Sensitization of Heat-Treated Listeria monocytogenes to Added Lysozyme in Milk

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Listeria monocytogenes was highly resistant to hen egg white lysozyme in whole milk but was sensitive in media and in phosphate buffer. Methods to sensitize the pathogen to lysozyme in milk were investigated. Treatment of whole milk by cation exchange to remove minerals, particularly Ca²⁺ and Mg²⁺, slightly promoted inactivation of L. monocytogenes by lysozyme at 4°C over a period of 6 days. Heat treatment (62.5°C for 15 s) strongly sensitized L. monocytogenes to lysozyme in demineralized milk and in MES [2-(N-morpholino)ethanesulfonic acid] buffer. Addition of Ca²⁺ or Mg²⁺ to the demineralized milk restored resistance to lysozyme. Cells were more rapidly heat inactivated at 55°C in demineralized milk containing lysozyme, and addition of Ca²⁺ to the demineralized milk restored the resistance to heat. The results indicate that minerals or mineral-associated components protect L. monocytogenes from inactivation by lysozyme and heat in milk, probably by increasing cell surface stability. The heat treatment of foods containing added lysozyme can probably play a significant role in producing microbiologically safe foods.

During the past decade, certain dairy products have been associated with major outbreaks of listeriosis (5, 11, 30). Listeria monocytogenes continues to be a serious concern in regard to food safety because of the high fatality rate of about 30% in infected humans and the high susceptibility of certain groups of humans (30). Humans susceptible to listeriosis include immunocompromised individuals with underlying diseases and pregnant women and their developing fetuses. These individuals should avoid eating foods that may be contaminated with the pathogen.

Avoiding contamination of many raw and minimally processed foods by L. monocytogenes is a difficult task for the food industry. L. monocytogenes is widespread in nature and has been isolated from soil, decaying vegetation (including silage), and human and animal feces. It has also been found in dairy foods, including raw milk and some ripened cheeses (28). L. monocytogenes has several phenotypic characteristics that may promote growth and survival in dairy products, including growth over the temperature range of 1 to 45°C, tolerance to >10% sodium chloride, and growth at pH 5.5 to 9.6 (31). Because of its pathogenicity and resistance properties, L. monocytogenes has been considered a relatively high-risk pathogen for the dairy industry (19).

Because of the ubiquity of L. monocytogenes and its relatively frequent contamination of raw and minimally processed foods, considerable attention has been given to developing antimicrobial agents that would kill the pathogen in these products. Our laboratory showed that egg white lysozyme inactivated L. monocytogenes in microbiological media and in various foods (16, 17). Inactivation of L. monocytogenes by lysozyme was highly dependent on the food system. L. monocytogenes was killed by lysozyme in vegetables, but it was relatively resistant in foods of animal origin, including milk and Camembert cheese (17). Further studies by us and by others (8) have shown that L. monocytogenes is strongly resistant to lysozyme in milk. No lysozyme activity against L. monocytogenes strains in whole milk was found, whereas killing in acid milk (pH 5.3) was detected (8). We undertook this study to determine the basis for this resistance and to develop methods to sensitize the pathogen to lysozyme in milk.

MATERIALS AND METHODS

Materials. The sources of egg white lysozyme hydrochloride and Micrococcus luteus were the same as previously described (17). Chemical reagents were of the purest grade available and were obtained from Sigma Chemical Co., St. Louis, Mo. Microbiological media were obtained from Difco Laboratories, Inc., Detroit, Mich. Whole pasteurized milk was purchased from the Department of Food Science dairy store, University of Wisconsin, Madison. Ultrapure water was prepared by passing distilled water through a Nanopure II mixed-bed ion-exchange system (Barnstead Co., Newton, Mass.). All reagents and buffers were prepared with ultrapure water.

Bacterial strains and culture conditions. L. monocytogenes Scott A was originally provided by E. H. Marsh, University of Wisconsin, Madison. L. monocytogenes was grown in static culture in tryptic soy broth [TSB] at 37°C and maintained on TSB agar plates at 4°C with monthly transfers. Growth in culture media was measured by optical density at 500 nm in a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, N.Y.).

L. monocytogenes was enumerated after exposure to lysozyme or heat by plating on tryptose-phosphate agar with 1% (wt/vol) sodium pyruvate (TPAP), because this medium effectively recovers healthy as well as heat-injured cells (32). TPAP contained, per liter, 20 g of tryptose, 2.0 g of glucose, 2.5 g of yeast extract, 2.5 g of NaCl, 2.5 g of Na₂HPO₄ · 7H₂O, 10 g of sodium pyruvate, and 15 g of agar. Plates were incubated at 37°C for 2 days.

