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Thermal Inactivation of *Listeria monocytogenes* within Bovine Milk Phagocytes

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Thermal resistance of intracellular and freely suspended *Listeria monocytogenes* that was associated with a milkborne outbreak of listeriosis was studied by using the sealed tube and slug flow heat exchanger methods. Test temperatures for the former method were 57.8, 62.8, 66.1, and 68.9°C (136, 145, 151, and 156°F, respectively); whereas those for the latter method were 66.1, 68.9, 71.7, and 74.4°C (151, 156, 161, and 166°F, respectively). The heating menstruum was sterile, whole milk. The intracellular inoculum was generated from an in vitro phagocytosis reaction by using endotoxin-induced bovine milk phagocytes. The phagocyte population consisted of 88% neutrophils, 8% macrophages, and 4% lymphocytes. Neutrophils harbored the majority of intracellular *L. monocytogenes*. The mean level of infectivity in the phagocyte population was 43%, and there were 26.1 ± 19.3 bacteria per cell (10^4 viable cells per ml of test milk). Initial bacterial counts for the freely suspended and intracellular experiments (the latter was based on a sonically disrupted sample) were 10^6 *L. monocytogenes* cells per ml. Heat-stressed bacteria were recovered by direct plating in parallel with recovery from an enrichment broth; both methods gave comparable results. The predicted $D_{62.8°C}$ (145°F) value for intracellular sealed tube studies was 53.8 s (z_D = 5.6°C [10.0°F]), indicating a safe 33.4 *D* margin of inactivation for vat pasteurization (62.8°C for 30 min). The predicted $D_{71.7°C}$ (161°F) value for intracellular slug flow heat exchanger studies was 4.1 s (z_D = 8.0°C [14.4°F]), indicating a potentially unsafe 3.7 *D* margin of inactivation for the high-temperature, short-time pasteurization minimum (71.7°C for 15 s). Results of extracellular and intracellular experiments were similar for both test systems. These data indicate that, under the defined parameters of this study, the intracellular position of *L. monocytogenes* does not significantly augment heat resistance.

Listeria monocytogenes is a gram-positive, non-spore-forming, aerobic to facultatively anaerobic, rod-shaped bacterium that is pathogenic to humans and animals. In humans, listeriosis is typically manifested as septicemia, meningitis, or abortion and occurs predominantly in neonates and immunosuppressed individuals. Infected animals that are symptomatic or asymptomatic may excrete *L. monocytogenes* in milk, blood, and feces (9). Humans presumably acquire listeriosis from direct contact with infected animals, but several recent outbreaks have confirmed an indirect transmission from animals to humans through consumption of contaminated food products (14, 27). The first of these outbreaks to be documented occurred in Nova Scotia, Canada, in 1981. Case studies revealed that cabbage used to prepare cole slaw, the implicated vehicle, was grown in soil fertilized with sheep manure from an infected herd (27).

Dairy products have since been identified as vehicles of infection in two recent human outbreaks of listeriosis. Between 30 June and 30 August 1983, 49 people in Massachusetts were hospitalized with septicemia or meningitis caused by *L. monocytogenes* serotype 4b; mortality was 29%. Results of epidemiological studies have shown that disease was strongly associated with the drinking of a certain brand of pasteurized whole or 2% fat milk, although *L. monocytogenes* was never detected in the finished dairy products. The implicated milk came from farms where listeriosis was detected in dairy cows, but neither evidence of faulty pasteurization nor postpasteurization contamination was found at the dairy plant where the milk was processed. These findings raised questions concerning the efficacy of pasteurization for eliminating *L. monocytogenes* in contaminated bovine milk (14). Another fatal outbreak of listeriosis (50 deaths) that was attributed to Mexican-style cheese occurred in Los Angeles and Orange counties, Calif., between January and June 1985. Sanitary conditions at the processing dairy, however, have strongly suggested the likelihood of postpasteurization contamination in this outbreak (17). These incidents have stimulated a reexamination of the thermal resistance of *L. monocytogenes* in fluid milk.

