

Stress-induced injury in *Listeria monocytogenes*

J.L. Smith

Eastern Regional Research Center, U.S. Department of Agriculture, Philadelphia, PA, U.S.A.

SUMMARY

Listeria monocytogenes cells, if subjected to sublethal stress, may be injured, i.e. they fail to grow in media containing selective agents even though non-injured cells normally grow in such media. Both injured and non-injured cells form colonies on non-selective agars. *L. monocytogenes* has been shown to undergo heat, acid, and freeze-thaw injury. Both acid and heat injured cells will not grow in the presence of phenylethanol, acriflavin, tellurite, polymyxin, NaCl, or a combination of these compounds when they are used in media as listerial selective agents. The addition of sugars, polyols, or salts to the heating menstruum protected *L. monocytogenes* against heat injury; however, fructose and NH₄Cl were unusual in not being protective and actually potentiated killing by the normally sublethal heat treatment. Repair of heat injured *L. monocytogenes* took place in 6 to 9 h in non-selective medium at temperatures ranging from 20 to 40°C but not at 5 or 12°C. Unlike repair in other microbial systems, the addition of pyruvate or catalase to selective media did not enhance repair of heat-injured *L. monocytogenes*. The paucity of information on the injury and repair phenomena in *Listeria* indicates that more studies are needed in order to adequately evaluate the importance of injured *L. monocytogenes*.

INTRODUCTION

The facultative intracellular bacterium, *Listeria monocytogenes*, is gaining recognition as a severe human pathogen. Due to the intracellular location of the organism, humoral antibodies produced in response to listerial infections are not completely protective and the infection must be combatted by cell-mediated immunity via T-lymphocytes and phagocytic cells [17]. Newborn babies are particularly susceptible to listerial infections since they do not have well developed immune systems. Adults who are immunocompromised because of pregnan-

cy or underlying malignancy are at risk to *Listeria* infections.

There is an increasing accumulation of evidence which indicates that foods may be the most significant means of transmission of the *Listeria* organism [14]. *Listeria* species are ubiquitous and grow reasonably well at refrigerated temperatures; therefore, a foodborne route of transmission is favored. In particular, modern food marketing systems with their emphasis on refrigeration and long shelf-life can lead to growth of *Listeria* if present in uncooked refrigerated food items or in refrigerated foods contaminated with *Listeria* after cooking.

Reports from the U.S., Canada, and the U.K. indicate that ingestion of various foods such as cole-slaw, milk, and cheeses has been correlated with severe listeriosis outbreaks [2,14,26]. Regular perusal of 'Food Chemical News' indicates that a number of products—ice cream, chocolate milk, and various types of cheeses—have been recalled because of *Listeria* contamination. However, the products involved in these recalls were not implicated in known cases of listeriosis.

INJURY IN *L. MONOCYTOGENES*

The following is an operational definition of microbial injury: microorganisms that have been treated with a sublethal stress are considered to be injured if they fail to grow and produce colonies when they are plated on agar media containing selective agents (bile salts, NaCl, antibiotics, etc.). Both injured and non-injured cells grow and produce colonies on non-selective agars whereas only the non-injured cells form colonies on selective agars [20]. If the injured microorganisms can escape the stressed environment or if the stress is removed, they may, under the proper conditions, repair the damage induced by the stress, grow, divide and behave normally, i.e. the cells resuscitate.

Since foods that have undergone some form of processing have been shown to contain *L. monocytogenes* or to have caused listeriosis, the organism is probably susceptible to stress-induced injury (with subsequent repair) in foods subjected to processing. Indeed, injury to *L. monocytogenes* has been shown utilizing model systems [3]. However, it is not known that any food-related outbreaks of listeriosis are correlated to repair and subsequent outgrowth of injured *L. monocytogenes*.

In *Listeria* the selective agent of choice that does not permit stressed cells to grow and produce colonies on agar media is NaCl. By plating stressed bacterial samples on a two-plate system—a nutrient agar medium containing 0.5% NaCl and one with 2 to 5% NaCl—the extent of stress injury in the *Listeria* sample can be determined.