Heat treatment of L. monocytogenes. To determine the susceptibility of heat-treated cells to lysozyme in milk, L. monocytogenes was grown statically overnight in TSB, 1 ml was
Genes in demineralized milk used for inoculation were harvested by centrifugation, and the cells washed in 1 ml of 67 mM sodium phosphate buffer (PB), pH 6.4, and resuspended in 100 μl of PB. The 100-μl cell suspension was added to 4.9 ml of PB at the desired temperature, and the suspension was mixed by vortexing and incubated for 13 s. The heated cell suspension was then transferred to 20 ml of ice-cold PB and mixed rapidly for immediate temperature cooling, and the cells were harvested by centrifugation. With the aid of an assistant, it was possible to transfer from heating meniscus to cold buffer in 2 s, resulting in a 15-s total heat treatment. These cells were used for inoculation to milk or buffer, which was incubated at 4°C. Survival was determined by plating on TPAP.

Resistance of *L. monocytogenes* to lysozyme in milk. A 100-fold stock solution of lysozyme hydrochloride was dissolved in PB, filter sterilized, and added to obtain a final concentration of 100 mg/liter of milk. *L. monocytogenes* was inoculated into the milk, samples were held at 4°C, and survivors were enumerated by plating on TPAP. Metals (CaCl<sub>2</sub>, MgCl<sub>2</sub>, and ZnCl<sub>2</sub>) were dissolved in distilled water, filter sterilized, and added back to demineralized milk to obtain concentrations similar to those found in whole milk. Metal analyses of whole milk, demineralized milk, and refortified milk were performed at the Soil and Plant Analysis Laboratory, University of Wisconsin, Madison, by using inductively coupled plasma spectroscopy as previously described for spores of *Clostridium botulinum* (20).

**FIG. 1.** Survival of *L. monocytogenes* in the presence of lysozyme (100 mg/liter) in whole milk and deminerlized whole milk at 4°C. Symbols: ○, unheated *L. monocytogenes* in whole milk with or without lysozyme; ●, unheated or heat-treated (60°C for 15 s) cells in demineralized whole milk without lysozyme; □, heat-treated cells in whole milk with or without lysozyme; ▼, unheated cells in demineralized whole milk containing lysozyme; Δ, heat-treated *L. monocytogenes* in demineralized milk containing lysozyme; ▲, unheated cells in 67 mM PB, pH 6.6, containing lysozyme.

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<sup>a</sup> Chelex was used at a concentration of 50 g/100 ml of milk.

**Resistance of *L. monocytogenes* to lysozyme in buffer.** The capacity of metals to restore resistance to lysozyme was evaluated by adding washed cells previously heat treated in PB to 5 ml of 50 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer, pH 6.4, containing 100 mg of lysozyme per liter. CaCl<sub>2</sub> and MgCl<sub>2</sub> were added to the buffer at the same levels as in whole milk (1,100 mg/liter for Ca<sup>2+</sup> and 110 mg/liter for Mg<sup>2+</sup>). The suspensions were held at 4°C, and viability over time was determined by plating on TPAP.

**Demineralization of whole milk:** To remove metals from whole milk, the pH of the milk was adjusted to 6.2 with concentrated citric acid, and the milk was dialyzed overnight at 4°C to two changes of 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid], pH 6.4, using dialysis tubing with a molecular weight cutoff of 6,000 to 8,000 (Spectrum Medical Industries, Inc., Los Angeles, Calif.). The milk was dialyzed the next day against two changes of 20 mM sodium phosphate buffer, pH 6.4. Certain batches of milk were further treated with Chelex 100 metal affinity exchange resin (Bio-Rad Laboratories, Richmond, Calif.). The Chelex resin was prepared by repeated washings in 0.5 M sodium acetate until a pH of 6.3 was reached and then washed five times with ultrapure water (500 to 800 ml of water per wash). The Chelex resin was used at 50 or 75 g/100 ml of milk. The milk was allowed to mix with the Chelex by stirring in a beaker overnight at 4°C and was stored refrigerated until use. The pH of the demineralized milk was 6.0 to 6.2. The removal of metals did not cause precipitation of proteins or other visible changes in the milk. To add CaCl<sub>2</sub> back to demineralized milk, the milk

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<sup>b</sup> Values for dialyzed preparations were generally lower than those in Table 1 because of longer dialysis and a higher concentration of Chelex (75 g/100 ml of milk compared with 50 g/100 ml of milk for Table 1).
was dialyzed against 20 mM sodium succinate buffer, pH 6.4, to avoid precipitation of calcium phosphate complexes.