The literature on the heat resistance of *L. monocytogenes* has expanded, with widely conflicting results. The unusual heat resistance of this organism in whole milk (1, 4, 7, 11, 25) and meat (19) has been reported. A pathogenic *L. monocytogenes* strain was isolated from 6 of 28 (21.4%) pasteurized milk samples marketed by a Spanish dairy (13). Results of thermal inactivation studies involving several strains associated with the Massachusetts outbreak have indicated that 10^5 to 10^6 *L. monocytogenes* cells per ml, which were freely suspended in whole milk, would not survive minimally recommended pasteurization standards (vat pasteurization, 62.8°C for 30 min; high-temperature, short-time [HTST] pasteurization, 71.7°C for 15 s) (3, 9, 10, 15). Results of thermal resistance studies on *L. monocytogenes* in cabbage juice that were performed under a wide range of physiological conditions have shown that 10^5 *L. monocytogenes* cells per ml would be inactivated within 10 min at 58°C (2).

The pathogenesis of *L. monocytogenes*, particularly the facultative intracellular characteristic, has led epidemiolo-

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gists to speculate that intracellular position may protect this organism from thermal inactivation during pasteurization (14). Mastitic cows excrete high levels of somatic cells compared with normal cows; and the phagocytic cells that are present, neutrophils and macrophages, could concentrate large numbers of organisms, obscuring bacterial CFU from milk. In an early study (18), naturally infected milk from cows containing 10^3 to 10^4 *L. monocytogenes* cells per ml was heated in a commercial-style pasteurizer; viable organisms were not detected in the end product. No attempt was made in that study to determine the level of intracellular *L. monocytogenes*, although the source infers an intracellular potential. Milk from experimentally infected cows containing 10^2 to 10^4 *L. monocytogenes* cells per ml was also processed in a commercial-type plate pasteurizer; survivors were detected in the pasteurized product on enrichment. A range of 1.5 to 9.2 *L. monocytogenes* cells was seen in each infected phagocyte (≤0.1% infectivity) before the heat studies (11). Parallel heat experiments in raw, whole milk in which freely suspended bacteria and bacteria internalized by murine peritoneal phagocytes were used were performed in sealed glass tubes (4). The thermal inactivation kinetics of these two bacterial cell treatments were analyzed statistically, and no significant difference was found; postheating survival was not detected for vat pasteurization, nor was it indicated for HTST pasteurization.

In this study we examined the thermal inactivation kinetics of freely suspended and intracellular *L. monocytogenes*; the latter was done within a homogeneic cell population by using both the sealed tube (ST) and slug flow heat exchanger (SF) methods. Sterile, whole milk was the heating menstruum because it afforded us the opportunity to maximally detect heat-stressed *L. monocytogenes*, which were free from a contaminating background, by using the best recovery system that we have determined to date.

(Results of this study were presented at the Annual Spring Meeting of the Food Research Institute in Madison, Wis., 27 and 28 May 1987.)

MATERIALS AND METHODS

Bacterial culture and culture conditions. *L. monocytogenes* F5069 was obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. This strain belongs to serotype 4b and was isolated from raw milk obtained from a farm that supplied the incriminated dairy products in Massachusetts (14). The identity and virulence of the organism were established by use of the criterion presented by Seeliger and Jones (28). Stock cultures were grown in Trypticase soy broth–0.6% yeast extract (TSBYE) (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h and maintained at 4°C with monthly transfer.

Phagocyte elicitation and harvest. Phagocyte production was stimulated by the intramammary infusion of 50 ml of a 1-μg/ml solution of *Escherichia coli* 0127:B8 lipopolysaccharide (Difco Laboratories, Detroit, Mich.) into the teat canal (one quarter only) of a healthy, lactating Jersey cow (5). Milk was collected from the infused quarter 22 to 24 h after treatment, diluted threefold in phosphate-buffered saline, and centrifuged at 300× *g* for 10 min. The somatic cell pellet was suspended in Hanks balanced salts solution (HBSS), filtered through 41-μm-pore-size nylon mesh, washed, and suspended again in HBSS to a concentration of 5×10^5 somatic cells per ml (C. W. Donnelly, E. H. Briggs, C. M. Beliveau, and W. L. Beeken, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, P27, p. 279). Viability was determined by trypan blue exclusion (4).

Preparation of bacteria. An 18-h culture of *L. monocytogenes* F5069 that was grown in tryptose phosphate broth (Difco) was washed twice with phosphate-buffered saline and suspended to a concentration of about 5×10^8 *L. monocytogenes* cells per ml in HBSS, as determined by fluorescent-activated cell sorter enumeration (8) and confirmed by viable plate count on tryptose phosphate agar (Difco). The standardized bacterial suspension was diluted 10-fold in HBSS, combined with pooled normal bovine serum (10% final concentration in the assay mixture), and incubated on a rotating shaker at 37°C for 15 min.

