

Heat Injury in *Listeria monocytogenes*: Prevention by Solutes

J. L. Smith and S. E. Hunter

U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 East Mermaid Lane,
Philadelphia, PA 19118 (U.S.A.)

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Cells of Listeria monocytogenes (Scott A strain) were heat-injured when held in phosphate buffer (0.1 M, pH 7.2) at 52°C for 1 h. Addition of salts (NaCl or KCl at 3 M), sugars (xylose, α -methylglucoside, mannose, glucose, galactose, lactose, or sucrose at 1 M), or polyols (glycerol or mannitol at 1 M) markedly decreased the injury, i.e. the presence of solutes in the heating buffer protected L. monocytogenes against heat injury. However, addition of NH₄Cl or fructose to the buffer did not lead to protection but rather increased cell death at 52°C. Utilization of carbohydrates for growth by L. monocytogenes was unrelated to their ability to protect the cells against heat injury. The survival of L. monocytogenes during heating when solutes were present has definite implication in terms of food safety.

Introduction

Listeria monocytogenes resembles other Gram-positive and Gram-negative bacteria in being injured when subjected to a stress such as sublethal heating. Injury is defined as the inability of bacterial cells which have been subjected to sublethal stress to form colonies on media containing bile salts, antibiotics, high concentrations of NaCl, etc. whereas cells not subjected to sublethal stress grow and produce colonies on such media. Beuchat *et al.* (5) found that two strains of *L. monocytogenes* were injured when heated in cabbage juice (pH 5.6) at 52°C. Injury was defined by them as the inability of the injured cells to form colonies on tryptic soy agar + 4% NaCl. Smith and Archer (18) showed that the Scott A strain of *L. monocytogenes* was injured when heated in potassium phosphate buffer (pH 7.2, 0.1 M) at 52°C. Injured cells were detected in that report by their inability to form colonies on tryptose phosphate agar + 5% NaCl, although unheated cells grow and form colonies in the presence of 5% NaCl. Therefore, injured cells appear to be unable to repair heat-induced damage in the presence of 4 to 5% NaCl.

Heat injury to *Staphylococcus aureus* was diminished or abolished when salts, sugars, polyols, or amino acids were present in the heating menstruum (20,22,23,24). In addition, sugars or polyols decreased the extent of acid injury in *S. aureus* (21). In the present study, the ability of various salts, sugars, and polyols to protect the Scott A strain of *L. monocytogenes* against heat injury was examined.

Material and Methods

Microorganism and preparation of cells for injury studies

L. monocytogenes Scott A strain, was maintained in brain heart infusion (BHI; Difco) stored at 5°C. Flasks (1000 ml Erlenmeyer) containing 100 ml BHI with a final glucose concentration of 0.5% w/v were inoculated with *L. monocytogenes* and incubated on a rotary shaker (200 r.p.m.) at 37°C for 20 h.

Cells were harvested by centrifugation, washed twice with sterile distilled water and resuspended in 5 ml sterile distilled water.

Heat injury procedure

For determination of heat injury, widemouth screw cap 160 ml dilution bottles containing 20 ml of sterile potassium phosphate buffer (pH 7.2, 0.1 M) or buffer solutions containing solutes were equilibrated to 52°C. A 1 ml aliquot of washed *L. monocytogenes* cells was added to each bottle to give approximately 5×10^9 cells/ml of buffer. When solutes were used, the appropriate concentration of solute was dissolved in 50 ml of double strength buffer (using heat if necessary). The pH was then adjusted to pH 7.2 after the solutions had cooled and the volume made to 100 ml with distilled water. Sterilization of buffer solutions was achieved by filtration using Nalgene Sterile Disposable Filterware flasks (115 ml, 0.45 μ m).

Plating procedure

At zero time and after 60 min incubation at 52°C, 0.1 ml of culture was removed from each injury vessel and placed into 9.9 ml sterile 0.1% (w/v) Bacto peptone water, which was used for preparation of successive dilutions. Appropriate dilutions were plated, using a Spiral Plater, on either tryptose phosphate broth containing 2% agar (TPBA) plus 1% (w/v) sodium pyruvate (TPBA + P) and on TPBA plus 5% (w/v) added NaCl (TPBA + S). Plates were incubated at 37°C and counted after 3 days.

