

Strains and Suspending Menstrua as Factors Affecting Death and Injury of *Listeria monocytogenes* During Freezing and Frozen Storage

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ABSTRACT

Cell suspensions of *Listeria monocytogenes* strains V7, California, and Ohio in phosphate buffer solution, tryptose broth, or milk were frozen and stored at -18°C . At appropriate intervals during storage, a sample was thawed at 35°C and surface-plated on suitable media to allow colony formation by noninjured or noninjured plus injured cells. Degrees of death and injury were calculated from the data. Cells of *L. monocytogenes* were more resistant to death and injury when they were suspended in milk or tryptose broth rather than phosphate buffer solution. There was a significant (two-way ANOVA) difference in resistance to death and injury during frozen storage among strains of *L. monocytogenes* suspended in tryptose broth. The difference was nonsignificant when the cells were suspended in phosphate buffer solution or milk. *Listeria monocytogenes* strain Ohio was more resistant to death and injury during frozen storage when cells were suspended in tryptose broth rather than milk. The opposite was true for strains V7 and California. Death and injury of *L. monocytogenes* strains V7, California, and Ohio suspended in phosphate buffer solution were 98.7, 97.9, and 91.2% and 77.5, 51.6, and 70.2%, respectively, after 4 wk of frozen storage. The values were 67.3, 91.6, and 42.3% and 44.4, 65.6, and 32.6%, respectively, when cells were suspended in tryptose broth, and they were

37.8, 40, and 60.7% and 10.8, 66.8, and 46%, respectively, when cells were suspended in milk.

(Key words: *Listeria monocytogenes*, freezing, death, injury)

Abbreviation key: PBS = phosphate buffer solution, TA = tryptose agar, TB = tryptose broth, TSA = tryptose salt agar.

INTRODUCTION

Listeria monocytogenes is a psychrotrophic bacterium that is widely distributed in nature (14). The mortality rate of listeriosis, the disease caused by *L. monocytogenes*, is about 30 to 45% (5). The annual incidence of listeriosis is approximately .7 case per million persons in the US (1).

There was a 4% overall incidence of *L. monocytogenes* in food sampled by the FDA, ranging from 7.5% for ice cream to .9% for milk (1). A study on *L. monocytogenes* in raw milk in three areas in the US revealed a 4.2% overall incidence of this pathogen in samples, with a range between 0 and 7% (9).

In a previous study, we determined that freezing killed and injured cells of *L. monocytogenes* strain Scott A. The extent of death and injury depended on freezing temperature, suspending menstrua, storage time, and number of freeze and thaw cycles (7). In another study, we found that milk fat, lactose, and casein (each at a concentration close to that in milk) protected *L. monocytogenes* strain Scott A from death and injury during frozen storage. This protection depended on the concentration of each milk component and the length of storage (6).

Different strains of *Salmonella* had variable resistance to death during storage at -17°C (8). Differences and similarities among strains of *L. monocytogenes* were exhibited in studies on 1)

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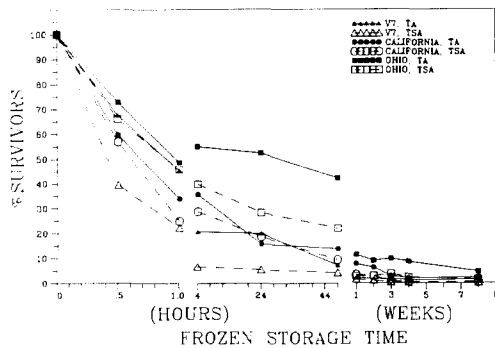


Figure 1. Survival, as determined with tryptose agar (TA) and tryptose salt agar (TSA), of different strains of *Listeria monocytogenes* during frozen storage when cells were suspended in phosphate buffer solution.

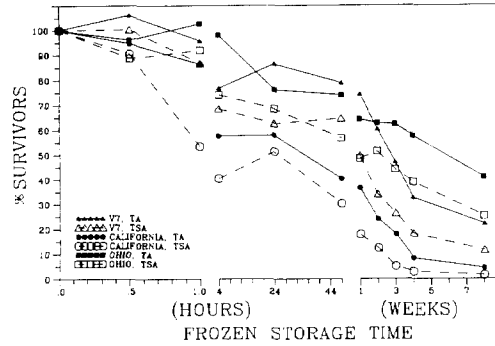


Figure 2. Survival, as determined with tryptose agar (TA) and tryptose salt agar (TSA), of different strains of *Listeria monocytogenes* during frozen storage when cells were suspended in tryptose broth.

survival after spray-drying in NDM (3), 2) resistance to heat treatment (2), 3) resistance to chlorine (4), 4) generation time and maximum population produced under similar circumstances (11), and 5) survival during cheese processing and ripening (12, 13).