Phagocytosis reaction. The phagocytosis reaction was initiated by mixing the standardized suspensions of opsonized *L. monocytogenes* F5069 (100 ml of 5×10^7 *L. monocytogenes* cells per ml) and endotoxin-induced bovine phagocytes (100 ml of 5×10^5 somatic cells per ml) at a ratio of 100:1, respectively, in a Boston round polypropylene flask. Phagocytosis was allowed to proceed by incubating this suspension for 15 min at 37°C on a shaking platform. The reaction was terminated by the addition of 1,900 ml of sterile, cold (4°C), homogenized whole (3.5% fat) milk. This milk was sterilized by autoclaving it at 121°C for 12 min, followed by immediate cooling to 4°C to prevent carmelization. The final 2-liter suspension was used for thermal resistance studies.

Determination of viable intracellular bacteria. A smaller-volume phagocytosis reaction was carried out to evaluate the level of intracellularity obtained after phagocytosis. Essentially, 0.1 ml of the bacterial suspension was added to 0.1 ml of the bovine somatic cell suspension in a Falcon tube (12 by 75 mm; Becton Dickinson Labware, Oxnard, Calif. [Div. Becton Dickinson and Co.]) in triplicate and incubated on a shaking platform for 15 min at 37°C. The assay was stopped by the addition of 2.5 ml of cold (4°C) phosphate-buffered saline. The somatic cells were washed four times with phosphate-buffered saline in a refrigerated centrifuge at 140 × *g* for 5 min. A portion (0.1 ml) of each phagocytosis replicate sample was used to make cytocentrifuge (Shandon Inc., Pittsburg, Pa.) preparations, which were stained with differential stain (Diff-Quick; American Scientific Products, McGaw Park, Ill.). After microscopic analysis, the percentage of infected phagocytes and the number of bacteria per infected cell was counted for 20 phagocytes. The range, mean, standard deviation, and standard error were calculated for these preparations. The distribution of phagocytic cell types was also determined from these smears.

The 2-liter milk suspension containing intracellular *L. monocytogenes* F5069 was also examined by the following procedure to determine the number of viable intracellular *L. monocytogenes* cells. Test milk in 4-ml portions was distributed to four 15-ml conical centrifuge tubes (Corning Glass Works, Corning, N. Y.) labeled A to D. A 1-ml portion was removed from tubes A and B, serially diluted, plated on Trypticase soy agar–0.6% yeast extract (TSAYE), incubated at 37°C for 48 h, and enumerated. The remaining 3 ml of test milk in tubes A and B was disrupted with a sonicator (Ultrasonic; Heat Systems, Plainview, N.Y.) equipped with a microtip (setting of 2.2 for 15 s at 60 W output) to release phagocytized bacteria and was enumerated as described above. The sonicator probe was sterilized with 0.5% NaOCl and rinsed with sterile distilled water between samples. Tubes C and D were centrifuged (CENTRA-7R; International Equipment Co., Div. Damon Corp., Needham Heights, Mass.) at 700 × *g* for 10 min, and 1 ml of decanted supernatant was also diluted and plated as described above. Somatic cell pellets were suspended in HBSS to the original

volume, and again, 1-ml portions from each tube were enumerated. The remaining 3 ml of each suspended pellet in tubes C and D was then sonicated and enumerated. The difference in bacterial count between sonicated and unsonicated pellets was used as an indicator of the number of viable intracellular *L. monocytogenes* cells.

Thermal inactivation studies. *L. monocytogenes* F5069 cells, which were internalized by bovine phagocytes, were thermally inactivated in an SF system (29) at temperatures of 66.1, 68.9, 71.7, and 74.4°C (151, 156, 161, and 166°F, respectively). For any given thermal inactivation experiment, a smaller portion (80 to 100 ml) of the original milk suspension (2,000 ml) was tested in parallel by using the ST method (3, 24) at temperatures of 57.8, 62.8, 66.1, and 68.9°C (136, 145, 151, and 156°F, respectively). Common temperatures for comparing these two procedures were 66.1 and 68.9°C. After heating and before direct plating, recovery enrichment, or both, all milk test suspensions were sonicated. Freely suspended *L. monocytogenes* F5069 milk suspensions were tested similarly, with the inclusion of a sonication step, for comparison with the inactivation kinetics of intracellular bacteria. Heat-stressed *L. monocytogenes* cells were recovered by direct plating on TSAYE in parallel with inoculation into a recovery enrichment broth (TSBYE); both systems were incubated for 7 days at 25°C. Cells grown on recovery enrichment broth were streaked on days 3, 5, and 7 onto modified McBride *Listeria* agar. The isolates from these systems were confirmed as *L. monocytogenes* as described previously (21).