The first study demonstrating heat injury in *L.*

monocytogenes was performed by Beuchat and co-workers [3]. Heating strains LCDC 81-861 and Scott A at 52°C in cabbage juice (pH 5.6) led to death of the cells (death is shown by subtracting the counts of TSA_{100 min} from counts on TSA_{0 min} in Fig. 1A and 1B). However, addition of the selective agent—NaCl at either 2 or 4%—to TSA indicated that injury was induced in *L. monocytogenes* by the heat treatment (extent of injury is calculated by subtracting the counts on TSAS from those on TSA in Fig. 1A and 1B). It is interesting that the extent of injury obtained was higher with TSA containing 4% NaCl than on TSA with 2% NaCl. Thus, the data obtained by Beuchat et al. [3] suggest that *Listeria* undergo different degrees of heat-induced injury and the difference can be shown by incorpo-

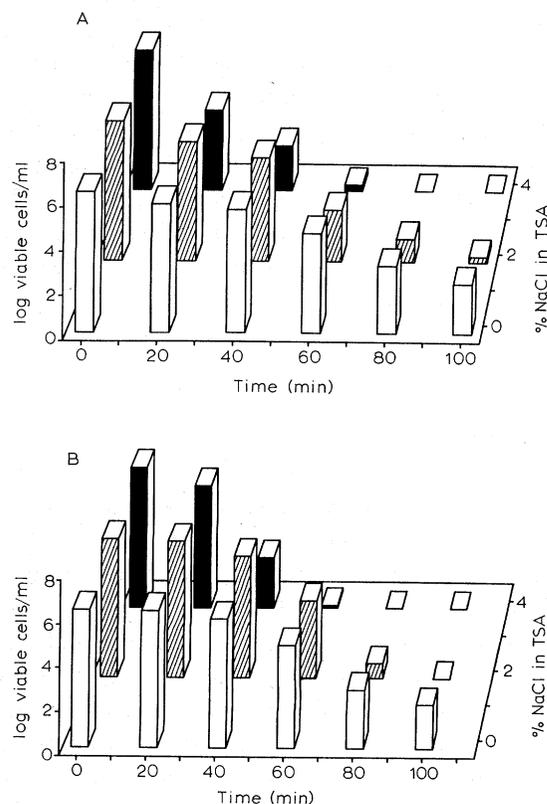


Fig. 1. Heat-induced injury in *L. monocytogenes* heated at 52°C in clarified cabbage juice (pH 5.6). At intervals, samples were plated on TSA containing 0, 2, and 4% added NaCl. Fig. 1A: Strain LCDC 81-861; Fig. 1B: Strain Scott A (modified from [3]).

rating varying levels of NaCl into the plating medium.

Dallmier and Martin [8] heated four strains (strains ATCC 7644, ATCC 115313, LCDC, and Scott A) of *L. monocytogenes* in 0.1 M phosphate buffer (pH 7.2) at 55°C using TSA and TSA + 5% NaCl (TSAS) as the differential plating media. They showed that heat-induced injury was produced in all four strains of *Listeria* (Fig. 2). Similarly, Smith and Archer (19) demonstrated injury (Fig. 3) in the Scott A strain heated in potassium phos-