**Influence of demineralization and lysozyme addition on thermal resistance of L. monocytogenes.** To determine the effect of demineralization of whole milk on heat resistance of *L. monocytogenes*, cells were added to 5 ml of whole milk or demineralized milk at 55°C to an initial cell concentration of approximately 10⁷/ml. Samples were taken every 2.5 min for up to 10 min and diluted immediately in cold PB, and survival was determined by plating on TAP. Lysozyme, when present, was added at a final concentration of 100 µg/liter of milk. Calcium, when present, was added at 1,100 µg/liter.

**Preparation of acid-precipitated whey.** The pH of whole pasteurized milk (pH 6.70) was lowered with 10 N HCl to 4.65. The milk was centrifuged at 15,000 × g for 15 min. The pH of the whey supernatant was raised to 6.70 with sodium hydroxide, and the supernatant was centrifuged again at 10,000 × g for 10 min.

**Analysis of lysozyme-treated L. monocytogenes by scanning electron microscopy.** *L. monocytogenes* was grown at 30°C to early log phase in TSB (Difco), a medium in which *L. monocytogenes* is highly susceptible to lysozyme (25). The cells were harvested, resuspended in PB (pH 6.2), and treated with 50 or 100 µg of lysozyme per liter for 45 min at 37°C. After treatment, the cells were harvested by centrifugation, fixed with 0.25% glutaraldehyde, and captured on a 0.45-µm-pore-size membrane. The membranes were fixed on a copper grid pretreated with 0.001% polylysine for 5 min and washed with distilled water. The cell-coated grids were washed three times with PB and distilled water. The cells were dehydrated with molecular sieved ethanol in a progressive series of 30, 50, 70, 80, 85, 90, 95, and 100%. The cells were super-critical-point dried with liquid CO₂ and coated with platinum to 1 nm thickness. Images were obtained with high-resolution low-voltage scanning electron microscopy at 1.5 kV. The magnification was ca. ×30,000 times.

**Statistical validation of the data.** Each datum point in the figures was the average of plate counts at three dilutions. Analysis of the variance of the data in the figures indicated that the average standard deviation for each datum point was approximately 14%. Because of the logarithmic plots, error bars in most cases are smaller than the figure symbols and are not presented. In our view, the significance of the graphs also is not reflected in the individual datum points but rather in the progressive trends in survival of *L. monocytogenes*. In several conditions tested, the differences in viability between the control and treatment groups were 4 to 6 log units, which we feel is significant compared with the magnitude of the standard deviation. Each experiment was replicated at least once.

**RESULTS**

**Inactivation of L. monocytogenes by lysozyme in milk.** In repeated trials, *L. monocytogenes* was found to be completely resistant to inactivation by lysozyme in whole or skim milk, but the same batches of cells were sensitive to lysozyme in PB (Fig. 1). *M. luteus* was rapidly lysed by lysozyme in milk (79% lysis in 40 min [data not shown]), indicating that lysozyme was not inactivated in milk. Experiments were carried out to understand the basis of resistance of *L. monocytogenes* to lysozyme in milk and to develop methods for sensitization of cells to the enzyme.

Heat treatment of *L. monocytogenes* at 60°C for 15 s prior to addition to milk did not significantly sensitize *L. monocytogenes* to lysozyme (Fig. 1). However, preliminary experiments suggested that sublethal heating of the cells sensitized them to lysozyme in buffer (data not shown; see also Fig. 3). Since divalent metals are known to stabilize the cell walls of gram-positive bacteria (10) and because EDTA sensitizes *L. monocytogenes* to lysozyme (16), we investigated whether the metal content of milk affected resistance to lysozyme. Removal of metals from the milk by treatment with a metal affinity resin and dialysis slightly promoted inactivation of *L. monocytogenes* by lysozyme (Fig. 1). When the cells were first heat treated in PB at 60°C for 15 s and then added to demineralized milk, inactivation by lysozyme was dramatically increased, giving more than a 4-log-unit decrease in cell numbers over 6 days at 4°C compared with control cells in milk without added lysozyme. The rate of inactivation of heat-treated cells in demineralized milk was similar to that observed in PB (Fig. 1).