Statistical analysis of the results of the thermal inactivation studies was performed by the procedure described by Bradshaw et al. (3). In addition, the residuals from the linear regression analysis were screened for outliers and influential values (12); the latter were defined as determinations that most affected the stability of the slope of the line and, consequentially, the *D* value.

RESULTS

Uptake of *L. monocytogenes* by endotoxin-induced phagocytes of bovine origin. To generate the large quantity of bovine phagocytes needed to conduct the phagocytic assays and the subsequent thermal inactivation studies, it was necessary to stimulate phagocyte production in milk through the intramammary infusion of *E. coli* endotoxin. Average yields of phagocytic cells collected 24 h after endotoxin infusion were 1.1×10^8/ml. Recovered cell populations were 89% viable and consisted of 88% neutrophils, 8% macrophages, and 4% lymphocytes. Resident bovine somatic cell populations were similar in composition. The uptake of *L. monocytogenes* cells by either resident or endotoxin-induced bovine phagocytes was dependent on the presence of 10% normal bovine serum. The phagocytic index (PI; percentage of cells that took up bacteria) and the capacity (PC; number of intracellular bacteria per infected phagocyte) were similar for resident (55.8%, 28.9 ± 22.9 bacteria per cell) and endotoxin-induced (56.1%; 24.7 ± 18.3 bacteria per cell) bovine phagocytes. The PI for endotoxin-induced neutrophils was 76.5%, and the PC was 19.6 ± 17.7 bacteria per cell. The PI for endotoxin-induced macrophages was 41%, and the PC was 18.2 ± 15.6 bacteria per cell. Bacteriocidal activities by resident and endotoxin-induced phagocytes were comparable, as *L. monocytogenes* cells appeared resistant to phagocytic killing on ingestion (C. W. Donnelly, manuscript in preparation).

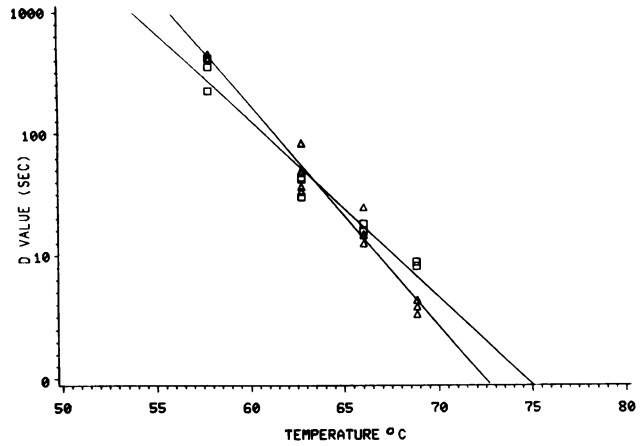

FIG. 1. Thermal death-time curves for intracellular (△; z_D = 5.6°C [10.0°F]) and freely suspended (□; z_D = 7.0°C [12.6°F]) *L. monocytogenes* F5069 determined by the ST heating method.

Determination of viable intracellular bacteria. The number of viable intracellular *L. monocytogenes* in the 2 liters of test milk used in each thermal inactivation experiment was taken to be the difference between the plate counts (CFU) of the sonicated cell pellet and those of the unsonicated cell pellet ($CD_{\text{sonicated}} - CD_{\text{unsonicated}}$). The number of intracellular *L. monocytogenes* per ml of test milk ranged from 5.0×10^5 to 8.5×10^6, with a mean of 2.5×10^6 (Table 1).