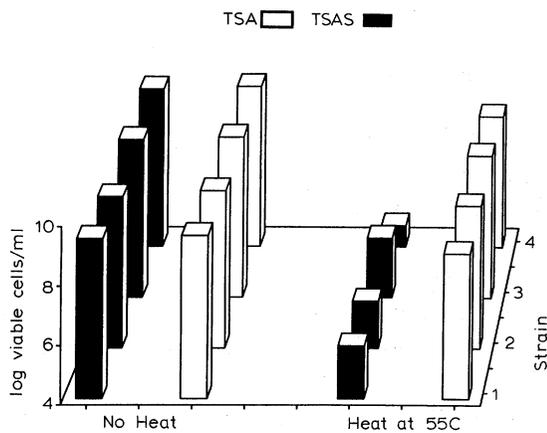


Fig. 2. Production of injury in four strains of *L. monocytogenes* in phosphate buffer (0.1 M, pH 7.2) heated at 55°C. Samples were plated on TSA and TSAS. Strain 1. ATCC 7644 (heated 35 min); Strain 2. ATCC 15313 (heated 10 min); Strain 3. LCDC (heated 30 min); Strain 4. Scott A (heated 20 min); (modified from [8]).

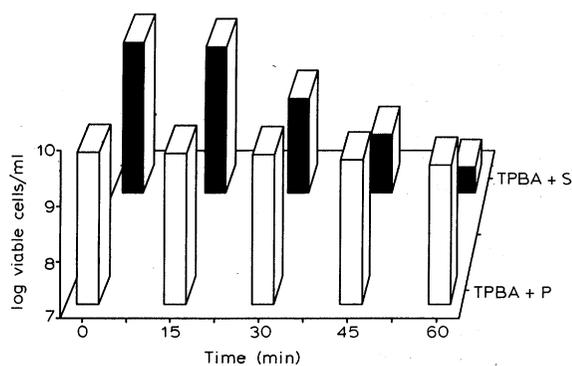


Fig. 3. Production of injured cells in *L. monocytogenes* Scott A strain in potassium phosphate buffer (0.1 M, pH 7.2) heated at 52°C. At intervals, dilutions of heated samples were plated on TPBA + P and TPBA + S. (modified from [19]).

phate buffer (0.1 M, pH 7.2). Their differential plating media consisted of TPBA + P and TPBA + S.

Other sublethal stresses have been shown to lead to injury in *L. monocytogenes*. When the Scott A strain was incubated at 36°C in acetate buffer (0.4 M, pH 4.2), there was at least a 2 log increase in the number of injured cells after 30 min treatment (unpublished observations). Freeze-injured *Listeria* resulted when the Scott A, Brie-1, LCDC 81-861, and DA-3 strains of *L. monocytogenes* were stored at -18°C for 14 days in tryptose phosphate broth [10a]. Thus, heating, acidification, and freezing led to injured cells in *L. monocytogenes* and it is probable that other types of stress induce injury in *Listeria*, also.

EFFECT OF SELECTIVE MEDIA ON REPAIR OF INJURED *L. MONOCYTOGENES*

While the use of selective media is necessary to isolate *Listeria* species, the selective agents generally prevent repair of injured cells, leading to underestimation of potential disease producing organisms. Smith and Archer [19] found that the selective agents used in media for the isolation of *Listeria* species such as 0.25% phenylethanol, 0.0012% acriflavin, 0.01% potassium tellurite, 0.01% polymyxin B sulfate, 5% NaCl or a combination of these ingredients did not allow resuscitation of heat injured Scott A strain of *L. monocytogenes*. Similarly, these selective agents prevented repair and colony formation of acid (acetate buffer, pH 4.2, 0.4 M, 36°C, 30 min) injured Scott A strain [6]. At the present time, a selective medium that allows resuscitation and outgrowth of injured *L. monocytogenes* is unavailable.

ROLE OF PYRUVATE IN REPAIR OF INJURED *L. MONOCYTOGENES*

The accumulation of H₂O₂ appears to be a universal response in cells undergoing injury and injured cells appear to have an increased sensitivity to the toxic effects of hydrogen peroxide [16]. Dallmier

and Martin [8] have shown that the catalase activity of heated cell extracts of four strains of *L. monocytogenes* decreased sharply at temperatures between 55 and 60°C and superoxide dismutase was even more heat labile than catalase.

