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Enhanced Thermal Destruction of *Listeria monocytogenes* and *Staphylococcus aureus* by the Lactoperoxidase System

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The lactoperoxidase system (LPS) enhanced thermal destruction of *Listeria monocytogenes* and *Staphylococcus aureus*. After LPS activation, biphasic survival curves were observed for *L. monocytogenes* at 57.8°C and for *S. aureus* at 55.2°C. The data were consistent with a model that assumed two bacterial populations differing in heat sensitivity. The more heat-sensitive fractions (93% of the *L. monocytogenes*, 92% of the *S. aureus*) were killed almost instantly. For these biphasic survival curves, *D* values were based on the much smaller, less-heat-sensitive fractions. For *L. monocytogenes*, the $D_{52.2°C}$ values were 30.2 min (untreated milk) and 10.7 min (LPS activated); corresponding $D_{55.2°C}$ values were 8.2 and 1.6 min; corresponding $D_{57.8°C}$ values were 2.3 and 0.5 min. For *S. aureus*, the $D_{52.2°C}$ values were 33.3 min (untreated milk) and 2.2 min (LPS activated), and the corresponding $D_{55.2°C}$ values were 7.6 and 1.1 min, respectively. The most rapid killing of *L. monocytogenes* occurred when samples were heated soon after activation of the LPS. Activation of the LPS followed by heating can increase the margin of safety with respect to milkborne pathogens.

Four major food-borne outbreaks of human listeriosis have been reported: one in Canada (35), two in the United States (14, 24), and one in Switzerland (19). Milk and milk products have been associated with three of these outbreaks (14, 19, 24). In addition to these outbreaks, several dairy products contaminated with *Listeria monocytogenes* have been recalled (15–18), costing the food industry millions of dollars.

The incidence of *L. monocytogenes* in raw milk has been documented (8, 25). The ability of this pathogen to multiply at refrigeration temperatures (39) and its relatively higher heat resistance compared with other nonsporeforming bacteria (2, 6, 11, 13) amplify the risk of milkborne listeriosis. Ironically, the main groups at risk include pregnant women and immunocompromised individuals for whom milk consumption is generally recommended (21, 24).

Staphylococci are a major cause of mastitis and may account for up to 30% of bovine mastitis (22). Thus, these bacteria can be shed into milk from udders of mastitic cows as well as from the milking environment. However, staphylococci are killed during pasteurization (20), and their presence in milk would be indicative of improper pasteurization.

The lactoperoxidase system (LPS) consists of the enzyme lactoperoxidase (LP), thiocyanate (SCN^-), and hydrogen peroxide (H_2O_2) and has a broad antimicrobial activity (31). The LP-catalyzed oxidation of SCN^- by H_2O_2 generates hypothiocyanite anion ($OSCN^-$) which exists in equilibrium with hypothiocyanous acid, pK_a 5.3 (1, 37). The hypothiocyanous acid plus $OSCN^-$ can oxidize essential sulfhydryl groups (−SH) in metabolic enzymes, thereby inhibiting bacterial growth (37, 38). Structural damage or changes in bacterial membranes with subsequent leakage or impaired uptake of nutrients, or both have also been reported (29, 31, 33).

In milk, the LPS can be bacteriostatic or bactericidal against a diversity of milkborne spoilage and pathogenic bacteria (5, 7, 12, 31, 33). However, there are no reports on the effect of the LPS on thermal inactivation of bacteria. Consequently, the present study was undertaken to determine the effect of the LPS on thermal resistance of bacteria suspended in milk. We report the dramatic increase in thermal destruction of *L. monocytogenes* and *Staphylococcus aureus* when exposed to the LPS prior to heating.

(Part of this research was previously presented [D. N. Kamau, S. Doores, and K. M. Pruitt, Abstr. Annu. Meet. Am. Soc. Microbiol., 1989, P50, p. 327].)

MATERIALS AND METHODS

Reagents. Bovine milk lactoperoxidase (EC 1.11.1.7), horseradish peroxidase (EC 1.11.1.7), bovine liver catalase (EC 1.11.1.6), 2-2′-azino-di-(3-ethyl-benzthiozoline-6-sulfonic acid) (ABTS), ferric nitrate, leucocrystal violet, 2-mercaptoethanol, and 5-5′-dithiobis-(-2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The purity index of LP was 0.76 (A_{412}/A_{280}) as reported by Sigma. Hydrogen peroxide (30%) and potassium thiocyanate were purchased from Fisher Scientific Co. (Pittsburgh, Pa.).