Thermal resistance of intracellular *L. monocytogenes*. The thermal inactivation kinetics of intracellular and freely suspended *L. monocytogenes* F5069 in sterile, whole milk was examined by heating these preparations at temperatures ranging from 57.8 to 74.4°C, either in sealed glass tubes immersed in a water bath or in an SF system. Mean *D* values were based on a total count from a sonicated initial sample (Tables 1 to 3). Regression line extrapolation of $D_{71.7°C}$ values from ST studies were 1.4 s for intracellular (z_D = 5.6°C [10.0°F]) and 2.7 s for freely suspended (z_D = 7.0°C [12.6°F]) *L. monocytogenes* (Fig. 1, Table 3). The predicted regression line $D_{71.7°C}$ values from SF studies were 4.1 s for intracellular (z_D = 8.0°C [14.4°F]) and 2.7 s for freely suspended (z_D = 7.3°C [13.1°F]) *L. monocytogenes* (Fig. 2 and Table 3). The measured $D_{71.7°C}$ value from the SF apparatus was 5.0 s (Table 1). These data were interpolated and extrapolated to the minimally expected HTST parameters obtained in a commercial pasteurization setting (Table 3).

Detection and enumeration of heat-stressed *L. monocytogenes*. ST thermal inactivation experiments were conducted at four temperatures (52.2, 57.8, 63.3, and 68.9°C) with several *Listeria* spp. that were freely suspended in sterile milk. Direct plating onto TSAYE was evaluated under two incubation conditions: 37°C for 48 h and 25°C for 168 h (7 days). Under the latter conditions, we were able to detect more organisms at higher heating times (3- to 10-fold) and positive samples which otherwise would have been negative (data not shown). This direct plating procedure was used to recover heat-stressed *L. monocytogenes* F5069 in this study, in parallel with recovery from an enrichment broth (TSBYE) that was inoculated with heated test milk; incubated at 25°C for 7 days; streaked onto modified McBrides agar at days 3, 5, and 7; and confirmed. The number of positive samples recovered by this enrichment method did not exceed the number of positive samples recovered by the direct plating procedure (data not shown).

TABLE 1. Thermal inactivation of intracellular *L. monocytogenes* F5069

Temp (°C)	Mean no. of intracellular bacteria/ml	Mean no. of bacteria/infected cell[a]	% Infected cells[b]	Test system[c]	*D* value (s)[d]	Avg *D* value	CV (%)
57.8	3.4×10^6	18.1	16	ST	453.0	429.8	7.6
	7.6×10^5	20.9	27	ST	406.5		
62.8	1.4×10^6			ST	83.1	55.2	40.0[e]
	1.4×10^6			ST	82.0		
	7.2×10^5			ST	36.3		
	5.0×10^5			ST	33.2		
	2.6×10^6	17.3	50	ST	47.3[e]		
	4.7×10^6	32.9	80	ST	49.3[e]		
66.1	7.6×10^5			ST	24.7	16.7	32.5
	4.8×10^6	26.3	54	ST	12.6		
	1.9×10^6	21.3	33	ST	14.6		
	2.0×10^6	25.3	47	ST	14.9		
	7.6×10^5			SF	17.2	18.4	6.0
	4.8×10^6	26.3	54	SF	19.4		
	1.9×10^6	21.3	33	SF	18.5		
68.9	2.3×10^6	25.2	20	ST	3.9	3.9	12.8
	2.4×10^6	30.8	75	ST	4.4		
	8.5×10^6	29.1	29	ST	3.4		
	2.3×10^6	25.2	20	SF	9.6	9.1	7.8
	2.4×10^6	30.8	75	SF	8.6		
71.7	2.5×10^6			SF	5.4	5.0	8.8
	3.2×10^6	40.8	48	SF	4.9		
	1.4×10^6			SF	5.3		
	1.4×10^6			SF	5.1		
	4.7×10^6	32.9	80	SF	4.3		
74.4	7.6×10^5	20.9	27	SF	1.5	1.5	13.3
	2.0×10^6	25.3	47	SF	1.7		
	2.6×10^6	17.3	50	SF	1.3		

[a] Range for the number of intracellular bacteria was 1 to 50, the mean was 26.1, and the standard deviation was 19.3.
[b] The mean number of phagocytes in each 2-liter test milk sample was 2.5×10^4 cells per ml. The mean percent infectivity was 42.5%.
[c] z_D = 5.6°C (10.0°F) in the ST system, and z_D = 8.0°C (14.4°F) in the SF system.
[d] *D* values were calculated from the total (intracellular + freely suspended) bacterial count.
[e] For the last two experiments reported at 62.8°C (145°F), samples were heated at statistically optimized intervals based on data obtained in the four previous runs at this temperature; therefore, this accounts somewhat for the high coefficient of variation.