These results suggested that minerals and mineral-associated components in milk (e.g., proteins) protect *L. monocytogenes* from inactivation by lysozyme. To investigate the possible role of proteins, they were partially removed from milk by acid precipitation at pH 4.65. Heat-treated *L. monocytogenes* was resistant to lysozyme in acid-precipitated whey; however, the cells were sensitive in acid-precipitated whey that was subsequently demineralized (data not shown). These results support the hypothesis that the metals rather than the protein components of milk protected *L. monocytogenes* against added lysozyme in milk.
Sensitivity of L. monocytogenes to lysozyme in metal-refortified milk. Analysis of demineralized milk for metal content by inductively coupled plasma spectroscopy indicated that the levels of Ca²⁺, Mg²⁺, and Zn²⁺ were decreased significantly compared with those in untreated whole milk (Table 1). Ca²⁺ was decreased from 1,170 to 100 mg/liter, Mg²⁺ was decreased from 118 to less than 1 mg/liter, and Zn²⁺ was decreased from 3.5 to less than 0.1 mg/liter (Table 1). We examined whether resistance of L. monocytogenes to lysozyme in demineralized milk could be restored by adding back these individual metal components. The metals were refortified in demineralized milk at concentrations that would approximate their original concentrations in whole milk. Analysis of metals in refortified milk by inductively coupled plasma spectroscopy indicated that Ca²⁺ was at about 63% of the original level (667 mg/liter), Mg²⁺ was at about 79% of the original level (82 mg/liter), and Zn²⁺ was at about 88% of the original level (3.0 mg/liter) (Table 2). Heat-treated L. monocytogenes (62.5°C for 15 s) in refortified milk became resistant to lysozyme when Ca²⁺ or Mg²⁺, but not Zn²⁺, was added back to the demineralized milk (Fig. 2). Calcium supplementation restored the resistance of L. monocytogenes to a degree that approached the resistance level in whole milk. These data support the hypothesis that the mineral components of milk protect L. monocytogenes from inactivation by lysozyme.

Influence of metals on lysis of L. monocytogenes in buffer. Milk is a complex food system which contains a variety of components that may interact with the cell and protect it from the lytic action of lysozyme. To specifically examine the influence of metals on the sensitivity of cells, inactivation by lysozyme in metal-free MES buffer was examined. PB was not used because of precipitation of calcium phosphate complexes. Unheated cells or cells heat treated for 15 s at 62.5°C were incubated in 50 mM MES containing lysozyme and Ca²⁺ or Mg²⁺ at 4°C. Unheated cells were only moderately sensitive to lysozyme, and the presence of Ca²⁺ or Mg²⁺ did not affect sensitivity (Fig. 3A). The addition of lysozyme resulted in a slow decline in cell numbers over 3 weeks at 4°C, and metal addition did not affect this slow decline. In contrast, heat-treated cells were very sensitive to lysozyme, and cell viability decreased by 4 to 6 log units over 24 days of exposure. The presence of Ca²⁺, Mg²⁺, or a combination of both protected heat-treated L. monocytogenes from inactivation by lysozyme in buffer (Fig. 3B). For cells heated at 62.5°C for 15 s, the greatest viability was maintained with added Ca²⁺ alone or the combination of added Ca²⁺ and Mg²⁺ (Fig. 3B). Calcium was more effective than Mg²⁺ in providing resistance to L. monocytogenes heated at 62.5°C for 15 s. Very similar results were observed with cells heated at 60°C for 15 s. Control incubations of L. monocytogenes with the metal addition but without lysozyme showed that the cells did not die off over the course of the experiment (data not shown). These experiments support the hypothesis that inactivation was caused by lysozyme and that Ca²⁺ and Mg²⁺ in milk provided resistance or recovery in response to challenge by lysozyme.
Tolerance of *L. monocytogenes* to thermal stress in milk. We examined whether the thermal sensitivity of *L. monocytogenes* was altered in demineralized milk. *L. monocytogenes* was very resistant to thermal inactivation at 55°C in whole milk with or without added lysozyme (Fig. 4A). In contrast, cell viability decreased rapidly during heating in the demineralized milk containing lysozyme, resulting in a 6-log-unit decrease in cell numbers after 5 min at 55°C (Fig. 4A). Interestingly, heat tolerance in demineralized milk containing lysozyme appeared to be partially restored by added Ca²⁺ (Fig. 4B). These results indicate that the metal composition and distribution within milk affects heat resistance of *L. monocytogenes* in the presence of lysozyme.