Assay for LPS components. SCN^- H_2O_2 and $OSCN^-$ concentrations were determined calorimetrically by using a Spectronic 21 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). A Gilford Response UV-VIS spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) was used for the LP activity assay.

SCN^- was assayed by the ferric nitrate method of Betts and Dainton (4). The assay for H_2O_2 was the leucocrystal violet method of Mottola et al. (30).

To measure the concentrations of $OSCN^-$, the method of Aune and Thomas (1) as modified by Månsson-Rahemtulla et al. (26) was used. For assay of $OSCN^-$ in milk, this method was modified as follows: to 6.0 ml of the 5-5′-dithiobis-(-2-nitrobenzoic acid) reagent was added 0.2 ml of the milk sample to be assayed, and the suspension was vortexed. To remove milk proteins, 0.1 ml of commercial cheese rennet (diluted 1:10 in distilled water) was added, followed by 0.1

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ml of $(NH_4)_2SO_4$ (20 mg/ml), and samples were held at 22°C for 5 min. The samples were then passed through 0.45-μm-pore-size filters (Metricel membrane filter no. 63068, Gelman Sciences Inc., Ann Arbor, Mich.), and the A_{412} of 3.0 ml of the filtrate was measured against a reference milk sample in which no $OSCN^-$ was generated.

The ABTS method of Schindler et al. (34) was used to assay for LP activity in tryptic soy-yeast extract broth (TSB-YE) and in milk. To eliminate turbidity which can interfere with this assay, 100-μl milk samples were added to 2.0 ml of ABTS reagent in 0.1 M sodium acetate buffer (pH 4.5) and passed through 0.45-μm-pore-size filters to remove acid-coagulated proteins. The mean LP activity in raw milk samples was 1.93 ABTS units per ml or 29.7 μg of LP per ml of milk. In the preheated milk samples, the mean residual LP activity was 0.60 ABTS units per ml (9.2 μg of LP per ml or 31%).

Growth media and culture preparation. *L. monocytogenes* Scott A (serotype 4b), an isolate from human listeriosis outbreak, was obtained from M. Doyle, University of Wisconsin, Madison, Wis. *S. aureus* ATCC 12600 was obtained from the American Type Culture Collection, Rockville, Md.

Tryptic soy broth and agar (Difco Laboratories, Detroit, Mich.) were prepared according to the instructions of the manufacturer with the addition of 0.6% yeast extract (Difco) to yield the TSB-YE or tryptic soy-yeast extract agar (TSA-YE). The pH of TSB-YE was adjusted to that of normal raw milk (pH 6.6) by the addition of sufficient amounts of 2 M sodium acetate. The volume of sodium acetate added was small (a few milliliters per liter of TSB-YE). The resulting increase in ionic strength would have no appreciable effect on the results since all cultures were grown in the same medium.

Stock cultures of *L. monocytogenes* on TSA-YE slants were incubated for 24 h at 35°C in the presence of 9.5% CO_2. Working cultures of *L. monocytogenes* were prepared by transferring a loopful from the stock cultures into TSB-YE followed by three consecutive 0.1-ml transfers and incubation for 18 to 20 h at 35°C in the presence of 9.5% CO_2.

Working cultures of *S. aureus* were prepared as for *L. monocytogenes* but with aerobic incubation at 37°C. The working cultures were maintained by daily transfers into fresh TSB-YE. Culture slants were refrigerated at 4°C.

Raw milk samples. Raw milk samples, obtained from The Pennsylvania State University dairy farm were aseptically drawn from healthy cows after the udders were thoroughly cleaned with an iodine solution followed by a 75% ethanol swabbing and a sterile water rinse. After discarding the first several discharges of milk from each teat, milk samples were collected by hand milking into 500-ml sterile bottles. These raw milk samples contained less than 100 CFU/ml as estimated by standard plate count procedures (28). These samples were preheated at 57°C for 20 min to eliminate the natural milk flora as confirmed by spread plating on TSA-YE with incubation for 48 h at 32°C.