In this study, we determined the lethal and sublethal effects of freezing on different strains of *L. monocytogenes*. Statistical analysis was done to determine whether the differences were significant. The effect of menstrua on behavior of the pathogen during freezing and frozen storage was studied by suspending cells of different strains in phosphate buffer solution (PBS), tryptose broth (TB), or raw milk.

MATERIALS AND METHODS

Microorganism

Listeria monocytogenes strains V7, California, and Ohio were used in this study. Strain V7 is a milk isolate obtained from the FDA. Strain California is a cheese isolate obtained from Silliker Laboratories (Carson, CA), and strain Ohio is another cheese isolate obtained from M. P. Doyle (Food Research Institute, University of Wisconsin, Madison, WI). Strains grown on tryptose agar (TA) slants were stored at 4°C. Each strain was activated in TB, cells were recovered by centrifugation, and they were washed three times as described earlier (7). The resulting cell pellet was used to prepare the cell suspension that was used to inoculate test samples.

Suspending Menstrua

Sufficient cell suspension was added to PBS (.1 M, pH 7.0), TB, or raw milk to give approximately 10^6 cells/ml. Raw milk obtained from the cooling tank of the dairy factory (Department of Food Science, University of Wisconsin-Madison) was placed in a sterile flask soon after the milk arrived at the factory. Sterile equipment was used so that no additional contamination was added to the milk. Inoculated test samples were dispensed, 1 ml per sterile vial.

Freezing and Thawing

Sufficient vials of each menstruum containing the cell suspension were frozen and stored at -18°C . At appropriate intervals, contents of one vial were thawed in a water bath at 35°C and tested immediately after thawing was completed, which required about 2 min.

Enumeration of *Listeria monocytogenes*

A suitable dilution (in .5% peptone solution) of thawed cells was surface-plated on TA and tryptose salt agar (TSA) to enumerate the injured plus noninjured and the noninjured cells, respectively. Incubation time and temperature for agar plates, counting aids, calculations, and terminology were as described previously (7).

Statistical Analysis

Slopes of survivor curves were determined from the regression equation for each curve and

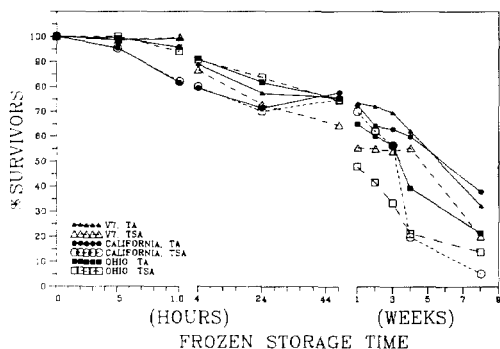


Figure 3. Survival, as determined with tryptose agar (TA) and tryptose salt agar (TSA), of different strains of *Listeria monocytogenes* during frozen storage when cells were suspended in milk.

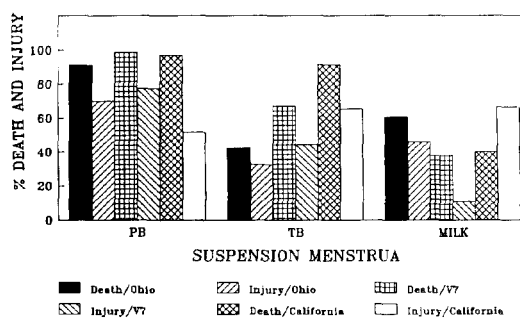


Figure 4. Death and injury of different strains of *Listeria monocytogenes* suspended in phosphate buffer solution (PBS), tryptose broth (TB), or milk after 4 wk of frozen storage at -18°C .

trial. The ANOVA (one-way) was applied to the slopes. The Minitab Computer Program (version 7, Minitab Statistical Software, Inc., State College, PA) was used for the regression and ANOVA calculations.

RESULTS AND DISCUSSION

The percentage of survivors, as determined with TA and TSA, were plotted against frozen storage time to illustrate the behavior of strains of *L. monocytogenes* during frozen storage (Figures 1, 2, and 3). Differences between numbers of colonies on TA and TSA represent the extent of injury to the bacterium. Death and injury of the bacterium after 1 mo of frozen storage were calculated, and those values are in Figure 4.