Scanning electron microscopy analysis of *L. monocytogenes* after treatment with lysozyme and chelator (EDTA). Scanning electron microscopy was performed to examine the influence of lysozyme and EDTA (which chelates Mg²⁺ and Ca²⁺) on the cell surface of *L. monocytogenes* Scott A. In the first analysis (Fig. 5), cells were grown for 16 h at 30°C in TSB. Lysozyme was added to 50 mg/liter, and the cells were incubated for 45 min. Lysozyme alone clearly caused digestion of the cell surface, producing depressions and removal of large portions of the wall material. The partially digested cells appeared to clump together, possibly through release of an adherent substance from the cell wall or membranes.

Treatment with EDTA appeared to soften the cells and reduce rigidity (Fig. 6b) compared with the control (Fig. 6a). Cells treated with 5 mM EDTA were slightly larger than control cells, indicating a loosening of the cell wall rigid structure (Fig. 6b). EDTA probably removed the metal ions from the structural framework and promoted the loosening of the rigid cell structure. Treatment with lysozyme at 100 ppm caused flattening and the release of globular substances from the cells (Fig. 6c), and this destruction was enhanced by EDTA (Fig. 6d). Cells treated with lysozyme and EDTA were extensively damaged as indicated by lysis and release of intracellular materials.

**DISCUSSION**

Although rigorous sanitation practices and improved quality control measures have reduced the incidence of *L. monocytogenes* in many raw and minimally processed foods, the pathogen continues to contaminate certain dairy foods, particularly raw milk and soft-ripened cheeses (11, 30). Dairy products appear to provide a unique environment which supports growth and survival of the pathogen (26, 28). There is considerable interest in controlling *L. monocytogenes* in dairy products, since this class of foods has been responsible for large outbreaks of listeriosis (11).

Postprocessing contamination is believed to be the major route of contamination of dairy products and some other foods, and it would be beneficial to incorporate antimicrobial agents that could eliminate the pathogen into foods. Lysozyme is attractive as a food preservative because it acts catalytically, thereby requiring very small quantities, and is safe in the
human diet. Lysozyme (murcopeptide glycohydrolase; EC 3.2.1.17) catalyzes the hydrolysis of the β-(1-4) linkage between N-acetylmuramic acid and N-acetylglucosamine residues, and its specific targets are the peptidoglycan and chitin components in the cell walls of bacteria and fungi (21, 29). In cheeses and other fermented foods, lysozyme is primarily active against specific groups of gram-positive bacteria (7). At the concentrations generally used, egg white lysozyme does not generally interfere with beneficial food fermentations carried out by lactic acid bacteria and yeasts. Egg white lysozyme has been used in the European cheese industry to prevent gas formation by clostridia in hard cheeses (7) but has not yet been approved as generally recognized as safe (GRAS) in the United States. Lysozyme is usually added to the vat milk at 20 to 35 mg/kg, and it associates nearly entirely with the curd, giving levels in the cheese of 200 to 400 mg/kg. The addition of lysozyme may also shorten the clotting times of milk, giving higher yields of cheese (14). In addition to being used in cheese, lysozyme has been used to prevent spoilage of various foods, including fresh vegetables, tofu, sausage, seafood, soy sauce, and alcoholic beverages (27). Recently lysozyme has been shown to have beneficial attributes in white and red wine production by preventing unwanted bacterial growth and as a replacement for sulfite (9).

Previous studies showed that egg white lysozyme effectively inactivated _L. monocytogenes_ in vegetables but was ineffective in Camembert cheese (17). The strong resistance of _L. monocytogenes_ to inactivation by lysozyme in milk and some cheeses was also demonstrated by Carminati and Carini (8). The basis for this unexpected resistance in dairy products was investigated in this study. We have found that certain metals in milk, particularly Ca²⁺ and Mg²⁺, increased the resistance of _L. monocytogenes_ to lysozyme and that sublethal heat treatment sensitized _L. monocytogenes_ to lytic activity.