Effect of the LPS on heat resistance of *L. monocytogenes* and *S. aureus*. An 18- to 20-h inoculum of *L. monocytogenes* or *S. aureus* was decimally diluted in phosphate buffer containing magnesium chloride (3), and 0.1 ml of each of these diluted inocula was pipetted into 10.0 ml of preheated milk to yield between 10^5 and 10^6 CFU/ml. These samples were separated into three groups: untreated milk, H_2O_2-treated milk, and LPS-treated milk.

Enough SCN^- and H_2O_2 to give initial concentrations of 2.4 mM SCN^- and 0.6 mM H_2O_2 were added to milk for *L. monocytogenes* studies. For *S. aureus* studies, initial concentrations of 1.2 mM SCN^- and 0.3 mM H_2O_2 were used. An inherent LP content of about 9.2 μg/ml was measured in preheated milk. To determine thermal resistance, 2.0-ml portions of each sample were pipetted into sterile glass ampoules (Wheaton no. 6202-12D; VWR Scientific, Bridgeport, N.J.) and flame sealed. A temperature-monitoring probe (Omega Engineering, Inc., Stamford, Conn.) was placed in an ampoule (containing uninoculated heating menstruum) and epoxy sealed. Samples were brought to an initial temperature of 35°C and then heated by complete immersion into a constant-temperature water bath (Blue M Electric Co., Blue Island, Ill.) to attain (come-up time of 2 to 3 min) holding temperatures of 52.2, 55.2, and 57.8°C. In preliminary studies, rapid destruction of *S. aureus* was observed at 57.8°C. Consequently, it was only practical to heat samples at 52.2 and 55.2°C, especially in the presence of the LPS.

At holding temperatures of 52.2, 55.2, and 57.8°C, untreated and H_2O_2-treated samples were removed at 10-, 5-, and 0.5-min intervals, respectively, for *L. monocytogenes*, while the LPS-treated samples were removed at 5-, 1-, and 0.17-min intervals for the above heating temperatures, respectively. For *S. aureus* heated at 52.2°C, untreated, H_2O_2-treated, and LPS-treated samples were removed at 10-, 5-, and 1-min intervals, respectively, while at 55.2°C, the respective time intervals were 5, 2.5, and 0.42 min. The ampoules were immediately cooled in an ice-water bath, wiped with 95% ethanol, and broken open, and 0.1-ml volumes were plated or transferred into 9.0 ml of dilution buffer for appropriate serial dilutions. Surviving bacteria were enumerated by spread plating onto TSA-YE and were incubated for 48 h at 35°C in the presence of 9.5% CO_2 for *L. monocytogenes* and for 48 h at 37°C (aerobically) for *S. aureus*. For each of these bacteria, these experiments were replicated four times, with a total of at least 14 observations per treatment.

Influence of holding time after exposure to the LPS on thermal resistance of *L. monocytogenes*. This experiment was designed to determine the length of time over which the LPS continued to affect the heat resistance of *L. monocytogenes*. Culture preparation, activation of LPS, and heat resistance procedures were followed as previously described. However, only LPS-treated milk samples were used since the goal was to determine whether recovery of *L. monocytogenes* from the LPS coincided with a return to normal heat resistance. After inoculation and activation of the LPS, milk samples were incubated at 35°C and decimal reduction times (D values) were determined after 0, 2, 6, 8, 14, and 16 h. This experiment was done in duplicate, with a total of at least 24 observations for each holding time.

RESULTS

Application of the logistic model to survival data analyses. D values are usually reported as the absolute values of the reciprocal of the slope of the plot of $\log_{10}$ survivors versus time (survival curve). However, our data did not fit the simple logarithmic model of death. Consequently, data were analyzed with the logistic model which can be used to analyze nonlogarithmic survival data (23). The general form of the logistic equation used to analyze our data was:

$$S = \mathrm{CFU}/\mathrm{CFU}_0 = 2/[1 + e(\beta t)] \quad (1)$$

where S is the surviving fraction at time t, CFU is the number of surviving bacteria, CFU_0 is the initial CFU, e is the base of the natural logarithm, and

$$\beta = 4[(d\mathrm{CFU}/dt)_{max}]/\mathrm{CFU}_0 \quad (2)$$

The differential $(dCFU/dt)_{max}$ is the slope of the survival curve at the point of inflection or the maximum killing rate, and β/4 is the maximum specific killing rate. The log transform of equation 1 is:

$$\log S = \log 2 - \log [1 + e(\beta t)] \quad (3)$$

where log S is the log (CFU/CFU_0) at any given time. The D value is given by:

$$D \text{ value} = 2/\beta \quad (4)$$

For survival curves with no initial lag in killing and only one distinct killing phase, data were fitted to equation 3.

