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High-Temperature Short-Time Pasteurization Inactivates *Listeria monocytogenes*

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ABSTRACT

Full scare commercial pasteurization equipment operated at 72-73°C with a holding time of 15-16 s was used to determine the ability of commercial thermal processing to inactivate *Listeria monocytogenes* strain Scott A. Three methods of providing *L. monocytogenes* concentration in raw milk were employed: freely suspended (extra-cellular), inside bovine phagocytes (*in vitro* procedure), and inside bovine phagocytes in experimentally infected cows (*in vivo*). Three enrichment methods were used to assay for *L. monocytogenes* after pasteurization: cold enrichment (4°C, 28 d), selective enrichment of Lovett et al. (FDA procedure) (*17*), and the USDA-FSIS two stage enrichment procedure. In addition, a 1-L sample taken just before the vacuum breaker was incubated undiluted in the original sample container (4°C, 4 weeks). None of the four assay methods could detect *Listeria* in the pasteurized milk.

Listeria monocytogenes has been the causative agent of five major foodborne epidemics in which dairy products, including milk and cheese, vegetables and coleslaw have been incriminated as the contaminated product (*15*).

The first outbreak in North America which incriminated dairy products occurred in Massachusetts during 1983. The outbreak involved a total of 49 cases, including seven perinatal cases and 42 immunosuppressed individuals, over a 3-month interval. Retrospective epidemiological analysis revealed that the patients who had consumed pasteurized whole or 2% milk from a single supermarket chain were more at risk than case controls matched for residence and predisposing conditions. A survey of dairy farms supplying the dairy plant in question revealed cases of bovine listeriosis in the herd. Although *L. monocytogenes* was never cultured from the pasteurized milk product, it was speculated that *L. monocytogenes* was shielded during commercial pasteurization (*11*) by their intracellular location within polymoromonullar leukocytes. However, Bunning et al. (*4*) determined that the intercellular location within bovine phagocytes did not significantly increase heat resistance.

Recently, two research groups have reported viable *L. monocytogenes* from pasteurized milk processed in plate type pasteurizers. Fernandez-Garayzabel et al. (*10*) used a pilot plant pasteurizer with a capacity of 100 L/h to heat raw milk inoculated with *L. monocytogenes* at concentrations of 3×10^6, 1×10^7, and 2×10^8 CFU/ml at temperatures of 69, 72, and 73°C for 15 s. The isolation procedures used were those of Dominguez-Rodriquez et al. (*6*), a complicated combination of nonselective and selective enrichments at 22 and 4°C. Heating at 69°C for 15 s inactivated 3×10^6 CFU/ml in three of five trials. *L. monocytogenes* was inactivated at 72°C for 15 s in one of three trials when the concentration was 1×10^7 CFU/ml and in one of four trials when the concentration was 2×10^8 CFU/ml. At all concentrations, 73°C for 15 s was effective in inactivating *L. monocytogenes (10).*

The successful operation of a pasteurization process is dependent upon temperature, time, and concentration of microorganism targeted for destruction. The failure of any pasteurizer operated at 69 to 72°C with a holding time of 15 s is predictable when the concentration of *Listeria* is 3×10^6 CFU/ml raw milk or greater (1×10^7 and 2×10^8 CFU/ml). The highest $D_{71.7°C}$ value reported for *L. monocytogenes* in raw milk is 2.3 s (unpublished). This means that 6.5 logs of *L. monocytogenes* is the operating capacity of the pasteurization process. The findings of Fernandez-Garayzabel et al. (*10*) do not represent a failure of pasteurization under simulated natural conditions but an experiment in which overload of the pasteurizer is predictable.

Based on their own data and that of others, Northolt et al. (*19*) assumed the concentration of *L. monocytogenes* in mixed milk at the processing plant to be one *L. monocytogenes* cell per 2 ml of raw milk. This is the same order of magnitude reported by Lovett et al. (*17*) and Farber et al. (*9*). With abusive temperature treatment for one week, the concentration of *L. monocytogenes* presented to the pasteurizer may be 10^3 - 10^4 CFU/ml (*19*). Even with this worst case scenario, the properly operated pasteurizer is theoretically capable of inactivating this concentration of *L. monocytogenes* in raw milk.