Effect of sugars and polyols on the growth of *L. monocytogenes*, Scott A strain

Washed cells were inoculated into flasks containing tryptic soy broth without glucose (Difco; TSB w/o glucose) with or without 1% sugars or polyols. Concentrated solutions of carbohydrates were sterilized by autoclaving and were added aseptically to the flasks. Flasks were incubated shaken (200 r.p.m.) and observed at 24, 48 and 72 h.

Data presented in the Figures and Tables represent means from 3-4 separate determinations.

Results

The number of injured cells was determined by using a two plate system consisting of TPBA + P and TPBA + S (18). Both injured and non-injured *L. monocytogenes* form colonies on TPBA + P. Since injured cells are apparently unable to repair heat induced damage in the presence of 5% NaCl, only non-injured cells form colonies on TPBA + S. The difference in bacterial count between TPBA + P and TPBA + S therefore indicates the number of injured cells.

When glycerol was added to the phosphate buffer heating menstruum, heat injury to *L. monocytogenes* was diminished at 0.5 M glycerol and was abolished at 1 M (Fig. 1). A Relative Injury Value (RIV) was developed to compare the effect of various salts, sugars and polyols on preventing injury to *L. monocytogenes* by heating. All experimental data were graphed as in Fig. 1 and the areas under the curve for TPBA + P and TPBA + S were determined. When the area for TPBA + S was subtracted from the area for TPBA + P, the resultant area is equivalent to the extent of injury. In Fig. 1, the area of injury obtained in the absence of glycerol was assigned the RIV of 100; thus the RIV for 0.5 M glycerol was 58.1 and that for 1 M was 4.8. The smaller the RIV, the greater the protection against heat injury afforded by the solute.

Data presented in Table 1 indicate that sugars, polyols, and salts either decreased or abolished the harmful effects of heat injury on *L. monocytogenes*, i.e. the RIV have low values. Generally, carbohydrates were more protective at the 1 M level than at 0.5 M. Addition of 1 M glycerol, sucrose, glucose, or α -methylglucoside to the heating buffer gave complete or almost complete protection against heat injury. Salts were not as protective as the carbohydrates on a molar basis. At 1 M concentration, NaCl was not protective and KCl was only partially protective; at 3 M, both salts were as effective as the carbohydrates (Table 1).

Ammonium chloride and fructose, however, behaved quite differently from other solutes tested. Addition of NH_4Cl (Fig. 2) or fructose (Fig. 3) to the heating menstruum not only did not lead to protection against heat injury but actually led to killing of *L. monocytogenes* at 52°C (as evidenced by a decrease in viable count on TPBA + P).

No relationship appeared to exist between stimulation of growth of *L. monocytogenes* by carbohydrates used in this study with protection against heat injury. Glucose, α -methylglucoside, fructose, mannose, lactose and sucrose stimulated bacterial growth whereas xylose, galactose, glycerol and mannitol did not increase the growth over the control lacking carbohydrate (data not presented). Even though galac-

Table 1 The effect of solutes on heat injury in *L. monocytogenes* (Scott A strain)

Solute	Relative Injury Value (RIV) ^a at solute concentration of:				
	0.5 M	1.0 M	(a_w) ^b	3.0 M	(a_w)
Xylose	54.5	21.3	(0.978)	— ^c	
α -Methylglucoside	23.1	4.0		—	
Glucose	18.4	0.0	(0.978)	—	
Mannose	15.9	29.1		—	
Galactose	51.7	28.0		—	
Lactose	25.8	—		—	
Sucrose	83.1	5.3	(0.968)	—	
Glycerol	58.1	4.8	(0.982)	—	
Mannitol	23.4	22.6	(0.990)	—	
NaCl	—	99.5	(0.967)	16.0	(0.893)
KCl	—	58.1	(0.968)	24.0	(0.904)

^a In absence of solute, there was no protection from heat injury (by definition), and the RIV was set arbitrarily at 100; the smaller the RIV, the more completely the solute protected *L. monocytogenes* against heat injury.

^b Water activity (a_w) values for sugars and polyols are from Chirife *et al.* (7); those for the salts are from Robinson and Stokes (17) and Troller and Christian (25).

