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Comparative Recovery of Uninjured and Heat-Injured *Listeria monocytogenes* Cells from Bovine Milk

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The standard selective enrichment protocols of the Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) were compared with an experimental nonselective broth enrichment (NSB) protocol and variations of the standard cold-enrichment (CE) protocol for the recovery of heat-injured *Listeria monocytogenes*. Bacterial cells (10^7/ml) were suspended in sterile milk and heated at 71.7°C in a slug-flow heat exchanger for holding times ranging from 1 to 30 s. Surviving cells were determined (50% endpoint) by the given protocols, and the following D values were obtained: NSB, $D = 2.0 \pm 0.5$ s; FDA, $D = 1.4 \pm 0.3$ s; USDA, $D = 0.6 \pm 0.2$ s; CE, $D \leq 1.2$ s. The respective direct-plating media used in these enrichments were also analyzed for recovery, and the following D values were calculated from the enumeration of surviving cells: NSB, $D = 2.7 \pm 0.8$ s; FDA, $D = 1.3 \pm 0.4$ s; USDA, $D = 0.7 \pm 0.2$ s. The low levels of heat-injured *L. monocytogenes* cells which were detected at inactivation endpoints on the optimal nonselective media (25°C for 7 days) failed to recover and multiply during experimental CEs (4°C for 28 days). Initial inactivation experiments in which raw whole milk was used as the heating menstruum gave much lower recoveries with all protocols. The detectable limits for uninjured cells that were suspended in raw milk were similar (0.35 to 3.2 cells per ml) for the standard CE, FDA, and USDA protocols. Recovery by the NSB procedure (68 cells per ml) was compromised by background flora. The above data suggest that any cells surviving high-temperature, short-time pasteurization will be injured and unable to multiply either during cold storage of milk or in the FDA or USDA systems. Thus, *L. monocytogenes* cells recovered in finished pasteurized milk products by these detection methods probably represent uninjured environmental contaminants.

The efficacy of high-temperature, short-time (HTST) pasteurization in eliminating *Listeria monocytogenes* cells from contaminated bovine milk has been questioned since a 1983 milk-borne outbreak of listeriosis was epidemiologically linked to the consumption of whole and 2% (fat) pasteurized milk (18). A critical problem with assessing the in vitro thermal resistance of *L. monocytogenes* is the detection of heat-injured cells, potentially at low levels, and their differentiation from the background flora of pasteurized milk (7, 13, 16, 21, 37). Several techniques for recovering *Listeria* spp. from food in the presence of background microflora have been published (1, 8–10, 13, 15, 16, 21, 22, 24, 26–28, 30, 33, 34, 36), but none have strictly addressed the heat injury variable (5, 7, 23, 37), and optimal conditions for recovery remain to be delineated. Cold enrichment (CE) (4°C for 7 to 28 days) of heated raw milk or of a portion of this milk which was inoculated into various selective and nonselective enrichment broths has been used to detect the survival of *L. monocytogenes* cells subjected to HTST processes (13, 16). However, these recovery methods are tedious and time-consuming and produce erratic results (13), and the pasteurization survival data have not been independently repeated (J. M. Farber, G. W. Sanders, D. B. Emmons, and R. C. McKellar, J. Food Prot. **50**:893, 1987; J. L. Lovett, J. G. Bradshaw, D. W. Francis, R. G. Crawford, C. W. Donnelly, G. K. Murthy, and I. V. Wesley, J. Food Prot. **51**:822, 1988).