The research of Doyle et al. (*8*) subjected experimentally infected cows' milk to commercial scale pasteurization. The concentration of 10^2 - 10^4 CFU/ml *L. monocytogenes* Scott A was induced by inoculation of 10^5 to 10^7 *L. monocytogenes* into the teat canal of lactating cows at weekly intervals. The heating process was 71.7 - 73.9°C for 16.4 s. In six of nine trials at that time and temperature, *L. monocytogenes* was isolated from the pasteurized milk.

Doyle et al. (*8*) employed three enrichment procedures to isolate *L. monocytogenes* from milk: cold enrichment, the enrichment techniques of Lovett et al. (*17*), and the enrichment techniques of Doyle and Shoeni (*7*). All three methods detected some positive samples, but not all positive samples were detected by all three methods.

The object of this study was to determine if *L. monocytogenes* in raw milk under natural conditions could survive high-temperature short-time pasteurization when the equipment and procedures complied with the Grade A Pasteurized Milk Ordinance (*12*).

MATERIALS AND METHODS

The pasteurization system, equipment calibration, and operation were in compliance with the pasteurized milk ordinance (*12,21*).

The bacterial inoculation

The bacterial strain used throughout this study was *L. monocytogenes* strain Scott A, serotype 4b, isolated from a patient involved in the 1983 foodborne outbreak of listeriosis in Massachusetts in which pasteurized milk was suggested as the contaminating source (*11*). The culture was obtained from the Centers for Disease Control, Atlanta, GA. On receipt in our laboratory, the culture was grown at 37°C for 24 h in trypticase soy agar-yeast extract broth (TSBYE) and stored at 20°C in a 40% glycerol solution.

To provide cultures to be used in experimentation, *L. monocytogenes* was grown in TSBYE for 18 h at 37°C and maintained at 40°C with monthly transfers.

Analysis for L. monocytogenes *freely suspended in raw whole milk*

L. monocytogenes was grown in TSBYE for 24 h at 37°C before it was used to inoculate 150 gal of raw milk to produce a concentration of 10^5 CFU/ml. A sample of the milk was analyzed before inoculation for standard plate count (SPC) (1). The concentration of *L. monocytogenes* in the inoculated milk was determined by direct plating on modified McBride agar (MMA) (*17*).

Analysis for L. monocytogenes *internalized in bovine phagocytes in vitro*

The Scott A strain of *L. monocytogenes* (100 ml of 5 x 10^7 CFU/ml) was incorporated *in vitro* with LPS-induced bovine phagocytes (100 ml of 5 x 10^5 somatic cells/ml) at ratio of 100:1, as described (*4*). The reaction was stopped by the addition of 1800 ml of cold (4°C) sterile milk to yield a final volume of 2 L.

The percentage of bovine cells infected, bacteria incorporated per bovine cell, and distribution of cell types were determined for the phagocytosis reaction as follows: A 0.1 ml bacterial suspension (5 x 10^7 CFU/ml) was incubated (37°C, 15 min) with 0.1 ml of endotoxin-induced somatic cells (5 x 10^5 cells/ml). The reaction was stopped by the addition of 2.5 ml PBS (4°C). The phagocytic cells were washed four times in phosphate-buffered saline (PBS) and centrifuged (140 x g for 5 min). A cytocentrifuge preparation (0.1 ml) was stained with a differential stain (Diff-Quick, American Scientific Products, McGaw Park, IL). The percentage of infected phagocytes and the number of bacteria per infected cell (mean and standard deviation) were determined for 20 phagocytes. The distribution of phagocytic cell types was also evaluated from these smears (*4*).

Just before the aforementioned 2-L suspension of phagocytic cells was added to the raw milk for pasteurization, the number of *L. monocytogenes* incorporated within bovine neutrophils was determined as follows: 4-ml were distributed to each of four 15-ml conical centrifuge tubes. A 1-ml aliquot from each tube was serially diluted, plated on trypticase soy agar-yeast extract (TSAYE), incubated at 37°C for 48 h, and enumerated. The tubes containing the remaining 3 ml were randomly assigned to two groups. Two tubes were sonicated to disrupt the phagoyctes and release the bacteria and two tubes were not. Both sonicated and unsonicated samples were centrifuged, and the pellets were resuspended to the original (3 ml) volume in Hanks' balanced salt solution (HBSS), serially diluted, and plated in TSAYE. The difference in the bacterial count between the sonicated and unsonicated pellets was used as an indicator of the number of viable *L. monocytogenes* cells incorporated within bovine phagocytic cells (*4*).

