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Adaptation to Sublethal Environmental Stresses Protects *Listeria monocytogenes* against Lethal Preservation Factors

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A sublethal dose of ethanol (5%, vol/vol), acid (HCl, pH 4.5 to 5.0), H_2O_2 (500 ppm), or NaCl (7%, wt/vol) was added to a *Listeria monocytogenes* culture at the exponential phase, and the cells were allowed to grow for 1 h. Exponential-phase cells also were heat shocked at 45°C for 1 h. The stress-adapted cells were then subjected to the following factors at the indicated lethal levels—NaCl (25%, wt/vol), ethanol (17.5%, vol/vol), hydrogen peroxide (0.1%, wt/vol), acid (pH 3.5), and starvation in 0.1 M phosphate buffer at pH 7.0 (up to 300 h). Viable counts of the pathogen, after the treatment, were determined on Trypticase soy agar-yeast extract, and survivor plots were constructed. The area ($h \cdot \log_{10}$ CFU/ml) between the control and treatment curves was calculated to represent the protective effect resulting from adaptation to the sublethal stress factor. Adaptation to pH 4.5 to 5.0 or 5% ethanol significantly ($P < 0.05$) increased the resistance of *L. monocytogenes* to lethal doses of acid, ethanol, and H_2O_2. Adaptation to ethanol significantly ($P < 0.05$) increased the resistance to 25% NaCl. When *L. monocytogenes* was adapted to 500 ppm of H_2O_2, 7% NaCl, or heat, resistance of the pathogen to 1% hydrogen peroxide increased significantly ($P < 0.05$). Heat shock significantly ($P < 0.05$) increased the resistance to ethanol and NaCl. Therefore, the occurrence of stress protection after adaptation of *L. monocytogenes* to environmental stresses depends on the type of stress encountered and the lethal factor applied. This "stress hardening" should be considered when current food processing technologies are modified or new ones are developed.

Food-borne pathogens are commonly stressed during food processing. In cheese manufacture, for example, pathogens existing in the raw milk undergo a series of stresses, which include heat during milk thermal treatment, hydrogen peroxide that may be added to the raw milk, acid produced by the starter cultures during the fermentation, and salt added to the curd. During sausage fermentation, food-borne pathogens are stressed by added salt, acid developed during fermentation, and heat during cooking and smoking. Food preservation and safety assurance are associated with these stresses, which are considered hurdles to the food-borne pathogens (12).

However, a phenomenon called stress hardening, which refers to the increased resistance to lethal factors after adaptation to environmental stresses, may counteract the effectiveness of food preservation hurdles and compromise food safety. In a previous study, we demonstrated that adaptation of *Listeria monocytogenes* to starvation, ethanol, hydrogen peroxide, and acid significantly increased the resistance of this pathogen to heat (16). Low heating rates, which mimic heat shock conditions, increased the thermotolerance of *L. monocytogenes* (20). This pathogen exhibited a higher thermotolerance when heated at less than 5°C/min than when heated at a greater rate (20). Acid-adapted *Escherichia coli* was more resistant to weak organic acids (propionic, lactic, acetic, benzoic, sorbic, and trans-cinnamic) than the nonadapted cells (6, 7). Acid-adapted *Salmonella typhimurium* had an increased acid resistance (4). Acid-adapted *Salmonella* spp. survived better during cheese ripening or storage than did the non-acid-adapted cells (13). Besides the increased resistance to inactivation during food processing, stress-hardened pathogens may also have increased virulence, since pathogens may sense environmental stresses as signals for the expression of virulence factors (1, 11, 17).

The phenomenon of stress adaptation and protection has been studied more extensively in model microorganisms like *E. coli* and *Saccharomyces cerevisiae* than in food-borne pathogenic bacteria. The objective of this research is to investigate if adaptation to certain environmental stresses (i.e., acid, ethanol, hydrogen peroxide, heat, and NaCl) increases the resistance of *L. monocytogenes* to lethal levels of the same (homologous) or different (heterologous) types of stresses. Relevance of these stresses and lethal factors to food processing and preservation are emphasized.