The limitations of detectability described above have necessitated the determination of the in vitro thermal resistance of *L. monocytogenes* in sterile whole milk (4, 7, 11, 12). Qualifiers in these experiments included the following: (i) initial large numbers of bacteria (10^6 to 10^7 cells per ml) were used to accurately assess the kinetics of death, and (ii) the small numbers of bacteria that survived treatment at 71.7°C for 15 s in some inactivations were detected only when using nonselective media that were incubated at 25°C for 7 days (7). Since some researchers (13, 16) have used CE and selective media that contain inhibitors in determining the survival of *L. monocytogenes* in raw milk after minimal HTST pasteurization (71.7°C for 15 s) (19), their data may not reflect the true resistance, because of the potential for nonrecovery of heat-injured cells (5, 20, 37). In this study, we compared the sensitivity of several standard and experimental selective and nonselective enrichment protocols, including variations of the standard CE protocol, for the ability to recover heat-injured cells that were obtained from typical laboratory HTST thermal inactivation experiments. We also examined the detectable limits for uninjured *L. monocytogenes* cells against a raw-milk background by using these methods.

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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 dairy processing plant in Massachusetts that was incriminated in the listeriosis outbreak mentioned above

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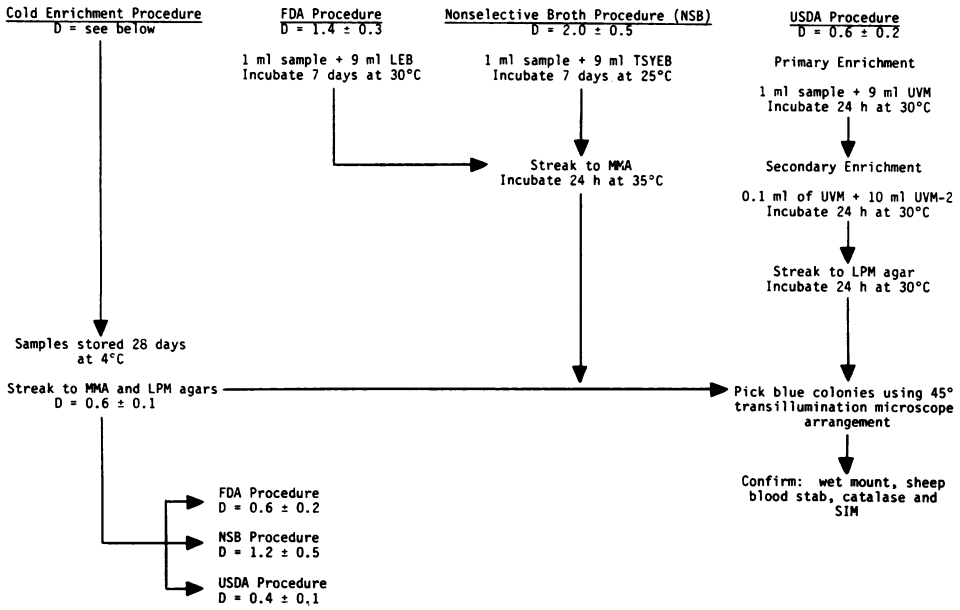

FIG. 1. Comparative studies by standard FDA, USDA, and experimental NSB and CE procedures for recovery of heat-injured *L. monocytogenes* cells. *D* values are expressed in seconds. UVM-2 broth contains twice the concentration of acriflavine (25 mg/liter) as UVM broth (30).

(18). Maintenance and characterization of this strain were as previously described (7).

Detection systems for *L. monocytogenes*. The standard Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) enrichment protocols have been previously described in detail (9, 24, 26–28, 30). Briefly, the FDA procedure used *Listeria* enrichment broth (LEB) and modified McBride agar (MMA) (Difco Laboratories, Detroit, Mich.), whereas the USDA procedure used Donnelly enrichment broth (UVM) and *Listeria* plating medium (LPM) (GIBCO Diagnostics, Madison, Wis.). Salt tolerance is an established criterion for assessing the recovery of heat-injured *L. monocytogenes* cells (2, 5, 37). The concentrations of NaCl in LEB, MMA, UVM, and LPM were 5, 5, 20, and 5 g/liter, respectively. Precise details of the various incubations and transfers for the standard FDA and USDA protocols are given in Fig. 1.