DISCUSSION

The thermal resistance properties of *L. monocytogenes* in fluid milk have been studied intensely and debated since the 1983 outbreak in Massachusetts, where epidemiologists indirectly implicated pasteurized whole and 2% fat milk and speculated that the intracellular position of this bacterium, within phagocytic cells shed in milk from cows, could afford heat protection (14). We examined this question of intracellular protection by determining the thermal inactivation kinetics of freely suspended and intracellular *L. monocytogenes* by using two heating techniques: the immersed ST method (3, 24) and the SF method (29). The inactivation rates for *L. monocytogenes* suspended in sterile milk, determined as *D* values at a range of constant temperatures, were essentially equivalent for intracellular and freely suspended test suspensions within each heating system (Tables 1 to 3 and Fig. 1 and 2). A significant difference occurred between *D* values measured at 71.7°C in the SF system, but this characteristic was inconsistent for all other temperatures in both systems; the mean $D_{71.7°C}$ value of 5.0 s had the largest deviation from the linear regression line and the subsequent intracellular z_D value obtained in the SF system. These inactivation data support the conclusion of Bunning et al. (4)

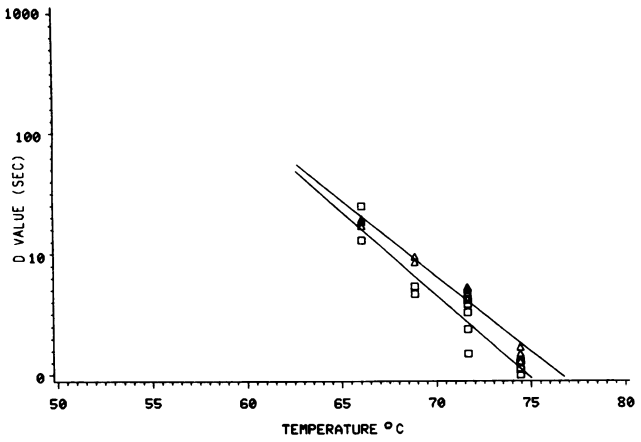

FIG. 2. Thermal death-time curves for intracellular (△; z_D = 8.0°C [14.4°F]) and freely suspended (□; z_D = 7.3°C [13.1°F]) *L. monocytogenes* F5069 determined by the SF method.

TABLE 2. Thermal inactivation of freely suspended *L. monocytogenes* F5069

Temp (°C)	Initial bacterial count	Test system[a]	*D* value (s)	Avg *D* value	CV (%)
57.8	3.8×10^6	ST	351.3	331.0	30.0
	3.0×10^6	ST	418.8		
	4.1×10^6	ST	223.0		
62.8	2.1×10^6	ST	41.9	38.3	19.1
	4.1×10^7	ST	29.9		
	1.6×10^8	ST	43.1		
66.1	2.4×10^6	ST	18.1	16.9	10.5
	1.5×10^6	ST	15.6		
	2.4×10^6	SF	25.0	19.1	44.1
	1.5×10^6	SF	13.1		
68.9	1.8×10^6	ST	8.3	8.6	4.9
	2.0×10^6	ST	8.9		
	1.8×10^6	SF	4.7	5.1	11.1
	2.0×10^6	SF	5.5		
71.7	1.4×10^6	SF	3.3	3.1	36.3
	2.0×10^6	SF	2.4		
	3.9×10^6	SF	3.9		
	2.0×10^6	SF	1.5		
	1.1×10^8	SF	4.2		
74.4	1.8×10^6	SF	1.1	1.1	13.9
	3.4×10^6	SF	1.3		
	2.7×10^6	SF	1.0		

[a] z_D = 7.0°C (12.6°F) in the ST system, and z_D = 7.3°C (13.1°F) in the SF system.

