening study**

For each of the five acids tested, five tubes of TSBYE (9
ml) at each test pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5) and five tubes of unacidified TSBYE (pH 6.8) were inoculated with 0.05 ml of a 24-h/30°C culture of L. monocytogenes then incubated at 30°C. Additional sets of tubes were inoculated and incubated at 4°C. Each of the four strains of L. monocytogenes was individually tested in this manner. Tubes were examined daily for turbidity over a 6-week period. After 6 weeks, tubes at 4°C were physically transferred and incubated at 30°C for an additional 2 weeks.

Enumeration procedure

Experimental samples and cultures were enumerated by pour plate technique. Each sample was diluted in phosphate buffered saline (pH 7.2), inoculated in Petri dishes, and mixed with tryptose phosphate agar (Difco) containing 1% pyruvic acid (Sigma Chemical, St. Louis, MO). Petri dishes were incubated at 30°C for 48 h before counting colonies.

Survival study

Based on results of the screening tests, the minimum tested pH at which no increase in turbidity occurred was determined for each acid. Flasks of TSBYE (100 ml) acidified to these pH values (acetic, pH 4.5; citric, pH 4.0; hydrochloric, pH 4.0; lactic, pH 4.5; propionic, pH 5.0) were prepared and inoculated with a composite suspension containing approximately equal numbers of cells of each of the four test strains of L. monocytogenes. Each flask of TSBYE was inoculated to give an initial population of approximately 10^5 CFU/ml. As a control, an additional flask containing unacidified TSBYE (pH 6.8) was also prepared and inoculated as described above. Duplicate flasks of medium at each pH plus control medium were prepared and one set was incubated at 30°C and one at 10°C. Immediately after inoculation, and every week thereafter, samples (1.0 ml) from each flask were aseptically withdrawn and viable L. monocytogenes were enumerated using the above procedure. Sampling of each pH variable was performed until no viable cells were recovered at two successive sampling periods or for 12 weeks. Data presented represent means of two replicate tests.

RESULTS

Results of the screening tests at 30°C indicated that all four strains of L. monocytogenes gave similar results. However, strains N7183 and N7095 grew in the presence of acetic acid at pH 5.5 but not at pH 5.0. Strains 7045 and V-37 grew in the presence of acetic acid at pH 5.0 but not at 4.5. Growth of all four strains was the same in the presence of all other acids tested; therefore, a composite of the four strains was used as inoculum in subsequent survival studies. The minimum test pH levels at which L. monocytogenes did not grow at 30°C as evidenced by no increase in turbidity are shown in Table 1.

At 4°C, the test strains of L. monocytogenes did not grow within 6 weeks in any test or control media. However, when these media were placed at 30°C, L. monocytogenes grew and the observed growth patterns were identical to those observed in media initially incubated at 30°C.

In survival studies conducted in TSBYE acidified with the test acids to the pH values listed in Table 1, L. monocytogenes was inactivated at a much higher rate at 30°C than at 10°C. This was true for all acids tested. At 30°C (data not shown), the initial L. monocytogenes population of approximately 10^4 CFU/ml was reduced to <10 CFU/ml within 1 week in TSBYE adjusted to pH 4.5 with acetic acid, to pH 4.5 with lactic acid, and to pH 4.0 with hydrochloric acid. Approximately 25 CFU/ml were detected at 1 week, while none (<10 CFU/ml) was detected at 2 and 3 weeks of incubation in TSBYE at pH 4.0 adjusted with citric acid. In TSBYE at pH 5.0 adjusted with propionic acid, the population of L. monocytogenes recovered was 2.8 x 10^4 CFU/ml immediately following inoculation, 1.4 x 10^4 CFU/ml at 1 week, 5.2 x 10^3 CFU/ml at 2 weeks, 3.8 x 10^2 CFU/ml at 3 weeks, and <10 CFU/ml at 4 weeks.

In contrast, viable cells of L. monocytogenes were recovered over a much longer period of storage at 10°C as compared to 30°C. In TSBYE containing no added acids, the population of L. monocytogenes increased from approximately 10^3 to approximately 10^6 CFU/ml and then remained at this level over the 12-week incubation period. In TSBYE at pH 4.0 adjusted with propionic acid, no change in viable population of L. monocytogenes was observed during the study (Fig. 1). The population of viable L. monocytogenes in TSBYE at pH 4.0 adjusted with citric acid gradually decreased from 1.8 x 10^5 to 27 CFU/ml over 12 weeks at 10°C (Fig. 2). Similar results were obtained in TSBYE at pH 4.5 adjusted with acetic acid; however,
no viable cells were detected in samples taken at 12 weeks (Fig. 3). In TSBYE at pH 4.5 adjusted with lactic acid and pH 4.0 adjusted with hydrochloric acid, the initial population of 10^4 CFU/ml was reduced to <10 CFU/ml within 6 weeks (Fig. 4 and 5).