MATERIALS AND METHODS

Microorganism. *L. monocytogenes* Scott A from the culture collection of the Food Microbiology Laboratory of Ohio State University was used throughout the experiment. Stock culture was stored at −20°C in Trypticase soy broth (BBL, Cockeysville, Md.) supplemented with 0.6% Bacto yeast extract (Difco, Detroit, Mich.) (TSBYE) and 10% (vol/vol) glycerol. The stock culture was transferred at least twice in 10 ml of TSBYE and incubated overnight at 35°C before use.

Heat shock. A portion (10 μl) of the *L. monocytogenes* Scott A culture was inoculated into 100 ml of TSBYE in a 250-ml flask. The inoculated medium was incubated at 35°C with shaking at 200 rpm in a shaker-incubator (model G24; New Brunswick Scientific Co., Inc., Edison, N.J.). When the optical density at 600 nm, measured with a spectrophotometer (Spectronic 1201; Milton Roy Co., Rochester, N.Y.), of the culture reached ca. 0.3, the cells were harvested by centrifugation at 3,000 × *g* in a refrigerated centrifuge (Sorvall RC-5B Superspeed Centrifuge; DuPont Instruments, Wilmington, Del.). The harvested cells were washed once in 10 ml of TSBYE and resuspended in 10 ml of the same medium. The cell suspension was heat shocked by holding it at 45°C for 1 h in a thermostatically controlled circulating water bath (Precision Scientific Inc., Chicago, Ill.). The cell suspension was then cooled in a bath of ice-water mixture and washed twice with 10 ml of prechilled (4°C) 0.1 M phosphate buffer (PB) or 0.85% saline at pH 7.0, before it was challenged with the lethal factors. For lethal acid challenge, only heat-shocked cells suspended in saline solution were used.

Adaptation to sublethal stress of ethanol, acid, H_2O_2, or NaCl. *L. monocytogenes* Scott A cultures were grown as described earlier. When *L. monocytogenes* in TSBYE grew to an optical density at 600 nm of 0.1 to 0.2, the cultures were treated with the following sublethal factors at the levels that earlier gave maximal thermotolerance (16): 5% (vol/vol) ethanol (95%; Aaper Alcohol and Chemical Co., Shelbyville, Ky.), hydrochloric acid (36.5%; Fisher Scientific, Fair Lawn,

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TABLE 1. Averages of protection areas as measures of resistance of *L. monocytogenes* to lethal factors, after the pathogen was adapted to sublethal stresses

Sublethal stress	Resistance to lethal factor[a]:			
	Acid (pH 3.5)	Ethanol (17.5%)	H_2O_2 (0.1%)	NaCl (25%)
pH 5.0	6.1*	9.7*	41*	337
pH 5.0, then 4.5	6.4***	7.5	47**	247
pH 4.5	3.1**	−3.3	54*	228
Ethanol (5%)	4.8*	8.0***	45*	563*
H_2O_2 (0.05%)	−2.0	−7.5	48**	−11
Heat shock	−3.2	16*	15*	401*
NaCl (7%)	−5.4	−0.4	50*	NA[b]

[a] Resistance data are averages of protection areas ($h \cdot \log_{10}$ CFU/ml). Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; values without superscripts are not significantly different from zero, which indicates no significant adaptive protection.

[b] NA, data not available.

N.J.) to adjust pH to 4.5 to 5.0, 500 ppm of H_2O_2 (3%; Cumberland, Smyrna, Tenn.), or 7% (wt/vol) NaCl (Jenneile Enterprises, Cincinnati, Ohio). *L. monocytogenes* cultures were incubated for 1 h in the presence of the stress factors (circa two doublings for the nonstressed culture), and the cells from both the stressed and nonstressed cultures were harvested, washed, and suspended in PB or saline solution at 4°C as indicated earlier.

Resistance to lethal doses of preservation factors. The lethal factors tested were 17.5% (vol/vol) ethanol, pH 3.5 (0.1 M citrate phosphate buffer), 0.1% (wt/vol) H_2O_2, 25% (wt/vol) NaCl in 0.1 M PB (pH 7.0), and starvation in 0.1 M PB (pH 7.0) at 30°C. The level of each lethal factor used in this study was determined by preliminary experiments, in which several levels of each lethal factor were tested.