The nonselective media used in the nonselective broth enrichment (NSB) protocol were Trypticase Soy (BBL Microbiology Systems, Cockeysville, Md.) plus 0.6% yeast extract (Difco) agar (TSYEA) and broth (TSYEB). Details of the NSB protocol are also given in Fig. 1.

In this study, the standard CE protocol was defined as a 10-fold dilution of test milk in tryptose broth (Difco) followed by incubation at 4°C for 28 days. After incubation, a loopful of this broth was streaked onto MMA.

The confirmation tests for isolated colonies on all plating media were previously described (7) and are indicated in Fig. 1.

Detectable concentration studies of uninjured *L. monocytogenes* cells suspended in raw bovine milk. The detectability level of uninjured *L. monocytogenes* cells in raw milk was determined by an endpoint dilution technique (17, 25, 32). A standardized raw-milk suspension containing about 10^5 CFU of *L. monocytogenes* F5069 per ml (mean aerobic plate count, 2.2×10^3 bacterial per ml) was 10-fold serially diluted to 10^{-2} CFU/ml with raw milk. Ten 1-ml fractions from each dilution were inoculated into the standard FDA, USDA, and CE media. Variations of the standard CE protocol used in these experiments are described in Table 1 and the Results section. The recovery on each medium was determined by estimating the concentration of *L. monocytogenes* cells obtained when 50% of the test aliquots were positive. The

TABLE 1. Fifty percent detectable concentrations of uninjured *L. monocytogenes* cells in raw bovine milk for 11 experimental recovery conditions

Condition no.	Exptl conditions	Detectable limit (organisms/ml)
1	USDA enrichment[a]	0.38[b]
2	FDA enrichment[a]	0.38[b]
3	NSB enrichment[a]	68[b]
4	CE, 7 days at 4°C undiluted; streak MMA, incubate 24 h at 35°C	320[c]
5	CE, 7 days at 4°C in tryptose broth[d]; streak MMA, incubate 24 h at 35°C	32[c]
6	CE, 28 days at 4°C undiluted; streak MMA, incubate 24 h at 35°C	32[c]
7	CE, 28 days at 4°C in tryptose broth[d]; streak MMA, incubate 24 h at 35°C	3.2[c]
8	CE, 28 days at 4°C undiluted; USDA enrichment	2.0[b]
9	CE, 28 days at 4°C in tryptose broth[d]; USDA enrichment	0.02[b]
10	CE, 28 days at 4°C undiluted; FDA enrichment	2.0[b]
11	CE, 28 days at 4°C in tryptose broth[d]; FDA enrichment	0.02[b]

[a] Detailed schema for the USDA, FDA, and NSB protocols are given in Fig. 1.
[b] Estimated from the Spearman-Karber 50% endpoint procedure (17, 25).
[c] Average of two concentrations at endpoints.
[d] One-milliliter aliquot of test milk diluted in 9 ml of tryptose broth (Difco).

lower the estimate of detection, the more efficient the recovery by a given protocol (17, 25, 32).

Thermal-inactivation studies. *L. monocytogenes* F5069 cells, which were either free living or internalized by bovine phagocytes (7), were thermally inactivated in a slug-flow heat exchanger (38) at the minimal HTST pasteurization standard temperature of 71.7°C. The heating menstruum was sterile whole milk (7). In some initial experiments, raw whole milk was used as the heating menstruum. Test suspensions were heated for holding times of 1, 2, 4, 8, 10, 15, 20, and 30 s and were sonically dispersed, as previously described (7). The pasteurized-milk ordinance requires a minimum holding time of 15 s at 71.7°C (19).

Statistical analysis of the inactivation results from direct-plating experiments was performed by the procedure described by Bradshaw et al. (3); i.e., regression analysis was used to estimate D values from plate count experiments. Quantal D values from enrichment detection systems were estimated by using the 50% endpoint statistical procedure (17, 25, 32). In addition, the residuals from the linear regression analysis were screened for outliers and influential values (14); the latter were defined as determinations that most strongly affected the stability of the slope of the line and, consequently, the D value.