The addition of catalase during the repair period generally is beneficial in removing toxic hydrogen peroxide. However, adding catalase to either solid or liquid media is not a simple matter since sterilization of catalase is difficult. The non-enzymatic H₂O₂ decomposer, sodium pyruvate, appears to be equally effective as catalase in removing hydrogen peroxide and is much easier to add to media since it can be autoclaved in situ. A number of workers have shown that the addition of pyruvate to selective media enhanced the recovery of injured microorganisms (Table 1). Thus, it would appear that the addition of pyruvate to the isolation media used for *L. monocytogenes* should be beneficial in the resuscitation of injured *Listeria*. Smith and Archer [19] used TPBA + P as the positive control medium in their studies since both injured-repaired and non-injured *L. monocytogenes* formed colonies on TPBA + P. However, they found that addition of 1 to 3% pyruvate to modified McBride medium did not enhance recovery of heat injured cells of the Scott A strain (i.e. pyruvate containing modified McBride medium did not give a bacterial count similar to that of TPBA + P). Why pyruvate addi-

tion is not effective in aiding resuscitation of heat-injured *L. monocytogenes* is puzzling, especially when it has been shown to be beneficial to recovery of other heat-injured microorganisms (Table 1). By decreasing the temperature of resuscitation and increasing the incubation time, Farber et al [10] indicated that addition of pyruvate (concentration not given) to modified McBride agar enhanced recovery of heat injured *L. monocytogenes*. It was necessary to incubate the plates at 22–25°C for 7 days in order to demonstrate enhancement of recovery by pyruvate; however, no data were presented. The substantial increase in incubation time necessary when using the lower temperatures does not appear to be a good approach.

EFFECT OF TEMPERATURE ON REPAIR OF HEAT-INJURED *L. MONOCYTOGENES*

Listeria monocytogenes grows well at temperatures ranging from 3 to 45°C [9] but little is known concerning the influence of temperature on the repair of injured cells. In preliminary studies on the effect of temperature on the repair of heat-injured Scott A strain (Smith and Archer, unpublished observations), *L. monocytogenes* was heat injured in potassium phosphate buffer (pH 7.2, 0.1 M) for one h at 52°C (2.0 to 2.5 log injury was achieved) and plated

Table 1

Microbial systems in which addition of sodium pyruvate enhanced recovery of injured cells.

Microorganism	Type of injury	Medium	Concentration of Na pyruvate added	Reference
<i>Staphylococcus aureus</i> strains M3, 11208, 196E, 1988, S6, 233	heat, drying, and freeze-drying injury	Staphylococcus medium 110 agar; Baird-Parker medium	1.0%	1
<i>S. aureus</i> , strain S-6	heat injury	tryptic soy agar + 7% added NaCl	0.2%	11
<i>S. aureus</i> , strain MF-31	heat injury	Vogel-Johnson agar	1.0%	16
<i>S. aureus</i> , strains MF-31, 196E, 181, 210	heat injury	trypticase soy broth + 10% NaCl	1.0%	5
<i>Salmonella typhimurium</i>	heat injury	Levine EMB agar + 2% NaCl	0.55%	7

onto TPBA. At zero time, 3 h, 6 h and 9 h after incubation at a given recovery temperature, the TPBA plates were overlaid with plain agar containing 10% NaCl (to prevent further repair of injured cells). After the agar overlay had hardened, the plates were incubated at 37°C and observed at 3 days. Complete or nearly complete recovery of heat-injured cells (i.e. the count was similar on the overlay plates to that of the control TPBA plates at 60 min heating) was observed between 6–9 h incubation at temperatures ranging from 20 to 40°C. Incubation for 9 h at 5 or 12°C before overlay with salt agar led to little detectable recovery. Lovett [13] found that 25°C was the optimum temperature for repair of sublethally heat injured *L. monocytogenes* and heat-injured *L. monocytogenes* incubated in pasteurized milk did not repair during a cold enrichment procedure (R.G. Crawford, C.M. Bellevue, C.W. Donnelly, J.T. Peeler and V.K. Bunning, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, P46, p. 281).