A portion (1 ml) of stress-adapted or non-stress-adapted *L. monocytogenes* culture (ca. 10^9 CFU/ml) was mixed with 10 ml of the medium containing the lethal factor in screw-capped test tubes (16 by 125 mm). In the case of starvation, 1 ml of the cell suspension was mixed with 10 ml of PB, and the tubes were incubated at 30°C. During the inactivation by the lethal factors, samples were drawn at predetermined intervals, and a portion (0.1 ml) of the sample, or dilution thereof, was surface plated onto Trypticase soy agar supplemented with 0.6% Bacto yeast extract. The plates were incubated at 35°C for 48 to 72 h, and colonies were counted. The total inactivation times, which depended on the lethal factors applied, were 4 h for acid, 10 h for ethanol or H_2O_2, 350 h for NaCl, and 300 h for starvation.

Data analysis. Survivor plots ($\log_{10}$ CFU/ml versus treatment time) were constructed. The plots were not always linear; thus, *D* values cannot be used to compare the treatments. Therefore, the protection area, which is analogous to the area of inhibition of Wenzel and Marth (24), was used to compare these treatments. The protection area is defined as the area ($h \cdot \log_{10}$ CFU/ml) between the treatment and the control curves, which equals the area under the treatment curve minus the area under the control curve. The size of the protection area is affected by the initial microbial level before the lethal challenge and the total treatment (inactivation) time. For all of the combinations of a single lethal factor with different sublethal stresses, we used the same initial level of the microorganism and the same total inactivation time. Therefore, only comparisons of the protection areas within a single lethal factor are meaningful (Table 1). A positive protection area means that preadaptation to stress increases the survival of *L. monocytogenes* under the lethal factor. The magnitude of this area indicates the degree of protection afforded by the stress factor. The experiments were independently repeated at least twice. The averages of the protection areas for each combination of adaptation and lethal factors were reported. Statistical significance of the treatments was determined with the Student's *t* test (Minitab software; Minitab Inc., State College, Pa.). When the average protection area is significantly greater than zero, this indicates that adaptation to the stress protects *L. monocytogenes* against the lethal factor.

RESULTS AND DISCUSSION

Resistance to acid. *L. monocytogenes* cultures were separately adapted to three acid conditions, (i) pH 5.0, (ii) pH 4.5, and (iii) pH 5.0, followed by additional incubation at pH 4.5. These treatments mimic the direct exposure to mild-acid, high-acid, and stepwise or gradual exposure to high-acid conditions, respectively. Preincubation of *L. monocytogenes* at any of the three acid conditions significantly ($P < 0.05$) increased the resistance of the pathogen to the lethal acid condition at pH 3.5 (Table 1; Fig. 1a). Treatments i and iii were not significantly different ($P > 0.05$) in increasing the resistance of *L. monocytogenes* to pH 3.5. However, resistance of the pathogen to the lethal acid condition was significantly ($P < 0.05$) greater after adaptation to the mild-acid condition (i) or the stepwise increase to the high-acid condition (iii) than to the high-acid condition (ii) (Fig. 1a).