Analysis for L. monocytogenes *internalized in bovine phagocytes in vivo*

Analysis for *L. monocytogenes* internalized in bovine phagocytes in raw milk from a herd of cows with an experimental infection, with shedding of *L. monocytogenes* into milk, was used for the final 11 trials. The establishment of this herd has been described (*3,13*). This was milk characterized by determining SPC, somatic cell count, *L. monocytogenes* concentration in cells/ml, and percentage of bacteria that was intracellular at time of pasteurization.

Bacterial assays

Three assay procedures were employed to isolate *L. monocytogenes* from milk: cold enrichment, the procedure of Lovett et al. (*17*), and the U.S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) procedure (*16,18*).

Cold enrichment

A 25-ml volume of milk was added to 225 ml tryptose broth and incubated at 4°C for 4 weeks. At 1 week and 4 weeks, the enrichment culture was streaked onto MMA and the plates were incubated at 35°C for 48 h before typical colonies were verified.

FDA procedure

According to the procedure of Lovett et al. (*17*), a 25-ml amount of milk was added to 225 ml enrichment broth (EB) composed of trypticase soy broth (BBL, Cockeysville, MD), 30 g/L; yeast extract (BBL), 6 g/l: acriflavin HCl (Sigma Chemical Co., St. Louis, MO), 15 mg/L; nalidixic acid (sodium salt) (Sigma), 40 mg/L; and cycloheximide (Sigma), 50 mg/L. The culture was incubated at 30°C for 7 d. At 1 and 7 d, the EB culture was streaked onto MMA for incubation at 35°C for 48 h before colonies were picked for verification. MMA contains phenylethanol agar (Difco Laboratories, Detroit, MI), 35.5 g/L; glycine anhydride (Sigma), 10 g/L; lithium chloride (Sigma), 0.5 g/L, and cycloheximide (Sigma), 200 mg/L.

The USDA-FSIS procedure

A 25-ml amount of milk was added to 225 ml primary enrichment broth composed of proteose peptone, 5 g/L; tryptone, 5 g/L; Lab Lemco powder (Oxide), 5 g/L; yeast extract, 5 g/L; NaCl, 20 g/L; KH_2PO4, 1.25 g/L; Na_2HPO4, 12 g/L; esculin, 1 g/L; nalidixic acid, 20 mg/L; acriflavin Hcl, 12 mg/L. The mixture was incubated at 30°C for 24 h. One-tenth ml of primary enrichment broth was pipetted into 10 ml of secondary enrichment broth (same as primary enrichment except that the concentration of acriflavin was 25 mg/L) and incubated at 30°C for 24 h. Following enrichment, the culture was streaked to LPM agar (Gibco, Grand Island, NY) supplemented with 2 ml/L of 1% filter sterilized moxalactam (Eli Lilly & Co., Indianapolis, IN) and incubated at 30°C for 24 h. Typical colonies were picked for confirmation.

Cold enrichment of whole raw milk

A 1-L sample was taken just before the vacuum breaker as a continuous bleed sample and was incubated undiluted in the original sample container at 4°C for 4 weeks. At the end of cold enrichment, the whole milk culture was sampled and analyzed by each of the three previously described enrichment methods.

Preliminary confirmation

Using 45°C incident transmitted light, the MMA and LPM plates were examined for blue colonies. Typical colonies were used in wet mount to determine motility and morphology. Additionally, a gram strain, a sheep blood stab for the expression of hemolysin, sulfide-indole-motility (SIM) for motility, and test for catalase were done.

Analysis of environmental sample

Sterile cotton swabs were used to take samples from the surface of the equipment, walls, floors, and from drains around the pasteurization equipment. The cotton swabs were placed in the EB of Lovett et al. (*17*), incubated 7 d at 30°C, and streaked onto MMA at 1 and 7 d. The MMA plates and the MMA culture strips from the air samples were incubated at 35°C for 48 h before being examined for presumptive *Listeria* colonies. Presumptive colonies were picked for confirmation as described for milk samples.

Water from the pilot plant supply and from the pasteurizer's heat exchanger were analyzed as described for milk.

The two-phase slug-flow heat exchanger was used to determine the effectiveness of pasteurization at the Pasteurized Milk Ordinance (PMO) minimum of 71.7°C and 15 s (*20*).

