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Effect of Food Processing-Related Stresses on Acid Tolerance of *Listeria monocytogenes*

Konstantinos P. Koutsoumanis,[1]† Patricia A. Kendall,[2] and John N. Sofos[1]*

Center for Red Meat Safety, Department of Animal Sciences,[1] and Department of Food Science and Human Nutrition,[2] Colorado State University, Fort Collins, Colorado 80523

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Stationary-phase cells of *Listeria monocytogenes* grown in glucose-free or glucose-containing media were exposed for 90 min to various stresses, including acid stress (pH 4.0 to 7.0), osmotic stress (10.5 to 20.5% NaCl), and various temperatures (−5 to 50°C), and were further exposed to pH 3.5. Exposure to a mildly acidic (pH 5.0 to 6.0) environment provided protection of the pathogen against acid upon subsequent exposure. This adaptive response, however, was found to be strongly dependent on other environmental conditions during the shock, such as temperature or the simultaneous presence of a second stress factor (NaCl). Growth of *L. monocytogenes* in the presence of glucose resulted in enhanced survival of the pathogen at pH 3.5. Sublethal stresses other than acidic stresses, i.e., osmotic, heat, and low-temperature stresses, did not affect the acid resistance of *L. monocytogenes* ($P > 0.5$). More-severe levels of these stresses, however, resulted in sensitization of the pathogen to acid.

Listeria monocytogenes is not remarkably acid tolerant and cannot grow at a pH below 4.5 to 4.6 (11). However, due to a phenomenon called stress hardening, i.e., increased tolerance after adaptation to stressful environments, the organism may become highly resistant to even extremely acidic conditions (10). Indeed, several studies have demonstrated that adaptation of *L. monocytogenes* cultures to stressing environments results in increased survival under acidic conditions that would be lethal for unadapted cells (6, 7, 10, 12, 14, 16). The potential of the pathogen to alter its cellular physiology during exposure to stress conditions (1, 4, 5, 12, 19) and become more resistant to further stresses may counteract the effectiveness of food preservation or hostile environments and compromise food safety. Although a number of studies addressing the stress hardening phenomenon have been reported (6, 7, 10, 12, 14, 16), there are still gaps in data on bacterial stress response. Most of the available data on adaptive responses to stresses refer to the effect of a single stress. During food processing, however, bacteria may be exposed to more than one stress at the same time or in sequence, applied as multiple hurdles (9). The objective of the present study was to evaluate the effects of selected single or combined environmental stresses relevant to food processing on the acid resistance of *L. monocytogenes*. The data on the adaptive responses of *L. monocytogenes* provided in the present study may lead to more-realistic estimates of potential risks associated with consumption of processed foods and to a better selection of processing conditions for enhanced food safety.

A mixture of five *L. monocytogenes* strains (Na-4 [serotype 4b], 163 [serotype 4], N-7143 [serotype 3a], N-7144 [serotype 1/2b], and N-7159 [serotype 1/2a]) was used throughout the study. Portions (100 μl each) of activated cultures were transferred to 10 ml of Trypticase soy broth (TSB) without dextrose (BBL, Becton Dickinson Co., Cockeysville, Md.) and incubated at 30°C for 24 h. Cultures were also grown in the presence of glucose by using TSB without dextrose supplemented with 1% glucose and exposed to pH 3.5. The five cultures were combined and centrifuged with a Beckman model J2-21 centrifuge at 6,000 rpm for 15 min at 4°C. The cells were then washed with 10 ml of phosphate-buffered saline (PBS), centrifuged, and resuspended in 10 ml of PBS. A portion (100 μl) of the suspension was used to inoculate 10 ml of TSB without dextrose. The cells in TSB without dextrose were exposed for 90 min to the following conditions: (i) pH 4.0, 4.5, 5.0, 5.5, 6.0, or 7.0 at 30°C; (ii) pH 5.5 at 30, 15, or 4°C; (iii) pH 5.5 with 10.5% NaCl at 30°C; (iv) pH 7.4 (the normal pH of TSB) with 10.5 or 20.5% NaCl at 30°C; (v) pH 7.4 at 45 or 50°C; and (vi) pH 7.4 at −5°C. The pH of the medium was adjusted to the tested values with lactic acid (85% [wt/wt] DL-lactic acid; Sigma, St. Louis, Mo.) and measured with a digital pH meter (Accumet 50; Fisher Scientific, Houston, Tex.) with a glass pH electrode (Hanna Instruments, Ann Arbor, Mich.). For heat stressing, a thermostatically controlled water bath (Isotemp 228; Fisher Scientific) was used.