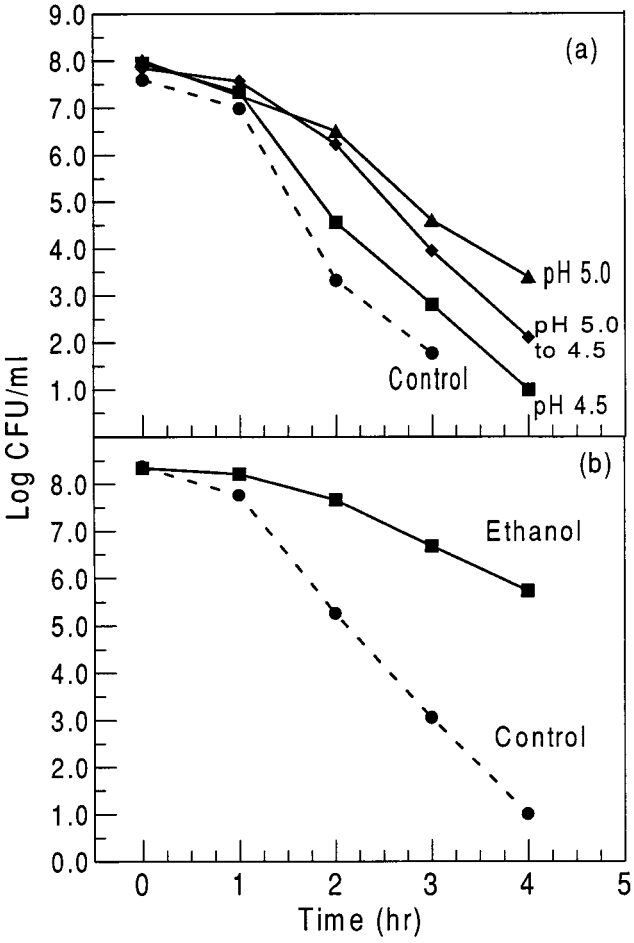

FIG. 1. Survival of *L. monocytogenes* at pH 3.5 after preincubation at pH 5.0, pH 4.5, or pH 5.0 followed by incubation at pH 4.5 (a) and in TSBYE containing 5% ethanol (b); data are for one of three to five trials.

Treatments i and iii are common in food production and processing. Food fermentation involves a gradual decrease in pH, which could lead to acid adaptation of *L. monocytogenes*. When this food is ingested, the acid-adapted *L. monocytogenes* may survive the low pH of the stomach and cause a food-transmitted illness. In nature, microorganisms present in fecal matter and acid rain-affected or sewage water undergo acid adaptation. Acid resistance resulting from acid adaptation was also demonstrated in other microorganisms (4, 6, 7).

Adaptation to ethanol significantly ($P < 0.05$) increased the resistance of *L. monocytogenes* to lethal doses of acid (Table 1; Fig. 1b). In *S. typhimurium*, adaptation to stresses other than acid (i.e., heat, hydrogen peroxide, and osmolarity) did not protect the microorganism against acid (11). There are at least two types of acid tolerance response in *S. typhimurium*, the log-phase and the stationary-phase acid tolerance responses (8). An acid-inducible pH homeostatic system, which was

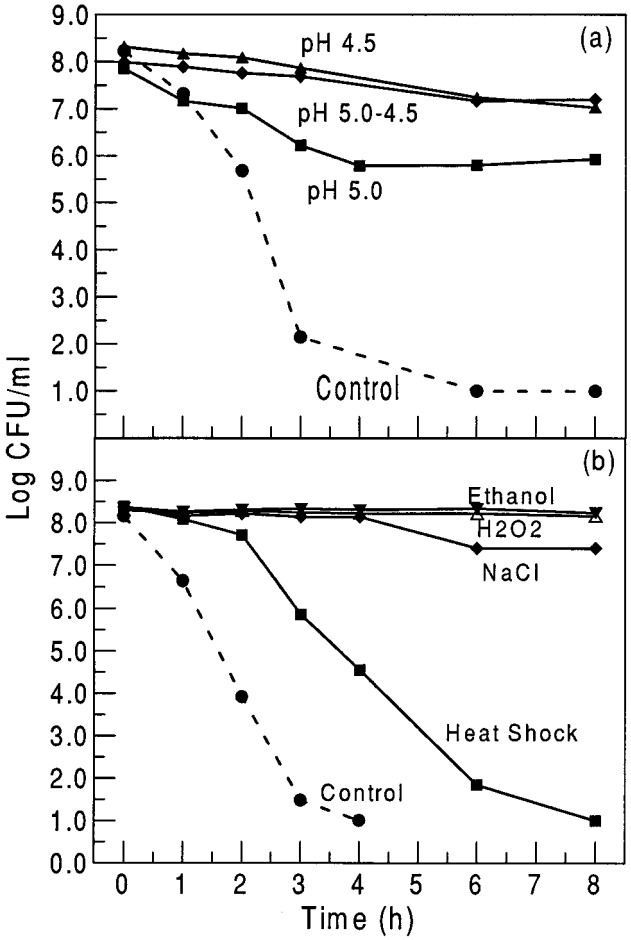

FIG. 2. Survival of *L. monocytogenes* in 0.1% hydrogen peroxide after preincubation at different pH values (a) or pretreatment with 5% ethanol, 0.05% hydrogen peroxide, or 7% sodium chloride and heat shocking at 45°C for 1 h (b); data are for one of two to four trials.