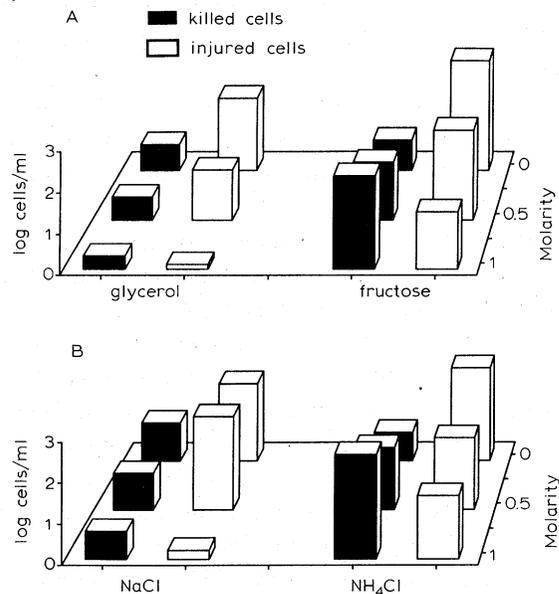
Thus, preliminary evidence indicates that heat-injured cells of *L. monocytogenes* do not repair at low temperatures; however, more studies are needed particularly at temperatures below 15°C. Attention should be paid to those types of foods that receive a heat treatment and then are kept for long periods in cold storage (e.g. milk and milk products).

EFFECT OF SOLUTES ON INDUCTION OF HEAT INJURY IN *L. MONOCYTOGENES*

Solutes, both inorganic and organic, can modify the injury process. Lee and Goepfert [12] showed that the addition of sucrose to the heating menstruum prevented injury to *Salmonella typhimurium*. Salts, sugars, polyols, or amino acids present in the heating medium decreased the extent of heat injury in *S. aureus* [21,23,24]. There did not appear to be a relationship between the protective effects of the solutes and the decrease in a_w produced by the solutes [21,23]. Non-metabolizable carbohydrates were just as protective against heat injury in *S. aureus* as were metabolizable carbohydrates [25]. In addition,

Smith et al. [22] demonstrated that sugars and polyols protected *S. aureus* against acid injury. Therefore, the protective effect given by a solute against heat or acid injury suggests that the presence of solutes in food products may change the pattern of injury in microorganisms subjected to the processing conditions which the food undergoes.

The Scott A strain of *L. monocytogenes* was also protected against injury when heated at 52°C in phosphate buffer containing sugars, polyols, or salts (Smith and Hunter, manuscript submitted for publication). The data presented in Fig. 4A show that glycerol protected *L. monocytogenes* against heat injury. Xylose, glucose, α -methylglucoside, mannose, galactose, sucrose, lactose, and mannitol behave quite similarly to glycerol. However, fructose did not prevent injury and in fact, it led to killing of the cells (Fig. 4A). Metabolism of a carbohydrate was not related to protection since neither xylose, galactose, glycerol, nor mannitol supported growth of the Scott A strain of *L. monocytogenes*, yet these compounds protected the cells against heat injury. Fructose, however, is utilized by the



Figs. 4A and 4B. The effect of solutes on heat injury in *L. monocytogenes* Scott A strain. Cells were heated in 0.1 M phosphate buffer with and without solute (buffer adjusted to pH 7.2) for one h. Samples were plated on TPBA + P and TPBA + S.

Scott A strain for growth but it was definitely not protective. It is not known why the combination of sublethal heat and fructose leads to death of the cells. Sodium chloride protected *L. monocytogenes* against heat injury (Fig. 4B); KCl behaved similarly to sodium chloride. However, protection was not found when NH₄Cl was added to the heating medium (Fig. 4B) and, like fructose, its presence led to increased death of *L. monocytogenes* at 52°C very much.

The mechanism of the decreased injury effect produced by the protective compounds is not understood nor is the mechanism of the killing effect produced by NH₄Cl or fructose during the injury process understood. The deleterious effect on *L. monocytogenes* shown by fructose has obvious implications for *Listeria* present in foods containing fructose as the sweetening and/or functional properties enhancing agent.