that intracellular *L. monocytogenes* cells are not shielded or protected from heat during pasteurization. Doyle et al. (11) have contended that intracellular position protected *L. monocytogenes* in milk that was obtained from artificially infected cows and that was heated in a commercial-scale pasteurizer at the HTST minimum-range parameter (71.7 to 73.9°C for 16.4 s). The level of intracellularity in the present study, however (42% infectivity at 26 bacteria per cell), was much higher than that in the latter study (11) (<0.1% infectivity at 1.5 to 9.2 bacteria per cell), suggesting that we should have detected a very significant difference in heat resistance between intracellular and freely suspended *L. monocytogenes*. The immunohistochemical approach of Doyle et al. (11) to the enumeration of intracellular *L. monocytogenes*, however, may not have been optimal. Moreover, we used sterile milk as the heating menstruum, whereas Doyle et al. (11) used raw milk. The absence of background flora afforded us the ability to optimally detect heat-stressed cells and to determine the actual number of viable intracellular *L. monocytogenes* cells by a differential plating method on nonselective agar. The findings of Doyle et al. (11), however, may be explained by physiological differences of the organism in vivo, either intracellular or free living, a circumstance that has not been definitively proven for the heat resistance of *L. monocytogenes* but that cannot be overlooked. Therefore, under the defined parameters used in this study, the intracellular position of *L. monocytogenes* did not significantly augment heat resistance.

The question of intracellular heat protection may be a moot point because in both this study and that of Doyle et al.

TABLE 3. Thermal inactivation of *L. monocytogenes* F5069 at minimum and expected HTST parameters[a]

Method	Condition[b]	$D_{71.7°C}$ (s)	15-s process[c]	$D_{72.2°C}$ (s)	19.5-s process[c]
ST	FS	2.7[d]	5.5	2.3[d]	8.6
ST	I	1.4[d]	10.9	1.1[d]	17.7
SF	FS	2.7[e]	5.5	2.3[f]	8.6
SF	I	4.1[e]	3.7	3.5[f]	5.6

[a] Minimum HTST pasteurization standard is 71.7°C for 15 s. Minimum expected parameter obtained in a commercial plate pasteurizer would approach 72.2°C for 19.5 s (W. H. Stroup, personal communication).
[b] FS, Freely suspended bacteria; I, intracellular bacteria.
[c] *D* process of inactivation for the given temperature at the indicated holding times.
[d] Extrapolated value.
[e] Predicted value from the line.
[f] Interpolated value.

(11), thermal inactivation at 71.7°C for 15 s did not provide an adequate *D* process (Tables 1 to 3), suggesting that a large inoculum of *L. monocytogenes* could survive commercial pasteurization whether it was intracellular or freely suspended. These data imply a greater heat resistance for this organism than has been reported previously. In general, the z_D values generated in this study for either the freely suspended or intracellular thermal death time curves (Fig. 1 and 2) are high (5.6 to 8.0°C), exceed typical values for vegetative cells, and approach a range normally found for spores (22, 24). These z_D values, however, are not tremendously higher than some values previously reported for *L. monocytogenes* (3, 4, 9) and other nonsporeformers (22, 31). Mean *D* values with higher coefficients of variation may be responsible for inordinately raising some z_D values. Other mitigating factors that also appear to affect thermal resistance studies for *L. monocytogenes* are discussed below.

Devices that are commonly used to study thermal inactivation of microorganisms differ in principles of function and provide optimal data only for certain holding times. For example, the ST method was used in this study to examine longer heating times, which are required for the lower heating temperatures needed in vat pasteurization. The SF method was used to study shorter heating times, which are required at the higher heating temperatures used in HTST processing (24, 29, 30). The ST method is valid for examining the thermal resistance of a milk pathogen at vat pasteurization time and temperature parameters (10, 14, 23), but extrapolation to the HTST pasteurization range, instead of actual measurement, is scientifically inappropriate. Therefore, direct methods to determine inactivation within the HTST pasteurization range in a laboratory setting, such as in the SF system, were developed (29, 30). Because come-up and come-down times are almost instantaneous (≅1.3 s) in the SF system, the data that were obtained were minimally affected by correction factors.