^c — = not done

tose, xylose, glycerol or mannitol did not stimulate bacterial growth, these compounds protected *L. monocytogenes* against heat injury (Table 1). In contrast, fructose, which does stimulate bacterial growth, appeared to be lethal to *L. monocytogenes* incubated at 52°C and did not protect against injury (Fig. 3).

Discussion

Previous workers have shown that microorganisms, during sub-lethal heat stress, are protected from injury if certain solutes are present in the heating menstruum. The amount of protection against heat injury and death in *Salmonella typhimurium* increased as the sucrose level in the heating medium increased (13). The addition of salts, polyols, sugars or amino acids to the buffer heating medium protected *S. aureus* against heat injury (20,23,24). Polyols or sugars also decreased the extent of acid injury in *S. aureus* (21). In this study, salts, polyols and sugars protected *L. monocytogenes* against heat injury. However, the mechanism by which solutes exert their protective effects on bacteria undergoing injury is unknown.

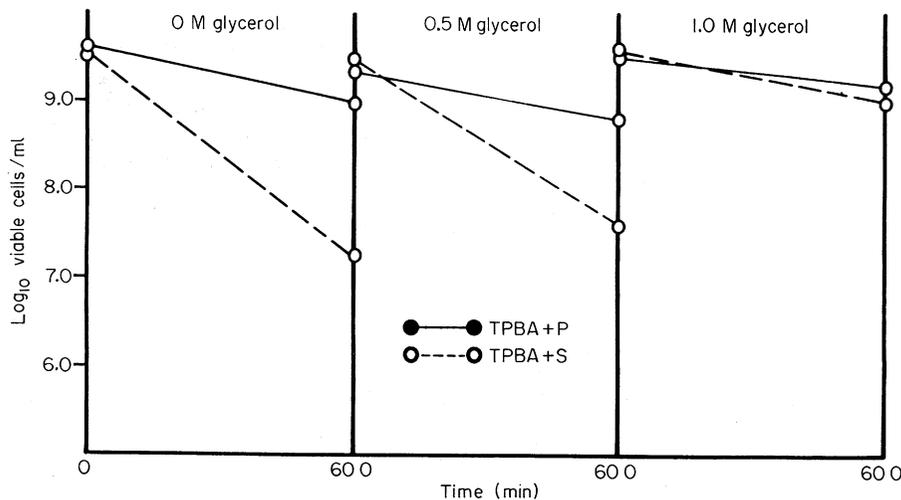


Fig. 1 Effect of glycerol on heat injury in *L. monocytogenes* (Scott A strain)

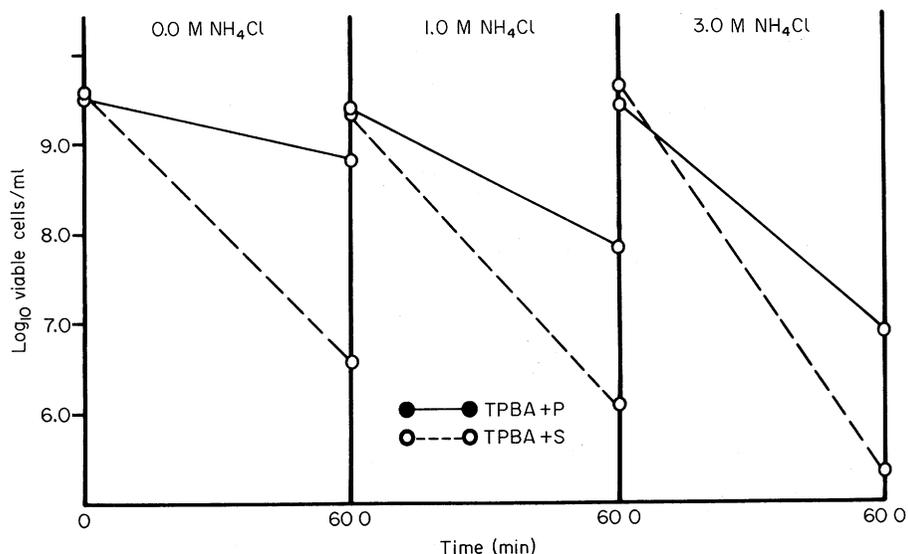


Fig. 2 Effect of NH₄Cl on heat injury in *L. monocytogenes* (Scott A strain)