DISCUSSION

In addition to providing information needed to conduct subsequent survival studies, the screening test results provided some useful information concerning the antilisterial activity of the test acids. Screening test results indicate that the minimum test pH at which *L. monocytogenes* did not grow in acidified TSBYE was different depending upon the acid tested. Based only on pH, propionic acid, which inhibited listerial growth at pH 5.0, was the most inhibitory against *L. monocytogenes*. Lactic and acetic acids were inhibitory at pH 4.5 and citric and hydrochloric acids at pH 4.0. These data are similar to the inhibitory pH values reported by Sorrells et al. (14) who also reported that, on an equivalent pH basis, acetic and lactic acids were more inhibitory against *L. monocytogenes* than citric and hydrochloric acids. These researchers did not test propionic acid. The screening test data also are in agreement with other reports that indicate factors other than pH, such as type of acid, temperature, concentration, and extent of dissociation, affect the antilisterial activity of many acidulants (4,12,14).

Because of the differences in dissociation properties of the test acids, different quantities of each acid were added to the medium to achieve these inhibitory pH levels. Therefore, the concentration at the pH values at which no growth of *L. monocytogenes* occurred was calculated and found to differ among the test acids. On this basis, the

![Figure 2. Population (CFU/ml) of *L. monocytogenes* incubated at 10°C in TSBYE and TSBYE acidified to pH 4.0 with citric acid.](image)

![Figure 3. Population (CFU/ml) of *L. monocytogenes* incubated at 10°C in TSBYE and TSBYE acidified to pH 4.5 with acetic acid.](image)

![Figure 4. Population (CFU/ml) of *L. monocytogenes* incubated at 10°C in TSBYE and TSBYE acidified to pH 4.5 with lactic acid.](image)

![Figure 5. Population (CFU/ml) of *L. monocytogenes* incubated at 10°C in TSBYE and TSBYE acidified to pH 4.0 with hydrochloric acid.](image)
inhibitory concentrations of citric acid (0.029 M) and propionic acid (0.031 M) were the lowest observed, while the concentration of acetic acid (0.068 M) was the highest (Table 1).

It has been indicated that the antimicrobial activity of many organic acids is attributed to the undissociated form of the acid molecule (1,10). Thus, the concentration of the undissociated form of each test acid at its respective inhibitory pH was calculated. Excluding hydrochloric acid, which is totally dissociated in aqueous environments, lactic (0.002 M) and citric (0.003 M) acids were found to have the lowest concentrations of undissociated acid at their respective inhibitory pH values. Acetic acid (0.044 M) was the highest (Table 1). Sorrells et al. (14) reported that, on an equal molar concentration basis, citric acid was more inhibitory than lactic acid which was more inhibitory than acetic acid.

Survival studies conducted over an extended period provided some additional insight into the response of L. monocytogenes to the acidic conditions described above. Although L. monocytogenes did not grow in TSBYE acidified to pH 5.0 with propionic acid, the cells were not inactivated and the population remained viable but static over the entire 12-week period at 10°C, indicating that this level of propionic acid was bacteriostatic rather than bactericidal under the test conditions. Acetic and citric acids at the tested levels exhibited a limited bactericidal effect on L. monocytogenes over 12 weeks at 10°C, whereas hydrochloric and lactic acids were lethal to 10^3-10^4 CFU/ml within 6 weeks of storage. In no instances during survival studies did the population of L. monocytogenes adapt to the acidic conditions and begin to multiply. At 30°C, L. monocytogenes is more sensitive to these acids at 30°C than at 10°C. This finding is in agreement with earlier reports (4,12,14).

Although L. monocytogenes is able to survive under many of the acidic conditions tested in this investigation, results of screening and survival studies indicate that lactic acid at very low concentrations of undissociated acid (0.002 M) can inactivate L. monocytogenes, albeit at a fairly low pH (4.5). This observation helps substantiate reports that indicate products such as properly fermented (good quality) silages do not support the development of L. monocytogenes (7). Citric acid, another commonly used food acidulant, also inhibited L. monocytogenes at a very low concentration of undissociated acid (0.003 M); however, its effect was primarily bacteriostatic. Although not tested here, propionic acid, which was bacteriostatic toward L. monocytogenes at pH 5.0 at 10°C, may be lethal to L. monocytogenes at a lower pH range. More research is needed in this area. Furthermore, the observation that the antilisterial activity of the acids was greater at 30°C than at 10°C indicates that environmental factors and/or treatments can influence an acid’s antibacterial activity. Thus, more research is needed to investigate the antilisterial activity of these acids when used in combination with other antimicrobial treatments, particularly at temperatures characteristic of refrigerated storage. Data collected in these types of studies will better define the antilisterial activity of these acids and possibly aid in developing means of eliminating L. monocytogenes from foods.

REFERENCES