When survival curves displayed an initial lag in death followed by a distinct one-phase killing, data were fitted to the following form of equation 3:

$$\log S = \log[1 + e(-\beta t_{1/2})] - \log \{1 + e[\beta(t - t_{1/2})]\} \quad (5)$$

where $t_{1/2}$ is the time at which the number of CFU is $CFU_0/2$ and is a measure of lag in killing, and the other terms are as defined for equations 1 through 3.

Data for survival curves with no initial lag in killing but having two distinct killing phases (biphasic) were fitted to the following two-term exponential form of equation 3:

$$\log S = \log (\{2F_1/[1 + e(\beta_1 t)]\} + \{2(1 - F_1)/[1 + e(\beta_2 t)]\}), \quad (6)$$

where F_1 and $(1 - F_1)$ represent two fractions of bacteria (differing with respect to heat resistance) and β_1 and β_2 are the specific killing rates for the two fractions, respectively. This model assumes that the two fractions (populations) are killed exponentially but at different, independent rates.

When the value of CFU is 1, then t is equal to t_1, and t_1 is the time required for CFU_0 to drop to 1. The value of t_1 can be calculated from the respective logistic equation by using the values of $t_{1/2}$ and β obtained by nonlinear regression. The value of t_1 is a good estimate of the time needed for almost complete killing of all of the inoculum.

Regression analysis of data from four separate experiments (a total of 40 observations) by the simple log model gave a mean square error of 1.09×10^{-2}. Analysis of the same data by use of the log transform of the logistic model gave a mean square error of 4.76×10^{-3}. Thus, data analysis by use of the logistic model reduced the mean square error by more than 40% compared with the results obtained by use of the simple log model. Therefore, we used the appropriate logistic equations (i.e., equations 3, 5, and 6) for data analyses.

Data were fitted to the various forms of the logistic equation by nonlinear regression (32). Analysis of variance with mean separation by the least significant difference procedure (36) was used to determine whether there were significant differences among parameter estimates ($P \leq 0.05$).

Influence of the LPS on thermal resistance of *L. monocytogenes*. Typical results on the effect of the LPS on thermal destruction of *L. monocytogenes* are shown in Table 1 and Fig. 1. In milk, the LPS significantly reduced the D values by 64.6% at 52.2°C, 80.5% at 55.2°C, and 78.3% at 57.8°C (Table 1). Alone, H_2O_2 treatment had no significant effect on the D values of *L. monocytogenes* in milk (Table 1).

An initial lag in killing was observed at 52.2 and 55.2°C for all survival data (Fig. 1A and B), and data were fitted to equation 5. At 57.8°C, survival curves for *L. monocytogenes* in H_2O_2- and LPS-treated milk revealed that there were two killing phases (biphasic), an initial rapid phase lasting a few seconds followed by a slower killing phase. To account for this observation, the two-term exponential equation 6 was used to analyze the data. It was assumed that there were two bacterial fractions, a heat-sensitive fraction (F_1) which was rapidly killed and a less-heat-sensitive fraction ($1 - F_1$) which was killed at a slower rate.