Solutes were used in the present study at levels that led to a decrease in water activity (a_w). In Table 1, a_w of NaCl or KCl that gave similar protection to that of polyols or sugars was 0.893 (NaCl) or 0.904 (KCl); the a_w values of the polyols or sugars ranged from 0.990 to 0.968 (for the 1 M level; Table 1). Protection from injury given by the solutes was associated with a_w values from 0.990 to 0.893. Thus, a_w appears not to be a major determinant of solute protection against heat injury. A similar conclusion was reached previously concerning solute protection against injury in *S. aureus* (21,22).

The observations that NH₄Cl and fructose did not protect *L. monocytogenes* against heat injury but led to killing of the cells at the injury temperature are interesting (Figs 2 and 3). Ammonium chloride at a_w values ranging from 0.900 to 0.975 (16.6–4.3%) protected *S. aureus* from heat injury (20,23). In contrast, similar levels of NH₄Cl did not protect *L. monocytogenes* against heat injury. Fructose was shown to protect *S. aureus* against heat-induced injury (20). In the present study, however, fructose, unlike the other sugars tested, was not protective and induced killing of the cells at 52°C. Fructose has a 35- to 350-fold higher carbonyl content than xylose, galactose, glucose or mannose in aqueous solution (12) and the carbonyl content increases with increased temperature. This type of instability may allow fructose to combine

with cell constituents to produce non-enzymatic browning reaction products (6). Einarsson *et al.* (9) have shown that browning reaction products may be bactericidal; this may explain why fructose did not protect *L. monocytogenes* during heating.

There does not appear to be a correlation between growth-promoting effect of sugars or polyols and protection against heat injury in *L. monocytogenes*. Xylose, galactose, glycerol or mannitol were not utilized for growth yet were protective. Fructose, which was utilized by *L. monocytogenes*, did not protect against heat injury. Smith and co-workers (24) showed no correlation with the ability of sugars or polyols to protect *S. aureus* against heat injury and the ability of *S. aureus* to metabolize those protective compounds. In addition, glucose protection of *S. aureus* against heat injury was not prevented when a number of metabolic inhibitors were also present during heating (24).

It is generally accepted that leakage of cellular material from the bacterial cell is concomitant with heat injury (19). Lee and Goepfert (13) suggested that sucrose protected *S. typhimurium* against heat injury and leakage of ultraviolet-absorbing materials from the cell interior by stabilizing the bacterial membrane much in the manner analogous to protoplast stabilization by solutes. McQuillen (15) indicated that at 1.5 molal

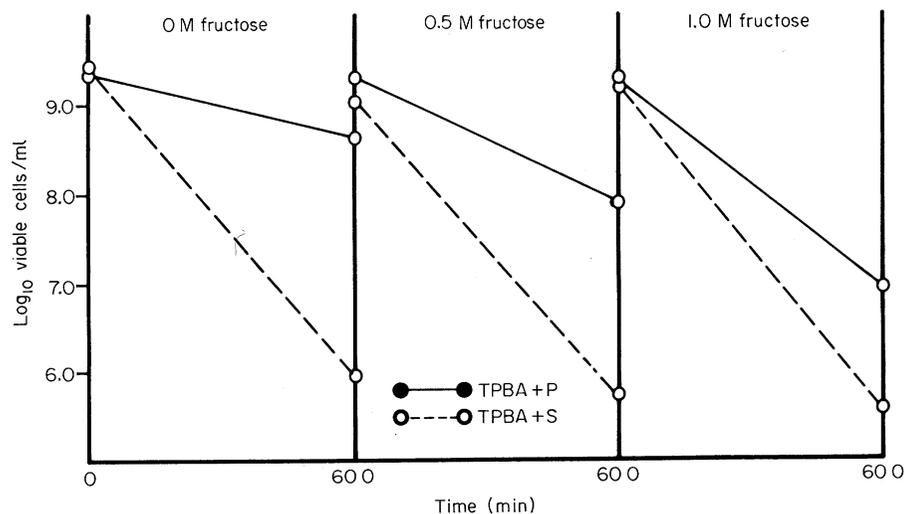


Fig. 3 Effect of fructose on heat injury in *L. monocytogenes* (Scott A strain)

NH₄Cl, KCl, NaCl, glucose, fructose, mannose, galactose and sucrose were very effective in stabilizing protoplasts of *Micrococcus lysodeikticus*, *Sarcina lutea* or *S. aureus* against lysis; glycerol, however, was not effective since it penetrated the protoplast rapidly.