CONCLUDING REMARKS

The importance of microbial injury of the food industry should not be underestimated. Sawyer and Pestka [18] made the following quite pointed statement:

A conservative assumption can be made that injured pathogenic bacteria in foods cause human disease at levels equivalent to their uninjured counterparts.

While their statement may be difficult to prove, it does emphasize the potential danger of microbial injury to the food consuming public.

A number of strains of *L. monocytogenes* have been shown to undergo injury when they are subjected to the stress of heating, freezing or acidification. Studies have been done, and are being done to determine the effects of microbial competition, chemicals, and physical agents on the survival of *Listeria*. Recent work has included the effect of chlorine [4], ultraviolet energy [27], and competition by pseudomonads [15] on the growth and survival of *L. monocytogenes*. However, injury effects were not considered. The effect of chemical and physical agents as well as microbial competition on

production of injury in *L. monocytogenes* must be investigated.

An effective solid medium for resuscitation of injured *Listeria*, which at the same time is selective for *Listeria* is desperately needed. At the enrichment level, suspect foods should first be inoculated into a non-selective broth and incubated 12 to 15 h at 37°C to allow repair of any injured cells. Then, with the addition of double-strength *Listeria*-selective broth followed by a 24 h further incubation period at 37°C, the procedure should allow isolation of both non-injured and injured-repaired *Listeria*. Quantitation of the enrichment would have to be done using an MPN procedure. But at the present time, there does not appear to be a selective solid medium that permits repair of injured *Listeria*. With other microbial species, addition of catalase or pyruvate to selective media allows repair of injured cells but these compounds do not appear to be effective with *Listeria*.

Basically nothing is known concerning the underlying mechanisms involved in the injury and repair processes nor is there much information concerning the nutritional and physical conditions necessary for repair of injured *Listeria* species. Importantly, more information is needed concerning the minimum temperature for repair since *Listeria* grows at low temperatures and the organism is found in refrigerated foods. No studies have been conducted of the effect of injury on virulence mechanisms of the cell. Considering the importance of *L. monocytogenes* today, the injury phenomenon and its associated repair is a problem that needs thorough study.

REFERENCES

- 1 Baird-Parker, A.C. and E. Davenport. 1965. The effect of recovery medium on the isolation of *Staphylococcus aureus* after heat treatment and after the storage of frozen or dried cells. *J. Appl. Bact.* 28: 390-402.
- 2 Bannister, B.A. 1987. *Listeria monocytogenes* meningitis associated with eating soft cheese. *J. Infection.* 15: 165-168.
- 3 Beuchat, L.R., R.E. Brackett, D.Y-Y. Hao and D.E. Conner. 1986. Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. *Can J Microbiol.* 32: 791-795.