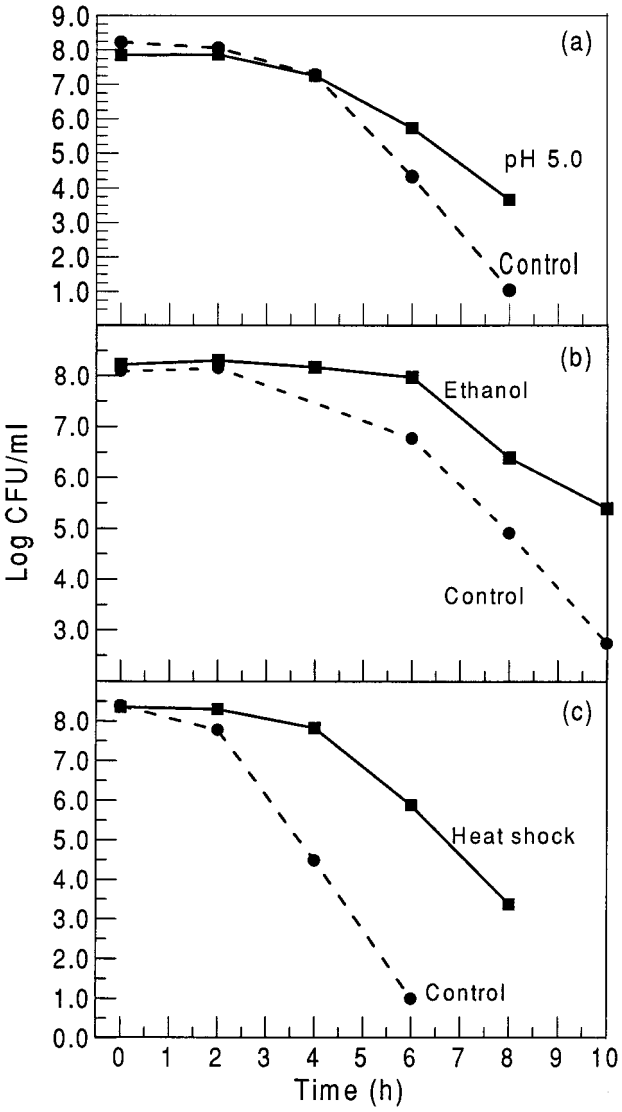

FIG. 3. Survival of *L. monocytogenes* in 17.5% (vol/vol) ethanol after preincubation at pH 5.0 (a), treatment with 5% ethanol (b), and heat shocking at 45°C for 1 h (c); data are for one of two to four trials.

found to increase the pH homeostatic capacity of *S. typhimurium* under lethal acid conditions, is induced during the adaptation (5). There are no reports indicating that similar acid adaptation mechanisms exist in *L. monocytogenes.*

Resistance to hydrogen peroxide. Adaptation to any of the stresses investigated in this study (acid, ethanol, H_2O_2, heat, or NaCl) significantly ($P < 0.05$) increased the resistance of *L. monocytogenes* to 0.1% H_2O_2 (Table 1; Fig. 2). Adaptation to 5% ethanol or 500 ppm of H_2O_2 provided the greatest protection against H_2O_2 (Fig. 2b). Protection against lethal levels of hydrogen peroxide may be partly explained by the induction of a sigma factor (σ^S, or KatF), which accounts for the general resistance to environmental stresses in microbial cells (9, 10, 15). Exposing *E. coli* cells to weak organic acids (acetate, propionate, and benzoate) increased the expression of σ^S and induced the synthesis of both HPI (*katG*) and HPII (*katE*) catalases (18). Acid-adapted *S. typhimurium* had an increased resistance to several environmental stresses, including oxidative stresses (11). Exposure of microorganisms to H_2O_2 induces the expression of OxyR (a sigma factor) regulon genes, which include *katG* encoding HPI catalase (3). Heat shock and oxidative stress may have similar mechanisms in inducing the oxidative adaptive response, because both stresses generate oxygen-derived free radicals, damage the cell membrane, and disrupt the electron transport system (19). This may account for the great protection to lethal factors after adaptation to H_2O_2 or ethanol (Fig. 2b).