In addition to Lee and Goepfert's (13) observation that sucrose decreased leakage of ultraviolet-absorbing materials while protecting *S. typhimurium* against heat injury, Smith *et al.* (20) found that most solutes—salts, sugars, and amino acids decreased the extent of leakage of 260- and 280-nm absorbing compounds while protecting *S. aureus* against heat injury. However, polyols (sorbitol, mannitol, xylitol and glycerol) and fructose protected *S. aureus* against heat injury but did not prevent leakage. These compounds actually increased leakage when compared to the unprotected control (20). Solutes may thus be protective against heat injury but may not necessarily prevent leakage of cellular materials. The hypothesis that solutes prevent bacterial injury (and concomitant leakage of cellular materials) by stabilizing the bacterial membrane is attractive. However, the observation that certain compounds protect against injury but not against leakage suggests that the hypothesis of solute protection against heat injury via membrane stabilization is not valid. Many sugars and polyols were found to stabilize aqueous solutions of purified proteins against heat denaturation (4). The stabilizing effects of glycerol, glucose, lactose, sucrose or NaCl resulted from preferential hydration of the proteins. Hydration hindered the unfolding (denaturation) of the proteins because of an increase in the energy of activation of the denaturation reaction. The native configuration was favored, and transition to the denatured state was prevented (2,3,10,14). The stabilizing effects of solutes on proteins result from an increase in the surface tension of the solvent water. Glycerol, however, actually decreases the surface tension of water (10); therefore, glycerol must have a different mechanism for hydrating and stabilizing proteins. Although solutions of pure proteins can be protected from heat denaturation by the presence of solutes, it can not be assumed that this type of stabilization can be applied to intact bacterial cells. Some of the protective sugars and polyols used in this study were not utilized by *L. monocytogenes*. In the absence of a mechanism to transport non-metabolizable carbohydrates across the bacterial membrane, the non-metabolizable protective compounds would be unable to prevent heat denaturation of internal proteins.

In the presence of solutes, dehydration of the microbial cell may occur. *Escherichia coli*, in NaCl- or sucrose-containing media, showed a decrease in cell volume (1); however, glycerol did not show such an effect since it was freely permeable. Passage of water across the cytoplasmic membrane led to plasmolysis (shrinkage of the protoplast) with resultant decrease in the water content of the cell. Bacteria are more resistant to the damaging effects of heat when they are in a dry state. Gibson (11) and Corry (8) showed that the increase in heat resistance of salmonellae and yeast in the presence of sugars or polyols was correlated with cell shrinkage induced by the solutes. Ng (16) suggested that the increase in heat resistance resulting when solutes were present was due to the dehydration of the bacterial cell. The cells internal a_w is decreased, leading to stabilization of the interior milieu against harmful effects of heat. If dehydration of *L. monocytogenes* occurs in the presence of high concentrations of solutes, such cells would be stable to sublethal heating and escape injury.

In terms of food microbiology, solute protection against heat injury in *L. monocytogenes* represents a concern to the consuming public because it indicates that foods containing certain solutes must be processed at higher temperatures in order to obtain complete destruction of the organism. Food processors need to monitor their heating processes carefully for solute-containing foods in order to ensure the absence of injured *L. monocytogenes*. On a more positive note, the induction of

death of *L. monocytogenes* during heating in the presence of fructose and the failure of that sugar to protect against heat injury suggest that *Listeria* may be more susceptible to the effects of heat in foods containing high-fructose syrups.

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Meetings, Exhibitions

20–24 February 1989: Stuttgart (West Germany)

IUFoST Symposium on Food and Biotechnology

Topics: Generation of food raw materials and additives by means of biotechnological methods; Bioengineering in food technology; Production and application of enzymes; Application of Microorganisms and cell cultures

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