- 4 Brackett, R.E. 1987. Antimicrobial effect of chlorine on *Listeria monocytogenes*. J. Food Prot. 50: 999–1003; 1008.
- 5 Brewer, D.G., S.E. Martin and Z.J. Ordal, 1977. Beneficial effects of catalase or pyruvate in a most-probable-number technique for the detection of *Staphylococcus aureus*, Appl. Environ. Microbiol. 34: 797–800.
- 6 Buchanan, R.L., J.L. Smith, H.G. Stahl and D.L. Archer. 1988. Listeria methods development research at the Eastern Regional Research Center, U.S. Department of Agriculture. J. Assoc. Off. Anal. Chem. 71: 651–654.
- 7 D'Aoust, J.Y. 1978. Recovery of sublethally heat-injured *Salmonella typhimurium* on supplemented plating media. Appl. Environ. Microbiol. 36: 483–486.
- 8 Dallmer, A.W. and S.E. Martin. 1988. Catalase and superoxide dismutase activities after heat injury of *Listeria monocytogenes* Appl. Environ. Microbiol. 54: 581–582.
- 9 Doyle, M.P. 1988. Effect of environmental and processing conditions on *Listeria monocytogenes*. Food Technol. 42 (4): 169–171.
- 10 Farber, J.M., G.W. Sanders and J.I. Speirs. 1988. Methodology for isolation of *Listeria* from foods—a Canadian perspective. J. Assoc. Off. Anal. Chem. 71: 675–678.
- 10a Golden, D.A., L.R. Beuchat and R.E. Brackett. 1988. Inactivation and injury of *Listeria monocytogenes* as affected by heating and freezing. Food Microbiol. 5: 17–23.
- 11 Hurst, A., G.S. Hendry, A. Hughes and B. Paley. 1976. Enumeration of sublethally heated staphylococci in some dried foods. Can. J. Microbiol. 22: 677–683.
- 12 Lee, A.C. and J.M. Goepfert. 1975. Influence of selected solutes on thermally induced death and injury of *Salmonella typhimurium*. J. Milk Food Technol. 38: 195–200.
- 13 Lovett, J. 1988. Isolation and enumeration of *Listeria monocytogenes*. Food Technol. 42 (4): 172–175.
- 14 Lovett, J. and R.M. Twedt. 1988. *Listeria*. Food Technol. 42 (4): 188–191.
- 15 Marshall, D.L. and R.H. Schmidt. 1988. Growth of *Listeria monocytogenes* in 10°C milk preincubated with selected pseudomonads. J. Food Prot. 51: 277–282.
- 16 Martin, S.C., R.S. Flowers and Z.J. Ordal. 1976. Catalase: its effect on microbial enumeration. Appl. Environ. Microbiol. 32: 731–734.
- 17 Rebhun, W.C. 1987. Listeriosis. Vet. Clinics North America: Food Animal Practice 3: 75–83.
- 18 Sawyer, C.A.D. and J.J. Pestka. 1985. Foodservice systems: presence of injured bacteria in foods during food product flow. Ann. Rev. Microbiol. 39: 51–67.
- 19 Smith, J.L. and D.L. Archer. 1988. Heat-induced injury in *Listeria monocytogenes*. J. Ind. Microbiol. 3: 105–110.
- 20 Smith, J.L. and S.A. Palumbo. 1982. Microbial injury reviewed for the sanitarian. Dairy Food Sanit. 2: 57–63.
- 21 Smith, J.L., R.C. Benedict and S.A. Palumbo. 1983. Relationship of water activity to prevention of heat injury in *Staphylococcus aureus*. Lebensm.-Wiss. u.-Technol. 16: 195–197.
- 22 Smith, J.L., R.C. Benedict and S.A. Palumbo. 1982. Acid injury in *Staphylococcus aureus* and its prevention by sugars and polyols. Develop. Indust. Microbiol. 23: 587–593.
- 23 Smith, J.L., R.C. Benedict and S.A. Palumbo. 1982. Protection against heat-injury in *Staphylococcus aureus* by solutes. J. Food Prot. 45: 54–58.
- 24 Smith, J.L., R.C. Benedict and S.M. Kalinowski. 1985. Solute that protect *Staphylococcus aureus* against heat-induced injury and their effect on cellular leakage. J. Food Prot. 48: 600–602.
- 25 Smith, J.L., R.C. Benedict, M. Haas and S.A. Palumbo. 1983. Heat injury in *Staphylococcus aureus* 196E: protection by metabolizable and non-metabolizable sugars and polyols. Appl. Environ. Microbiol. 46: 1417–1419.
- 26 Twedt, R.M. 1986. Listeria. In: (D.O. Cliver and B.A. Cochrane, eds.), pp. 113–120, Progress in food safety. Food Research Institute, Univ. Wisconsin, Madison, WI.
- 27 Yousef, A.E. and E.H. Marth. 1988. Inactivation of *Listeria monocytogenes* by ultraviolet energy. J. Food Sci. 53: 571–573.