Resistance to ethanol and NaCl. Heat shocking and adaptation to pH 5.0 and ethanol significantly ($P < 0.05$) increased the resistance of *L. monocytogenes* to a lethal level (17.5%) of ethanol (Table 1; Fig. 3). Heat shocking and adaptation to ethanol also significantly ($P < 0.05$) increased the resistance of *L. monocytogenes* to 25% NaCl (Table 1; Fig. 4). A study showed that adaptation of *S. typhimurium* to acid induced a general protection against various environmental stresses (11). Heat shocking cross-protected *Bacillus subtilis* against lethal salt stress (22). Ethanol adaptation and heat shock increased the resistance of *L. monocytogenes* to lethal doses of heat (14, 16). Exposure of microorganisms to sublethal levels of ethanol can induce stress proteins with a profile similar to that of stress proteins induced by heat shock (21, 23). Therefore, heat shock and adaptation to sublethal levels of ethanol may have similar effects on the behavior of *L. monocytogenes.*

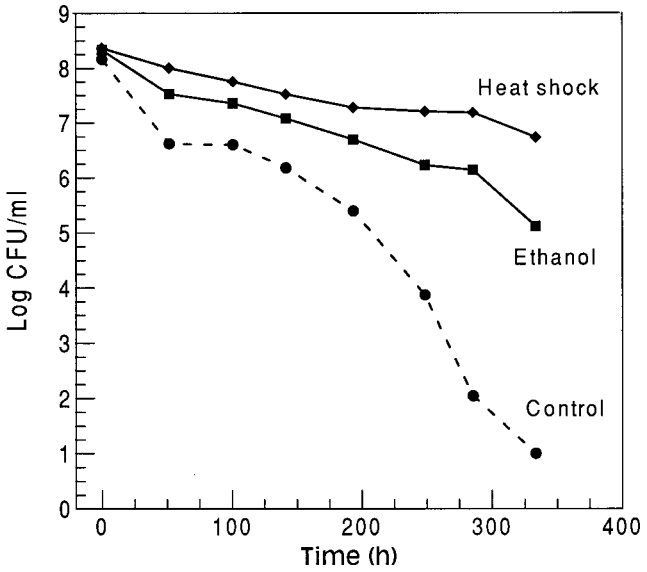

FIG. 4. Survival of *L. monocytogenes* in 25% (wt/vol) NaCl after pretreatment with 5% ethanol or heat shocking at 45°C for 1 h; data are for one of three or four trials.

Survival during starvation. Adaptation to any of the stresses tested in this study did not significantly ($P > 0.05$) increase the survival of *L. monocytogenes* during starvation in 0.1 M PB (pH 7.0) at 30°C (data not shown). Counts of stress-adapted and nonadapted *L. monocytogenes* cells decreased rapidly at the beginning of starvation. During this stage, the population of the nonstressed *L. monocytogenes* appeared to decrease more rapidly than that of the stressed cells. However, after prolonged starvation (300 h), the difference between the two treatments diminished, possibly because adaptation to the starvation stress became dominant in both treatments (data not shown).

Findings in this study support the stress hardening concept (16). The food industry relies on the hurdle concept, i.e., the combination of inhibitory factors (hurdles), for food preservation and safety (12). Hurdles in food systems can be applied simultaneously or sequentially. When applied sequentially, the hurdles may not deliver the desired effect. Stress adaptation to the first encountered hurdle, which "hardens" the pathogens and increases their resistance to subsequent preservation factors, may counteract the hurdle buildup. Therefore, the stepwise addition of hurdles (e.g., sublethal stresses) may compromise the safety of certain foods. Accordingly, studies on stress adaptation and cross-protection should help in ensuring food safety when new processing and preservation protocols are developed.

Consumers are increasingly demanding natural, healthy, and convenient foods that are low in salt and low or lacking in preservatives. The stress hardening phenomenon is particularly significant to the safety of these minimally processed foods in which food-borne pathogens are more likely to be stressed than inactivated. In these food systems, preservation hurdles of small magnitude are applied, and the pathogens may become adapted to these low hurdles. Baird-Parker (2) pointed out that these new foods are "less microbiologically robust," which may account for the rising incidence of food-borne illnesses. The stress hardening concept may also be beneficially applicable in situations where survival of useful microorganisms (e.g., *Bifidobacteria* and lactic acid bacteria) during severe stress conditions is desired.

In conclusion, we demonstrated that adaptation to one stress can protect *L. monocytogenes* against the same or certain different types of lethal stresses. This stress hardening should be considered when current food processing technologies are modified or new ones are introduced.

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