After exposure to stress, the cultures were centrifuged again with the Beckman model J2-21 centrifuge at 6,000 rpm for 15 min at 4°C. The harvested cells were resuspended in 1 ml of PBS, which was used to inoculate 10 ml of TSB without dextrose that had been adjusted to pH 3.5 with lactic acid (85%; Mallinckrodt). The inoculum contained approximately 10^7 to 10^8 CFU/ml, and exposure of the cultures to the different stresses for 90 min did not result in significant ($P > 0.05$) changes in the pathogen population. The samples were incubated at 30°C for 6 h. Every hour, 1 ml of the sample was removed and 0.1 ml of the appropriate dilution was surface plated on duplicate plates of tryptic soy agar (Difco, Becton Dickinson Co., Sparks, Md.). The plates were incubated at 30°C for 48 h, and colonies were counted. Two replicates of the experiments, each involving the analysis of two samples, were conducted. With SAS software (Statistical Analysis

* Corresponding author. Mailing address: Center for Red Meat Safety, Department of Animal Sciences, Colorado State University, Fort Collins, CO 80523. Phone: (970) 491-7703. Fax: (970) 491-0278. E-mail: john.sofos@colostate.edu.

† Present address: Aristotle University of Thessaloniki, Faculty of Agriculture, Department of Food Science and Technology, Thessaloniki 54124, Greece.

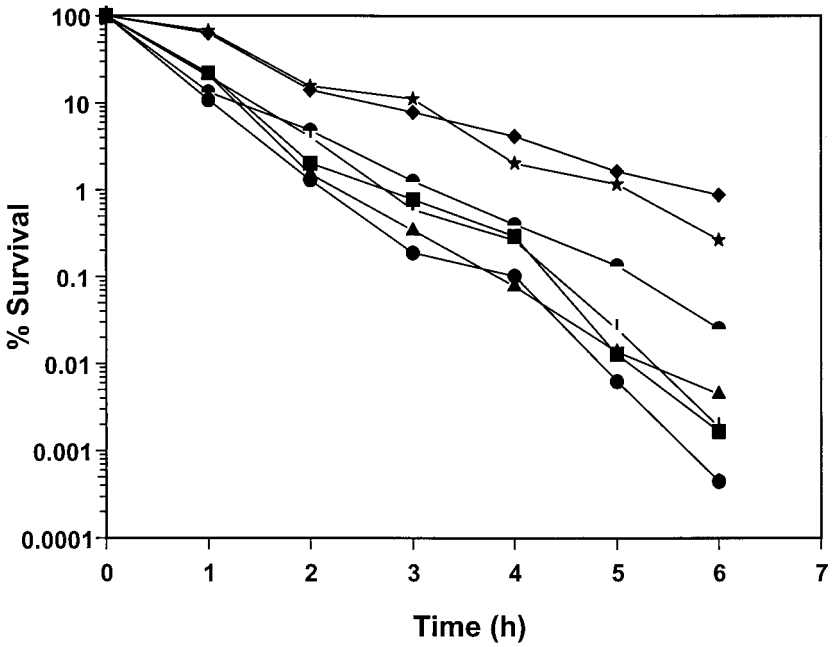

FIG. 1. Survival of *L. monocytogenes* (grown in TSB without glucose) at pH 3.5 after exposure to different pH shocks at 30°C for 90 min. ■, control (no shock); ●, pH 4.0; ▲, pH 4.5; ★, pH 5.0; ◆, pH 5.5; [◓], pH 6.0; |, pH 7.0. Each point is a mean of four values.

System for Windows, version 8.0; Statistical Analysis Systems Institute, Cary, N.C.), the data were fitted to the exponential model, $N_t = N_0 \times e^{-kt}$, where t is time (in hours), N_t is the population in number of CFU per milliliter at time t, N_0 is the population at time zero, and k is the death rate (per hour). The statistical significance of the different treatments was determined on the basis of the death rate by Student's t test with the SAS software.

Although the increased acid resistance of *L. monocytogenes* after exposure to mildly acidic conditions has been reported previously (6, 7, 10, 12), data on the range of pH values to which the organism can adapt after exposure or the optimum pH for adaptation are limited. In the present study, a mild acid shock at pH 5.0, 5.5, or 6.0 resulted in an increase in the survival of the pathogen ($P < 0.05$) at normally lethal acid conditions (pH 3.5) compared to that observed for nonshocked cultures (control) (Fig. 1 and Table 1). As shown in Fig. 1, *L. monocytogenes* at pH 3.5 followed a very close to linear logarithmic reduction. The acid resistance levels of cultures exposed to pHs of 4.0, 4.5, and 7.0 were not different ($P > 0.05$) from that of the control (Table 1).