TABLE 1. Effect of the LPS on survival curve parameters[a] for *L. monocytogenes* heated in milk at 52.2, 55.2, and 57.8°C

Sample treatment[b]	Temp (°C)	β/4 (±SD) (10^{-2}/min)[c]	$t_{1/2}$ (±SD) (min)[d]	t_1[e] (min)	D value (±SD) (min)[f]
Milk	52.2	1.7 ± 0.2	42.0 ± 2.1	198	30.2 ± 3.7
Milk + HP	52.2	1.7 ± 0.3	25.8 ± 5.3	195	29.4 ± 3.3
Milk + LPS	52.2	4.7 ± 0.2	0	70.3	10.7 ± 0.4
Milk	55.2	6.1 ± 0.8	16.6 ± 1.2	52.8	8.2 ± 1.1
Milk + HP	55.2	4.5 ± 0.6	12.8 ± 1.7	71.4	11.1 ± 1.4
Milk + LPS	55.2	30.4 ± 3.5	3.2 ± 0.3	10.6	1.6 ± 0.9
Milk	57.8	21.3 ± 1.0	0	15.1	2.3 ± 0.1
Milk + HP	57.8	19.2 ± 2.1[g]	0	15.7	2.6 ± 0.5
Milk + LPS	57.8	104.2 ± 14.7[g]	0	2.6	0.5 ± 0.1

[a] Parameter estimates were obtained by fitting survival data to the appropriate logistic equation by nonlinear regression (32).
[b] Abbreviations: Milk, untreated milk; Milk + HP, milk + 0.6 mM H_2O_2; Milk + LPS, milk + 2.4 mM SCN^- + 0.6 mM H_2O_2 + 9.2 μg of inherent LP per ml.
[c] Maximum specific killing rate (β/4) = $(dCFU/dt)_{max}/CFU_0$.
[d] Time at which CFU = $CFU_0/2$, where CFU_0 = initial CFU/ml.
[e] Predicted time for surviving CFU/ml = 1.
[f] D value = 2/β at $(dCFU/dt)_{max}$.
[g] These are $\beta_2/4$ values, where D value = $2/\beta_2$ based on the less-heat-sensitive fraction $1 - F_1$ = 0.4 for Milk + HP and 0.07 for Milk + LPS.

For the LPS- and H_2O_2-treated samples at 57.8°C, the two fractions were as follows: $F_1 = 0.93$ and $1 - F_1 = 0.07$ in LPS-treated milk and $F_1 = 0.6$ and $1 - F_1 = 0.4$ in H_2O_2-treated milk. The $\beta_1/4$ and $\beta_2/4$ values represent the maximum specific death rates for the F_1 and $1 - F_1$ fractions, respectively. The $\beta_1/4$ values were so large that the term $\{2F_1/[1 + e(\beta_1 t)]\}$ in equation 6 was zero for all values of $t > 0$, suggesting instant killing of fraction F_1. This fraction was greater in the LPS-treated (93% of population) than in H_2O_2-treated (60%) milk. The D values in LPS- and H_2O_2-treated milk heated at 57.8°C were estimated from β_2, based on the less-heat-sensitive fraction (Table 1).

The LPS also significantly increased the maximum specific killing rates of *L. monocytogenes* in milk by 176% at 52.2°C, 398% at 55.2°C, and 388% at 57.8°C (Table 1). In all cases, the $t_{1/2}$ and t_1 values were the lowest in the presence of the LPS.

Effect of holding time after exposure to the LPS on heat resistance of *L. monocytogenes* in milk. The data for 0 and 2 h of holding time were consistent with equation 5; 6-, 14-, and 16-h holding data fit equation 3; and 8-h holding data fit equation 6. These variations can be attributed to different kinetics of heat injury and recovery. Data were fitted to the appropriate equations by nonlinear regression (32). When the LPS was activated and samples were held at 35°C prior to heating, the resulting D values increased gradually from 1.4 min at 0 h to 6.7 min (a 378.6% increase) after 16 h (Table 2). After an 8-h holding time, the D values approached those observed for H_2O_2-treated and untreated controls (Table 1). With prolonged holding, a decrease in the maximum specific killing rate was also observed (Table 2). The highest killing rates and the lowest D values were observed when samples were heated immediately after activation of the LPS. The predicted time needed to reduce the initial number of CFU per milliliter by half ($t_{1/2}$) after 6 h of holding was 0 min,