The difference in the *D* process of inactivation of *L. monocytogenes* at 71.7°C for 15 s versus that at 62.8°C for 30 min, which are the pasteurized milk ordinance parameters for HTST and vat pasteurization, respectively (15), was expected in this study since, in terms of pasteurization guideline history, the two sets of parameters were never deemed synonomous. The HTST minimal standard resulted from considerations of high temperature on flavor and texture, practical experience, and numerous inactivation experiments with several microorganisms, particularly *Mycobacterium tuberculosis* and *Brucella abortus*, in milk at high processing temperatures. The parameters for vat pasteurization resulted from heat studies with *Coxiella burnetii* in raw milk. The pasteurization curve for milk generated with these two parameters has a slope (z = 4.3°C [7.7°F]) that is lower than some values that are known for vegetative cells. When the thermal death time curves for *M. tuberculosis* in milk were updated in 1959, reported z values ranged from 4.8°C (8.6°F) to 5.2°C (9.4°F), which were much lower than a z value of 6.7°C (12.0°F) determined earlier. A margin of safety of about 28.5 min at 61.7°C and approximately 14 s at 71.7°C was calculated based on these new z values (31). The slopes of the thermal death curves for *L. monocytogenes* in this study and others (3, 4, 9) exceed these newer z values for *M. tuberculosis* and approach those calculated originally. These data also indicate that the *D* process of inactivation at 71.7°C for 15 s is inadequate. Extrapolation of the milk pasteurization curve from the point at 62.8°C for 30 min, assuming a z_D value range of 5.6 to 8.0°C for *L. monocytogenes* obtained in this study, would put the time range for 71.7°C at 45 to 140 s. Alternatively, the high-temperature parameter could be raised to provide a *D* process with a large safety margin. The dairy in Massachusetts that processed the milk incriminated in the 1983 outbreak operated its pasteurizer at approximately 76.1°C (170°F) for 19.5 s. These parameters should have inactivated a high level of *L. monocytogenes*, based on the data from this study and that of Doyle et al. (11).

Sterile milk was used as the heating menstruum in this study to analyze specifically the question of intracellular protection, free from a contaminating background. This circumstance also facilitated the recovery of heat-stressed organisms. Raw milk carries a variable background flora which could compete with *L. monocytogenes* during and after heat treatment; it also possesses inhibitory substances (e.g., lysozyme, the lactoperoxidase-thiocyanate-hydrogen peroxide system) which could affect the infectivity levels and the ability of *L. monocytogenes* to recover after heat processing. We are currently examining the thermal inactivation of intracellular *L. monocytogenes* within a homogeneic phagocyte population that is suspended in raw milk to address any potential discrepancy.

Finally, recovery and detection systems play a strong role in determining the heat resistance of *L. monocytogenes*. Several techniques for detecting *Listeria* spp. from a highly contaminated background in food have been published (3, 6, 8, 11, 20, 21, 26); but none have strictly addressed the heat-stressed variable (16), and optimal conditions for recovery remain to be delineated. In this regard, the highest count of *L. monocytogenes* shed by a naturally or artificially infected cow remains 1×10^4 to 2×10^4 bacteria per ml (7, 11, 18). When examined in light of a detailed risk analysis model (D. A. A. Mossel, Proceedings of the World Health Organization Conference on Listeriosis, Agenda Item 8, 1986), most notably including a high bulk tank dilution effect, commercial pasteurization does not appear to be a problem. Compliance with the HTST pasteurization minimum parameter then becomes an academic question. Indeed, a survey of raw milk for the incidence of *L. monocytogenes* in bulk tank samples from California and Ohio indicated that, when found, this organism was present at a level of one bacterium per ml (21). The ultimate optimization of a recovery system would probably not raise this level significantly.

Therefore, intracellular position does not protect *L. monocytogenes* from pasteurization, but the heat resistance of this organism suggests that a large inoculum could survive minimal HTST pasteurization treatment. Vat pasteurization, however, could eliminate such an inoculum. While further studies to examine a safe minimum holding time at 71.7°C for *L. monocytogenes* appear to be warranted, in commercial practice it is unlikely that levels of *L. monocytogenes* would approach those necessary to provide a sufficiently high level to enable survival of bacteria during HTST processing. The risk of postpasteurization contamination of processed dairy products by *L. monocytogenes* poses a far more serious threat than the risk of survival during thermal processing. This consequence has been documented by the Dairy Safety Initiatives Program of the Food and Drug Administration, in which to date in all instances of *Listeria* contamination involving processed dairy products, contamination has resulted from an environmental source that is located within a processing plant and not from deficiencies in the actual pasteurization process (J. Kozak, personal communication). Prevention of postprocess contamination in dairy products deserves renewed attention and should be of higher priority than concerns of inadequacies in the present minimum HTST pasteurization standards.

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