Strain Behavior in Phosphate Buffer Solution

Data in Figure 1 show survival of *L. monocytogenes* strains during frozen storage of cells suspended in PBS. These data suggest that strain Ohio was more resistant to death and injury during frozen storage for up to 8 wk than were strains V7 and California. However, statistical analysis indicated that the differences in survival among the three strains are significant at $P = .1$ but not at $P = .05$. Thus, there is only weak evidence that our strains of *L. monocytogenes* differed in their resistance to death and injury during frozen storage when cells were suspended in PBS. Death and injury of cells of

strains V7, California, and Ohio were 55, 66, and 51% and 51, 27, and 6%, respectively, after the initial hour of frozen storage and 93, 86, and 58% and 41, 32, and 48% after 48 h of frozen storage. Death and injury of the three strains occurred primarily during the initial hours of frozen storage. Results of a previous study revealed that death and injury of cells of strain Scott A (under similar conditions) were 27 and 34% after the initial hour of frozen storage and 55 and 50%, respectively, after 48 h of frozen storage (7). Strain Scott A, a patient isolate, seemed to be more resistant to death and injury during frozen storage than were strains V7, California, and Ohio, all isolated from dairy foods.

Strain Behavior in Tryptose Broth

Strain California was more susceptible to death and injury during frozen storage in TB than were strains V7 and Ohio (Figure 2). Differences in percentage of survivors among the strains were significant at $P = .05$. Thus, there is strong evidence that our strains of *L. monocytogenes* differed in their resistance to death and injury during frozen storage when cells were suspended in TB. Death and injury of strains V7, California, and Ohio were 4.3, 13.8, and .0% and 9.4, 28, and 10%, respectively, after the initial hour of frozen storage and 21, 60, and 26% and 18, 25, and 23%, respectively, after 48 h of frozen storage. El-Kest et al. (7), in another study with strain Scott A,

found that death and injury were 15.5 and 7% after 1 h and 19.3 and 29.5% after 48 h of frozen storage. Death and injury during freezing and frozen storage of different strains of *L. monocytogenes* suspended in TB were less than when cells were suspended in PBS. Presence of solutes around cells of *L. monocytogenes* may eliminate crushing of the cells between ice crystals during frozen storage (7).

Strain Behavior in Milk

There was almost no death or injury for strains V7 and Ohio during the initial hour of frozen storage in milk (Figure 3). Strain California seemed to be more susceptible to death and injury during short-term (the initial 48 h) frozen storage than were strains V7 and Ohio. Strain Ohio seemed to be more susceptible to death and injury during long-term (the interval between 1 and 8 wk) frozen storage than were strains V7 and California. Differences in percentage of survivors among strains were significant at $P = .1$ but not at $P = .05$. Hence, the evidence is weak that the strains of *L. monocytogenes* that we studied differed in resistance to death and injury during frozen storage when cells were suspended in milk. Death of the three strains after 48 h of frozen storage was about 25%, and from 1 to 15% of the surviving cells were injured. Presence of milk around cells of *L. monocytogenes* strains V7, California, and Ohio apparently protected the cells from death and injury during the initial 2 d of frozen storage. El-Kest and Marth (6) also found that casein, lactose, and milk fat protected *L. monocytogenes* strain Scott A against death and injury during frozen storage. The milk components also may have protected strains V7, California, and Ohio in these experiments.

Death and Injury of Strains After Frozen Storage

Frozen foods are often stored for at least 4 wk. Data in Figure 4 show that death and injury of the three strains of *L. monocytogenes* were greater during a similar period when cells were suspended in PBS rather than in TB or milk. Death and injury of strains V7 and California suspended in milk were less than when cells were suspended in PBS or TB. The cryoprotec-

tive effect of milk components for *L. monocytogenes* strain Scott A was discussed elsewhere (6). More death and injury of frozen cells of *Streptococcus lactis* occurred when suspended in PBS than in NDM (10). However, *L. monocytogenes* strain Ohio was less resistant to death and injury when cells were suspended in milk than in TB.

Implications for Frozen Dairy Foods

One should recognize that *L. monocytogenes* suspended in milk resisted death and injury during frozen storage, but there was no significant difference in resistance among strains. This resistance decreased after an initial 2 d of frozen storage. Accordingly, care should be given to eliminate contamination of frozen dairy foods with *L. monocytogenes* before freezing. Storage of frozen dairy products for at least 4 wk before consumption could reduce the population of the pathogen, if present, by up to 50%, depending on how various components of a product interact to protect the pathogen during freezing and frozen storage.

ACKNOWLEDGMENT

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