Comparative recovery of heat-injured *L. monocytogenes*. Replicate aliquots (4- to 8-1 ml fractions) from each inactivation holding time were inoculated into the NSB, FDA, and USDA media (Fig. 1). Duplicate aliquots (1 ml) from each holding time were also enumerated directly onto the respective plating medium (TSYEA, MMA, and LPM) for each enrichment procedure.

The NSB protocol (7 days of enrichment at 25°C) was previously shown to give greater recovery than either a 2- to 7-day enrichment at 37°C or a standard CE process for *L. monocytogenes* cells that were suspended in sterile milk and heated in our inactivation system (7; V. K. Bunning and R. G. Crawford, unpublished observations). For this reason, heated test milk in this study was not subjected to the standard CE. Instead, the original undiluted test milk from each holding time was subjected to CE and then streaked on to MMA and LPM. This cold-enriched test milk was further analyzed by the FDA, USDA, and NSB protocols (Fig. 1). Since several investigators have shown that uninjured *L. monocytogenes* will grow in sterile milk at temperatures ranging from 4 to 37°C (11, 34), our approach allowed the assessment of recovery and growth of heat-injured *L. monocytogenes* cells during cold storage.

Detection method comparisons for the recovery of heated cells in the inactivation studies are presented as D values (Fig. 1). Higher D values indicate greater recovery. Similar recovery studies were performed with a standardized intracellular *L. monocytogenes* inoculum, which was prepared as previously described (7).

Additionally, initial comparative recovery experiments were performed in our inactivation system with standardized intracellular and freely suspended *L. monocytogenes* inocula (1×10^7/ml) that were suspended in raw whole milk. Experimentation with both raw and sterile milk as the heating menstruum allowed the examination of the effects of selective media and of competition of pasteurized-milk background flora on the recovery of heat-injured *L. monocytogenes*.

RESULTS

Comparative recovery of uninjured *L. monocytogenes* cells from raw bovine milk. The detectable limits for uninjured *L. monocytogenes* cells that were suspended in raw milk (mean aerobic plate count, 2.2×10^3/ml) were similar (0.38 to 3.2 cells per ml) for the standard CE, FDA, and USDA protocols (Table 1, conditions 1, 2, and 7). Recovery by the NSB procedure (68 cells per ml), however, was compromised by competition and overgrowth from background flora (Table 1, condition 3). CE of undiluted milk for 28 days followed by streaking on MMA was 10-fold less sensitive (32 cells per ml) than standard CE (Table 1, conditions 6 and 7). Seven-day CE of undiluted milk and milk diluted in tryptose broth were 10-fold less sensitive in recovery (320 and 32 cells/ml, respectively) than the respective 28-day CEs (Table 1, conditions 4 to 7). Maximal recovery of uninjured cells (0.02 cells per ml) was found following standard CE with subsequent processing by either the FDA or USDA protocol (Table 1, conditions 9 and 11). CE of undiluted milk for the same time (28 days) followed by processing by either the FDA or USDA protocol was 100-fold less sensitive (2.0 cells per ml) than the latter approach (Table 1, conditions 8 and 10).

Comparative recovery of heat-injured *L. monocytogenes* cells from sterile bovine milk. A standardized suspension of *L. monocytogenes* cells (10^7/ml) in sterile milk was heated in a slug-flow heat exchanger for the given holding times. Aliquots (1 ml) from each holding time were inoculated into the NSB, FDA, and USDA media (Fig. 1). Recovery by the NSB procedure ($D_{71.7°C} = 2.0 \pm 0.5$ s) was significantly greater than by the FDA ($D_{71.7°C} = 1.4 \pm 0.3$ s) and USDA ($D_{71.7°C} = 0.6 \pm 0.2$ s) protocols (Fig. 1). Aliquots (1 ml) from each holding time were also enumerated directly onto the respective plating medium for each enrichment procedure. Initial counts (holding time = 0) were statistically equal on all three direct-plating media. Recovery on TSYEA ($D_{71.7°C} = 2.7 \pm 0.8$ s) was significantly greater than on either MMA ($D_{71.7°C} = 1.3 \pm 0.4$ s) or LPM ($D_{71.7°C} = 0.7 \pm 0.2$ s). By experimental design, recovery by either the NSB protocol or direct plating on TSYEA provided the upper limit of recovery for control purposes (5; Bunning and Crawford, unpublished). The holding time between all positive and all negative recoveries (endpoint) was ≤15 s with these media in the set of comparative-recovery experiments given in this report. Survivors at the 15-s holding time were exceptional, with direct counts of ≤5 CFU/ml.