RESULTS AND DISCUSSION

The general protocol of the experimental work presented three phases. Phase I, composed of five pasteurization trials, examined the ability of pasteurization to inactivate freely suspended *L. monocytogenes* Scott A in raw milk at a concentration of 10^5 CFU/ml. This is one log greater than our worst case scenario of 10^4 CFU/ml following gross mishandling and within the theoretical capacity of the system using a $D_{71.7°C}$ value of 2.3 s. No *L. monocytogenes* cells were detected in any sample of pasteurized milk. The standard plate count (SPC) and the freely suspended *L. monocytogenes* concentration in the raw milk for each of five trials are given in Table 1. The background flora of raw milk never exceeded 10^4 CFU/ml, making enumeration of the *L. monocytogenes* in raw milk by direct plating on MMA possible.

TABLE 1. *Characterization of raw milk to be pasteurized following inoculation with freely suspended* L. monocytogenes *Scott A.*

Trial No.	SPC* before inoculation	*L. monocytogenes* (CFU/ml)
1	3.5×10^4	3.0×10^5
2	4.8×10^3	2.0×10^5
3	6.8×10^4	3.1×10^5
4	1.0×10^4	2.1×10^5
5	1.5×10^4	2.6×10^5

*Standard plate count.

The second phase consisted of seven trials using an inoculum of *L. monocytogenes* internalized by bovine phagocytes *in vitro* (Table 2). On the basis of earlier small scale production of *L. monocytogenes* in bovine phagocytes (*4*), we attempted to produce an inoculum of 10^7 CFU/ml in order to achieve 10^5 CFU/ml in raw milk; however, scale-up to the larger volume needed to inoculate 150 gal of raw milk proved difficult. The actual concentration achieved in seven trials was 10^3 to 10^5 CFU/ml. The percentage of bovine cells, predominantly neutrophils, with internalized bacterial cells ranged from 22 to 91% (Table 2). The number of bacteria per bovine cell was 20-30 in the first five trials but decreased in the last two trials to 2-9 bacteria/bovine cell. With the exception of trials 6 and 7, the percentage of intracellular *L. monocytogenes* was >50%. In trial no. 2, both the concentration of *L. monocytogenes* in raw milk and the number internalized were low. We focused on intracellularity in Phase II because some researchers have hypothesized that this increases the heat resistance of *L. monocytogenes (7,11)*. No viable *Listeria* cells were detected in any sample of pasteurized milk assayed during Phase II.

Phase III included 11 trials involving pasteurization of raw milk from experimentally infected cows (Table 3). *L. monocytogenes* concentration varied from 1.4×10^3 to 9.5×10^3 (CFU/ml). Intracellularity was good, although some of the milk in each shipment was 4 d old by the time it was presented to the pasteurizer. The SPC was similar to that of the milk in the first 12 trials. The somatic cell count was indicative of mastitis induced by the replication of the *L. monocytogenes* at the inoculation site. These 11 trials simulated natural infection of lactating cows with shedding of *Listeria* into milk, largely in the intracellular state as determined by sonication. No viable *L. monocytogenes* cells were detected in any sample of pasteurized milk analyzed during Phase III.

A continuous sample was taken in each of the Phase III runs. The 1-L undiluted pasteurized milk sample was incubated at 4°C for 28 d before it was assayed by all three procedures used in these trials. This provided both primary enrichment at 4°C followed by secondary enrichment at 30°C. No *Listeria* cells were detected in pasteurized milk from the continuously monitored sampling port by any of the methods used to assay for *L. monocytogenes*.

TABLE 2. *Characterization of intracellular* L. monocytogenes *Scott A inoculum and the raw milk containing the inoculum.*

Trial	% Cells infected	Bacteria/bovine cell		Concentration in raw milk (cells/ml)	% Intracellular at pasteurization
		Mean	Std. Dev.		
1	63	27.7	19.0	5.6×10^4	64
	64	25.4	19.8		
2	63	21.3	17.6	1.1×10^3	3
3	75	21.4	19.7	3.4×10^4	81
	80	27.5	19.1		
4	63	20.3	19.9	2.1×10^4	50
5	23	32.7	20.5	3.2×10^4	91
	26	28.9	21.1		
6	79	9.4	6.9	1.3×10^5	22
	66	9.3	5.7		
7	28	2.3	1.3	7.4×10^4	67
	34	4.1	5.9		