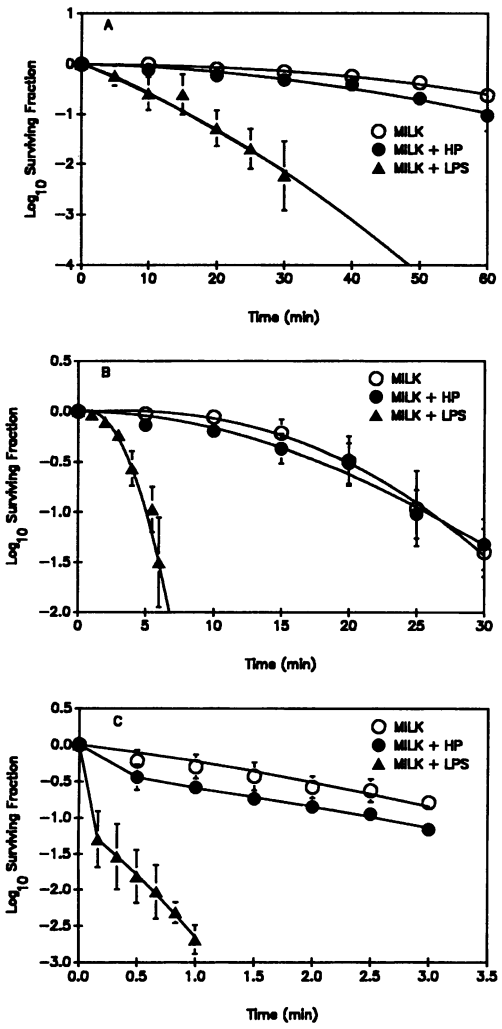

FIG. 1. Survival curves for *L. monocytogenes* heated in milk at 52.2°C (A), 55.2°C (B), and 57.8°C (C). Abbreviations: MILK, untreated milk; MILK + HP, milk + 0.6 mM H_2O_2; MILK + LPS, milk + 2.4 mM SCN^- + 0.6 mM H_2O_2 + 9.2 μg of inherent LP per ml. Plotted points are the means of at least 14 observations per experiment. The lines are the predicted survival curves obtained by fitting the data to the logistic equations by nonlinear regression (32). Vertical bars indicate one standard deviation.

suggesting there was no lag in killing of *L. monocytogenes* after this period of exposure to the LPS (Table 2).

Effect of LPS on heat resistance of *S. aureus* in milk. The LPS significantly increased the heat destruction of *S. aureus* both at 52.2 and 55.2°C (Table 3 and Fig. 2). The *D* values for *S. aureus* at 52.2 and 55.2°C, respectively, were 33.3 and 7.6 min in untreated milk, 20.4 and 10.0 min in H_2O_2-treated milk, and 2.2 and 1.1 min in LPS-treated milk (Table 3). Compared with untreated controls, H_2O_2 treatment alone significantly reduced the *D* value of *S. aureus* by 35% at 52.2°C but had no significant effect at 55.2°C. In contrast, the LPS significantly reduced the *D* values by 93.3% at 52.2°C and by 85.5% at 55.2°C (Table 3).

At 55.2°C, survival curves for *S. aureus* revealed that there were two killing phases (biphasic) as observed for *L. monocytogenes* at 57.8°C. The data followed the predicted curves very well (Fig. 2B). The two fractions observed at 55.2°C were as follows: $F_1 = 0.92$ and $1 - F_1 = 0.08$ in LPS-treated milk, and $F_1 = 0.2$ and $1 - F_1 = 0.8$ in H_2O_2-treated milk (Fig. 2B). For fraction F_1, the $\beta_1/4$ values were 3.33 in LPS-treated milk and 0.33 in H_2O_2-treated milk. The *D* values were obtained from the second killing phase (using β_2; Table 3) and are therefore not representative of the whole population.

TABLE 2. Effect of holding time after exposure to LPS on survival curve parameters[a] for *L. monocytogenes* heated in milk at 55.2°C

Holding time (h)	Log CFU_0 (±SD)[b]	β/4 (±SD) (10^{-2}/min)[c]	$t_{1/2}$ (±SD) (min)[d]	t_1 (±SD) (min)[e]	*D* value (±SD) (min)[f]
0	5.36 ± 0.05	36.9 ± 2.0	4.2 ± 0.1	8.8	1.4 ± 0.1
2	5.30 ± 0.05	19.8 ± 1.5	3.2 ± 0.2	16.3	2.5 ± 0.2
6	5.24 ± 0.04	12.8 ± 0.3	0.0	24.9	3.9 ± 0.1
8	5.15 ± 0.12	7.4 ± 1.6[g]	0.0	41.8	6.9 ± 1.7
14	5.81 ± 0.05	10.2 ± 0.2	0.0	34.6	4.9 ± 0.1
16	6.75 ± 0.04	7.4 ± 0.2	0.0	54.7	6.8 ± 0.2