CE recovery from undiluted milk samples that were incubated for 28 days at 4°C ($D_{71.7°C} = 0.6 \pm 0.1$ s) was not enhanced relative to recovery by the NSB, FDA, and USDA protocols (Fig. 1). This result was still evident even after these cold-enriched samples were subsequently analyzed by the NSB, FDA, and USDA enrichment protocols, although one experimental run with the NSB procedure did result in significantly higher recovery, thereby accounting for the higher D value and variation ($D_{71.7°C} = 1.2 \pm 0.5$ s) (Fig. 1).

Initial inactivation studies with an intracellular inoculum produced a similar trend in recovery results (data not shown), supporting earlier conclusions of a lack of significant intracellular protection from heat (6, 7).

Initial inactivation studies in which raw whole milk was used as the heating menstruum showed that recovery by the standard CE, FDA, and USDA protocols and the NSB protocol (data not shown) was significantly lower than the upper limits established with these systems when sterile milk was used in this study. Indeed, it was often difficult to detect *L. monocytogenes* cells by these recovery systems at the earliest holding times when raw milk was the heating menstruum. Corresponding holding times from sterile-milk ex-

periments gave a range of 10^2 to 10^4 cells per ml when aliquots were directly plated on TSYEA.

DISCUSSION

Two theoretical possibilities exist when *L. monocytogenes* cells are detected in an HTST-pasteurized milk product: either the prepasteurized raw milk was contaminated with a high enough level of the pathogen that some organisms survived the pasteurization process, or the postpasteurized product was contaminated from the surrounding environment. The microbiological protocols that have been used both to detect *Listeria* cells in adulterated milk products and to determine the thermal resistance properties of the organism may provide insight into this pasteurization survival–environmental contamination dilemma, because of the potential different abilities of these systems to detect uninjured and heat-injured cells (5, 37).

Although *L. monocytogenes* is considered halotolerant (35), several investigators (2, 5, 37) have observed that heat-injured cells lose tolerance to high salt concentrations. In regard to the USDA and FDA protocols, it is clear that UVM contains a higher salt concentration than LEB (2 and 0.5%, respectively). Both of these protocols provided comparable and excellent recovery (0.38 organisms per ml) of uninjured *L. monocytogenes* cells against a raw-milk background (Table 1). The USDA method, however, was consistently less efficient than the FDA method in detecting heat-injured *L. monocytogenes* cells in the thermal inactivation experiments in which sterile milk was used as the heating menstruum (Fig. 1). In addition, direct plating of heated test milk on MMA (FDA method) or LPM (USDA method), followed by extensive incubation for up to 7 days, found the latter medium to be quite inferior in recovery efficiency, suggesting that the inhibitors in LPM agar were also detrimental to heat-injured *L. monocytogenes* cells. Extended incubation of LEB for 7 days, with eventual streaking onto MMA for confirmation, often gave recoveries that approached those obtained by NSB protocol. This result implies that LEB, which is essentially TSYEB plus selective agents, was less inhibitory to injured *Listeria* cells, allowing for increased recovery over the longer incubation period. A similar trend in recovery of injured cells has been found by others with these systems (5, 37). The dual components of the FDA detection scheme, then, appear less inhibitory to heat-injured *L. monocytogenes* cells in milk than do their respective counterparts in the USDA scheme.