TABLE 3. *Characterization of the intracellular* L. monocytogenes *Scott A in contaminated milk from infected cows.*

Trial	SPC/ml	Somatic cell/ml	*L. monocytogenes*/ml	Intracellular %
1	1.6×10^4	4.8×10^6	9.5×10^3	52
2	1.7×10^4	5.0×10^6	2.0×10^3	45
3	2.4×10^4	4.5×10^6	3.5×10^3	65
4	3.4×10^3	3.8×10^6	1.4×10^3	37
5	3.1×10^3	4.2×10^6	2.2×10^3	62
6	3.9×10^3	4.8×10^6	1.4×10^3	51
7	1.7×10^3	3.8×10^6	1.6×10^3	52
8	4.4×10^3	3.7×10^6	3.6×10^3	53
9	3.0×10^3	3.6×10^6	2.1×10^3	55
10	2.4×10^3	1.8×10^6	3.2×10^3	28
11	2.8×10^3	1.8×10^6	3.6×10^3	38
Mean	8.0×10^3	3.8×10^6	3.1×10^3	49

The slug-flow heat exchanger was used in these experiments to determine the effectiveness of pasteurization at the minimum conditions of 71.7°C for 15 s, as specified in the Grade A Pasteurized Milk Ordinance (*12*). This is possible because the slug-flow heat exchanger has negligible "come-up" and "come-down" time used in calculating the lethality of the total heat exposure. No viable *L. monocytogenes* cells were detected when raw milk experimentally contaminated *in vivo* at a concentration of 10^3 CFU/ml was held at 71.1°C for 15 s.

For assay of *L. monocytogenes* Scott A from raw and pasteurized milk, we chose two of the three methods used by Doyle et al. (*8*), cold enrichment, and the selective enrichment method of Lovett et al. (*17*). The third method used was that of the USDA-FSIS which employs a two-stage enrichment procedure. The usefulness of the first two methods has been demonstrated (*5,8*).

Environmental sampling produced no *L. monocytogenes*. The *Listeria*-free environment of the pilot plant evidences the satisfactory cleanup and sanitization of the operation area.

In summary, *L. monocytogenes* in freely suspended state, internalized in bovine phagocytes *in vitro* or in milk from experimentally infected lactating cows was inactivated following legal pasteurization procedures (71.7°C, 15 s). In the first five pasteurization trials, the concentration of *L. monocytogenes* cells/ml in raw milk averaged 2.6×10^5. The next seven trials employed *L. monocytogenes* internalized in bovine phagocytes *in vitro*. Fifty-four percent of the cells were internalized at pasteurization, with 3% being the

lowest concentration encountered and 91% the highest. The concentration of *L. monocytogenes* in raw milk averaged 5 x 10^4 CFU/ml. In the last 11 trials, the concentration of *L. monocytogenes* in milk from experimentally infected cows averaged 3.4 x 10^3 CFU/ml with 53% of the bacteria associated with the bovine phagocytes at pasteurization as determined by sonication.

The analysis of pasteurized milk for *L. monocytogenes* employed two methods used successfully by Doyle et al. (*8*) plus the USDA-FSIS procedure. Additionally, a 1-L sample of undiluted pasteurized milk from each trial was cold-enriched at 4°C for 28 d before analysis by all three of the enrichment procedures. Knabel et al. (*14*) have shown that heat-shocked (43°C, 18 h) *L. monocytogenes* subsequently exposed to 62.8°C, 5 min survive thermal inactivation. In our studies, no *L. monocytogenes* was recovered from any of the 23 undiluted 1-L pasteurized milk samples incubated undisturbed (4 weeks, 4°C). This included 1-L samples from 11 trials with milk from experimentally infected cows whose udder temperatures during acute mastitis would theoretically induce heat-shock proteins to favor survival of *Listeria* during pasteurization (*14*).

The slug-flow heat exchanger was used to provide the minimum heating of *L. monocytogenes* (71.7°C for 15 s) for high-temperature, short-time pasteurization as defined by the Grade A Pasteurized Milk Ordinance (*12*).

No *L. monocytogenes* Scott A survived any pasteurization procedure. Therefore, *L. monocytogenes* will not survive high-temperature, short-time pasteurization when equipment and procedure comply with the Grade A Pasteurized Milk Ordinance.

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