Heat treatment of _L. monocytogenes_ may have disrupted wall integrity sufficiently to allow lysozyme to reach its target peptidoglycan and lyse the cell. The cell surface of _L. monocytogenes_ is composed of peptidoglycan, teichoic acids, and lipoteichoic acids (12, 13, 15). No outer membrane or capsule is known to be present in the cell wall structure. The isolated cell walls are composed of about 30 to 40% peptidoglycan, while the remainder of the dry matter is composed of teichoic and lipoteichoic acids (13) which are covalently attached to the peptidoglycan. Whereas teichoic acids generally constitute 30 to 70% of the walls of most gram-positive organisms, they are more prevalent in _Listeria_ spp., providing up to 75% of the dry wall mass (1, 3, 4). A proposed function of the teichoic acids is the control of cation concentration in the wall-membrane region of the cell (2, 3). The cell walls of gram-positive bacteria can be thought of as functioning partly as a cation exchanger, with Ca²⁺ and Mg²⁺ ions having a higher affinity towards cells walls than K⁺, Na⁺, and Li⁺ ions but having a lesser affinity than H⁺, La³⁺, Cd²⁺, or Sr²⁺ ions (23). Metal ions in the teichoic acid and peptidoglycan polymers have been shown to cause a contraction in cell walls, giving a more rigid structure (10, 22, 24). In the present study, treatment of _L. monocytogenes_ with EDTA promoted an enlargement of the cells, suggesting that removal of the metals allowed an expansion of the woven cell surface structure. Marquis et al. (23) reported that Mg²⁺ or Ca²⁺ ions pair with two anionic groups (mostly phosphate and carboxylate groups) in peptidoglycan as well as teichoic acids in bacterial cell walls, and that laboratory showed that magnesium can be displaced by high concentrations of other cations, such as sodium. Marquis and others have contributed to our conceptual understanding of the cell walls of gram-positive bacteria as comprising relatively loosely knit, elastic networks of polyelectrolyte polymers. These macrostructures have the ability to expand and contract, depending
FIG. 6. Scanning electron microscopy analysis of *L. monocytogenes* treated for 45 min at 37°C as follows: (a) control; (b) EDTA (5 mM); (c) lysozyme (100 mg/liter); (d) EDTA plus lysozyme. See Materials and Methods and Results for details.

on the ionic composition and pH of the medium. Sensitivity to lysozyme would therefore depend on the medium composition, particularly the presence of phosphate, organic chelators, and minerals, which would affect the porosity and charge of the gram-positive cell surface.

The ionic bonds between divalent metals and anionic side groups in teichoic acids probably contribute to protection of the peptidoglycan from lysozyme. *Micrococcus lysodeikticus*, which is extremely sensitive to lysozyme, has a wall composed almost entirely of peptidoglycan. Various chemical and physical treatments may disrupt the polymeric network surrounding the cell to a sufficient degree to allow penetration of large molecules such as lysozyme (45 by 30 by 30 Å [45 by 30 by 30 × 10⁻¹⁰ m]). Heat treatment can disrupt the integrity, but if enough divalent metal cations are present, the cell wall integrity can probably be repaired in heat-injured cells, thereby excluding penetration by lysozyme. D-Alanine was shown to be lost from cells of *Staphylococcus aureus* during heating, and magnesium binding was required for repair (18). Magnesium and iron cations were found to have a role in repair of heat-injured *L. monocytogenes* (6). Our results support the hypothesis that repair of injured cells and resistance to lysozyme in milk are dependent on the presence of Ca²⁺ or Mg²⁺. For whole bovine milk, Zhang and Allen (33) reported that magnesium and zinc were associated with different fractions: 2.3 and 1.7%, respectively, with fat, 62.0 and 8.2% with whey, and 30.5 and 88.4% with casein. Hence, removal or partitioning of mineral components in milk by fractionation or other technologies could influence the susceptibility of *L. monocytogenes* or other gram-positive pathogens to lysozyme in dairy products.

It was interesting that unheated *L. monocytogenes* did not appear to have increased resistance to lysozyme in the presence of minerals. In contrast, sublethally heated cells were able to markedly restore lysozyme resistance and maintained viability in the presence of Mg²⁺ or Ca²⁺. It is possible that...
heating may trigger a stress response inducing the capacity to repair cell surface lesions. It has been reported that divalent cations are necessary for repair of heat-injured Listeria spp. (6). These results support the hypothesis of a dynamic interchange of metals with the cell surface and the important role that metals have in promoting survival of bacteria in foods.

Our data support the conclusion that heat treatment of milk or other foods containing added lysozyme can exert a significant role in enhancing the microbiological safety of foods. Moreover, chemical and physical processing methods that are compatible with food composition and disrupt the cell surface or chelate metals would be valuable in the application of lysozyme and other lytic enzymes as food preservatives.

ACKNOWLEDGMENTS

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