The significance of this detection system study obviously lies in the comparison of selective and nonselective approaches. The use of sterile milk in the thermal-inactivation experiments allowed us to determine the recovery of heat-injured cells on selective media relative to what was simultaneously known to be the superior detection system (7; Bunning and Crawford, unpublished), i.e., the control or nonselective media. Previous studies failed to examine recovery relative to nonselective media, because the scope of the experiments involved only raw food products that have interfering background flora (13, 16, 21). The use of raw milk as the heating menstruum in our inactivation system greatly compromised the detection of *L. monocytogenes* in all protocols, relative to experiments in which sterile milk was used. This result was also evident even at the earliest holding times. Overall, these observations reveal that heat-injured *Listeria* cells are inhibited not only by selective-media factors and conditions (Fig. 1) but also by competition from thermoduric milk flora.

Coculture and conditioned-medium experiments with known milk-borne organisms have shown a small but significant enhancement of *L. monocytogenes* growth by *Pseudomonas* spp. (29), whereas *Lactobacillus* spp. (M. A. Daeschel, L. J. Harris, M. E. Stiles, and T. R. Klaenhammer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, P43, p. 281) and *Streptococcus lactis* (J. M. Wenzel and E. H. Marth, Abstr. Annu. Meet. Am. Dairy Sci. Assoc., D74, J. Dairy Sci. **71**:86, 1988) strongly inhibit the growth of *L. monocytogenes*. These competition studies were done with uninjured *L. monocytogenes* cultures. The variability of raw-milk background flora, then, may interfere with the growth and recovery of *L. monocytogenes*.

Key risk analysis factors (7, 31) in the pasteurization survival issue include (i) the highest contamination levels of *L. monocytogenes* found either in raw-milk bulk tanks (1.3 to 4.2% incidence at <1 organism per ml) (15, 28) or in naturally or artificially infected milk from a single cow (10^4 organisms per ml) (7, 13), and (ii) the thermal inactivation kinetics for various strains (3, 4, 6, 7, 11–13), which were determined by valid heating techniques (12). In view of these facts, it is unlikely that organisms will escape HTST pasteurization (7, 39). The data from this report suggest that any cells surviving HTST minimal processing (detected only by NSB or on TSYEA after a 71.7°C exposure to an inoculum of 1×10^7 cells per ml for ≤15 s in the absence of competitors) will be injured and unable to multiply either during cold storage of milk or in the FDA or USDA systems. Uninjured cells can clearly grow in sterile bovine milk at 4°C (11, 34). The public health hazard, then, is further minimalized not only by competition from background flora but also by the inability of the low levels of cells exposed to the longer heating times to recover during cold storage.

Uninjured *L. monocytogenes* cells are detected at relatively low levels (0.38 to 3.2 organisms per ml) by the standard CE, FDA, and USDA protocols against a typical raw-milk background level (Table 1, conditions 1, 2, and 7). CE of undiluted milk for 28 days provided adequate recovery (32 organisms per ml), but subsequent processing by either the FDA or USDA protocol increased this recovery (2.0 organisms per ml) (Table 1, conditions 6, 8, and 10). The standard FDA, USDA, and experimental CE protocols were highly inhibitory to the recovery of heat-injured *Listeria* cells, relative to the NSB procedure, in our inactivation experiments with sterile milk (Fig. 1). Recovery from inactivations in which raw whole milk was used as the heating menstruum was much lower than these upper-limit values. It can be reasoned from these observations, then, that *L. monocytogenes* cells detected in finished pasteurized milk products by the FDA, USDA, or CE protocol probably represent uninjured environmental contaminants. Indeed, the high risk of postpasteurization contamination has been documented by the FDAs Dairy Safety Initiatives Program (7; J. J. Kozak, Dairy Food Sanit. **6**:184–185, 1986) and is clearly more compatible with accurate risk analysis models (7, 31; C. W. Donnelly, Dairy Food Sanit. **8**:297–299, 1988).

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