[a] Parameter estimates were obtained as described in Table 1, footnote *a*.
[b] Initial log CFU/ml after activation of LPS and holding samples at 35°C for the given times before heating.
[c,d,e,f] Parameters are as defined in Table 1, footnotes *c* through *f*, respectively.
[g] This is the value of $\beta_2/4$, where *D* value = $2/\beta_2$ based on the less-heat-sensitive fraction $1 - F_1 = 0.79$.

DISCUSSION

The *D* values obtained for *L. monocytogenes* in untreated milk are comparable to those reported by others (6, 9). At holding temperatures between 52.2 and 57.8°C, the LPS reduced the *D* values by between 64.6 and 80.5%. The LPS also dramatically reduced or eliminated the initial lag in killing and increased the killing rates of this pathogen. The lower *D* values and the higher killing rates are indicative of increased heat sensitivity of *L. monocytogenes* in LPS-treated milk, as compared with untreated and H_2O_2-treated milk.

Biphasic survival curves were observed at 57.8°C for *L. monocytogenes* and at 55.2°C for *S. aureus*, both in H_2O_2- and LPS-treated samples. The biphasic curves suggest two populations (fractions) with respect to heat resistance, or a dual killing mechanism, after exposure of these bacteria to either H_2O_2 or the LPS. The good fit obtained by using the two-term exponential equation further supports these postulations. The heat-sensitive fraction (F_1) in H_2O_2- and LPS-

TABLE 3. Effect of LPS on survival curve parameters[a] for *S. aureus* heated in milk at 52.2 and 55.2°C

Sample treatment[b]	Temp (°C)	β/4 (±SD) (10^{-2}/min)[c]	t_1 (±SD) (min)[d]	*D* value (±SD) (min)[e]
Milk	52.2	1.5 ± 0.03	208.6	33.3 ± 0.56
Milk + HP	52.2	2.5 ± 0.1	120.6	20.4 ± 0.83
Milk + LPS	52.2	23.0 ± 0.73	14.0	2.2 ± 0.07
Milk	55.2	6.6 ± 2.23	52.8	7.6 ± 0.26
Milk + HP	55.2	7.4 ± 1.5[f]	54.9	10.0 ± 3.30
Milk + LPS	55.2	46.0 ± 9.0[f]	4.5	1.1 ± 0.22

[a] Parameter estimates were obtained as described in Table 1, footnote *a*.
[b] Milk, Untreated milk; Milk + HP, milk + 0.3 mM H_2O_2; Milk + LPS, milk + 1.2 mM SCN^- + 0.3 mM H_2O_2 + 9.2 μg of inherent LP per ml.
[c,d,e] Parameters are as defined in Table 1, footnotes *c*, *d*, and *f*, respectively.
[f] These are $2/\beta_2$ values, where *D* value = $2/\beta_2$, based on the less-heat-sensitive fraction $1 - F_1 = 0.80$ in Milk + HP and 0.08 in Milk + LPS.