In most of the published studies referring to stress adaptation of microorganisms, cultures are exposed to the stress under optimum-temperature conditions (6, 7, 10, 12). However, during processing, distribution, and storage, the temperature of foods

TABLE 1. Death rates of *L. monocytogenes* at pH 3.5 after exposure to different types of stressing treatments for 90 min

Treatment	Initial shock	Death rate (h^{-1})[a]	SD	r^2 (minimum-maximum)[c]
1	No shock (control)	1.81	0.14	0.976–0.980
2	pH 4.0 at 30°C	1.93	0.09	0.978–0.986
3	pH 4.5 at 30°C	1.75	0.16	0.988–0.990
4	pH 5.0 at 30°C	0.92*	0.01	0.941–0.987
5	pH 5.5 at 30°C	0.81*	0.08	0.938–0.978
6	pH 6.0 at 30°C	1.32*	0.08	0.972–0.994
7	pH 7.0 at 30°C	1.79	0.18	0.982–0.986
8	pH 5.5 at 15°C	1.20*	0.01	0.974–0.983
9	pH 5.5 at 4°C	1.51	0.16	0.992–0.996
10	pH 5.5 + 10.5% NaCl at 30°C	1.24*	0.04	0.969–0.977
11	Gradual decrease of pH[b]	0.34*	0.03	0.908–0.944
12	10.5% NaCl at 30°C	1.83	0.14	0.951–0.961
13	20.5% NaCl at 30°C	2.06*	0.02	0.982–0.985
14	45°C	1.60	0.20	0.964–0.987
15	50°C	2.13*	0.01	0.984–0.993
16	−5°C	1.85	0.06	0.981–0.991

[a] Each number is a mean of four values. Values without an asterisk are not statistically different ($P > 0.05$) from values for nonshocked (control) cultures. An asterisk indicates that the value is statistically different ($P < 0.05$) from that for the nonshocked (control) culture.
[b] Cultures grown in TSB with 1% glucose at 30°C for 24 h.
[c] Correlation coefficient of data fit to the exponential model.

may vary significantly. Thus, investigation of the temperature effect on the stress hardening phenomenon is of great interest. *L. monocytogenes* was exposed for 90 min to a mild acid shock (pH 5.5) at different temperatures (4, 15, and 30°C). The results obtained showed that the acid tolerance induced by the acidic shock was strongly dependent on temperature, since cells became less resistant to acid as the temperature during the shock decreased from 30 to 4°C (Table 1). These results could lead to the conclusion that limited acid-adaptive responses should be expected at refrigeration temperatures. However, more research is needed to determine the effect of time of exposure to stress at low temperatures on stress adaptation.

A common combination of hurdles applied during food processing is acid-osmotic stress (17). The presence of 10.5% NaCl during exposure of *L. monocytogenes* to pH 5.5 resulted in a decrease in the acid resistance of the pathogen, although its resistance level was still significantly ($P < 0.05$) higher than that of nonshocked cells (Table 1).

Fermentation is another food process that leads to stressful conditions under which acid-adaptive responses by pathogens may occur. During fermentation, bacteria are exposed to a gradual decrease in pH rather than a stable low-pH environment (18). Growth of the pathogen in the presence of glucose resulted in enhanced survival at pH 3.5 compared to survival levels observed for acid-shocked and nonshocked (control) cultures (Table 1). The induction of acid tolerance due to glucose metabolism has been studied extensively during the last decade (2, 3, 13). However, in studies of bacterial survival in acidic environments and stress-adaptive responses, the presence of glucose in the growth medium has been ignored. Since even small amounts of glucose or other fermentable carbohydrates can lead to acid-adapted cells (2, 3, 14–16), it is important to take this effect into account when the acid resistance of microorganisms is being investigated.

In addition to acid stress, pathogens may be exposed to a series of other stresses during food processing, such as osmotic shock when salts are used or during drying, heat shock during cooking, or low-temperature shock during refrigerated storage. In the present study, exposure of nonadapted *L. monocytogenes* to 10.5% NaCl did not enhance the survival of the pathogen at pH 3.5 (Table 1). In contrast, an increase in the concentration of NaCl to 20.5% resulted in more acid-sensitive cells (Table 1). Similar responses were also obtained when cultures were exposed to heat stress. Nonadapted cultures exposed to mild heat (45°C) did not show any increase in resistance to acid, while exposure to a higher-temperature (50°C) shock resulted in faster death at pH 3.5 (Table 1). A 90-min shock at −5°C did not affect the acid resistance of the pathogen (Table 1). Overall, the data derived from exposure of the organism to osmotic, heat, and low-temperature stresses showed no significant induction of acid tolerance in *L. monocytogenes*. This finding is in agreement with those of other *L. monocytogenes* and salmonella studies, which found that stresses other than acid (heat, H_2O_2, and osmolarity) do not protect the pathogens against acid (8, 10). In the present study, however, exposure of *L. monocytogenes* to more-severe stresses (close to lethal levels; 20.5% NaCl, 50°C) resulted in sensitization of the pathogen to acid.

In conclusion, the results of the present study demonstrate that prior adaptation of *L. monocytogenes* to mildly acidic environments may result in an increase in the acid resistance of the pathogen. The adaptive response, however, is a complicated process and depends on a number of factors, such as the pH of the stress, the temperature during stress, the presence of additional stress factors, and previous growth conditions. These findings may have important implications in the food industry, since they can lead to more-realistic estimates of risks and help in the development of effective critical control points to enhance the safety of processed food products.

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