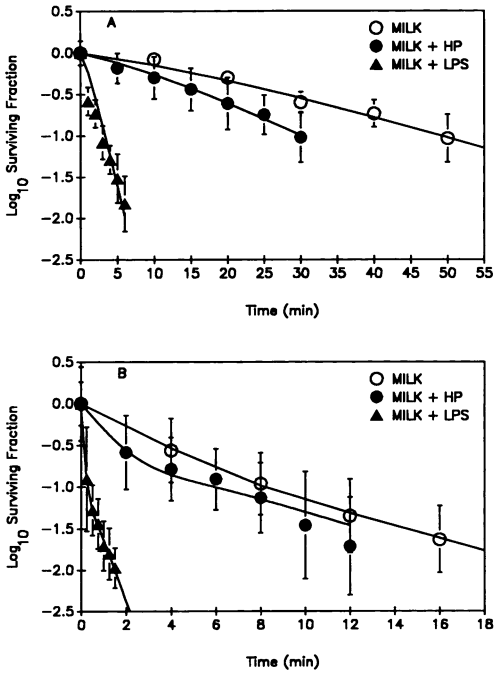

FIG. 2. Survival curves for *S. aureus* heated at 52.2°C (A) and 55.2°C. (B). Abbreviations: MILK, untreated milk; MILK + HP, milk + 0.3 mM H_2O_2; MILK + LPS, milk + 1.2 mM SCN^- + 0.3 mM H_2O_2 + 9.2 μg of inherent LP per ml. Plotted points are the means of at least 14 observations per experiment. The lines are the predicted survival curves obtained by fitting the data to the logistic equations by nonlinear regression (32). Vertical bars indicate one standard deviation.

treated milk can be attributed to the effects of the treatments, since this fraction was not observed in the untreated control. Fraction F_1 represented most of the bacterial population at 57.8°C for *L. monocytogenes* and at 55.2°C for *S. aureus*. After less than 1 min of heating at 57.8°C, no viable cells of *S. aureus* could be recovered from the LPS-treated milk, suggesting that all cells were killed instantly as fraction F_1. It is conceivable that activation of the LPS followed by heating to pasteurization temperatures would also result in the instant killing of *L. monocytogenes*. The increase in heat sensitivity was more pronounced in the presence of the LPS than with H_2O_2 treatment, as evident from the higher maximum specific killing rate for the less-heat-sensitive fraction ($1 - F_1$) in LPS-treated milk. Since the heat-sensitive fractions were killed almost instantly, the reported D values for *L. monocytogenes* at 57.8°C and for *S. aureus* at 55.2°C are based on the smaller, less-heat-sensitive fraction and are not a true reflection of the killing rates for the entire population.

Unlike the effects of the LPS, the effects of H_2O_2 alone were diminished as the heating temperature was increased from 52.2 and 57.8°C. Thus, in the absence of added SCN^-, low concentrations of H_2O_2 (less than 1 mM) may have no significant effect on thermal destruction of *L. monocytogenes* in milk at pasteurization temperatures.

After exposure of *L. monocytogenes* to the LPS, the lowest D values and the most rapid killing were observed when samples were heated immediately following activation of the LPS. With increasing holding time, there was a gradual increase in the D value and a decrease in the killing rate. Thus, *L. monocytogenes* can recover from the effects of the LPS and eventually regain normal heat resistance. After 8 h, most of the cells may have recovered from the LPS as reflected in the higher D values which approach those obtained for untreated bacteria. The D values would be expected to remain about the same during the log phase and probably shift to higher values once the cells reached the stationary phase. Martinez et al. (27) reported increased shelf life of fluid milk which had been preserved by the LPS 4 days prior to pasteurization, followed by postpasteurization reactivation of the LPS. However, during the 4-day storage period, bacteria may have recovered from the LPS, and the observed increase in shelf life probably resulted from the postpasteurization reactivation of the LPS.

The bactericidal effect of the LPS against *L. monocytogenes* even in the absence of heating has been reported (7, 12). Borch et al. (5) also reported killing of *Campylobacter* spp. on exposure to the LPS. It would therefore be expected that the fraction of bacteria that survives the bacteriostatic or bactericidal effects of the LPS are more susceptible to thermal destruction as observed in the present study. The LPS may enhance thermal destruction of bacteria by damaging bacterial membranes, among other effects (29, 31). Similar reductions in thermal resistance can be produced by exposing bacteria to various stress factors (salts, acids, etc.). However, such treatments are not practical for milk. Activation of the LPS is feasible because H_2O_2 is approved for milk preservation and both LP and SCN^- are natural milk components (31, 33).

The two-phase killing model can be a useful tool for analyzing biphasic survival curve data and for comparing treatment effects. For example, the values of $\beta_1/4$, $\beta_2/4$, $t_{1/2}$, t_1, F_1, and $1 - F_1$ can be used to compare the effects of different treatments and to provide information complementary to D values.

In conclusion, the use of the LPS in conjunction with thermal processing can increase the margin of safety with respect to milkborne pathogens and can extend shelf life. Furthermore, low-temperature LPS processes can be designed to replace high-temperature processes. Such low-temperature LPS processes could result in better retention of nutrients and higher quality in heat-sensitive foods.

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