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Acid Adaptation Promotes Survival of *Salmonella* spp. in Cheese

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***Salmonella typhimurium* was adapted to acid by exposure to hydrochloric acid at pH 5.8 for one to two doublings. Acid-adapted cells had increased resistance to inactivation by organic acids commonly present in cheese, including lactic, propionic, and acetic acids. Recovery of cells during the treatment with organic acids was increased 1,000-fold by inclusion of 0.1% sodium pyruvate in the recovery medium. Acid-adapted *S. typhimurium* cells survived better than nonadapted cells during a milk fermentation by a lactic acid culture. Acid-adapted cells also showed enhanced survival over a period of two months in cheddar, Swiss, and mozzarella cheeses kept at 5°C. Acid adaptation was found in *Salmonella* spp., including *Salmonella enteritidis*, *Salmonella choleraesuis* subsp. *choleraesuis* serotype heidelberg, and *Salmonella choleraesuis* subsp. *choleraesuis* serotype javiana, associated with food poisoning. These observations support the theory that acid adaptation is an important survival mechanism enabling *Salmonella* spp. to persist in fermented dairy products and possibly other acidic food products.**

Salmonella spp. have been implicated as the causative agent of food-borne illness involving several dairy foods, including natural cheese, and continue to be a concern to the dairy industry (16, 19). In 1989, *Salmonella choleraesuis* subsp. *choleraesuis* serotype javiana was epidemiologically implicated in an outbreak in Minnesota and Wisconsin of salmonellosis caused by the consumption of mozzarella cheese (24). The *S. choleraesuis* subsp. *choleraesuis* serotype javiana isolates from the contaminated cheese did not have unusual resistance characteristics and would not survive pasteurization or the stretching and molding procedure used in the manufacture of mozzarella cheese (6). The serotype javiana outbreak and most other outbreaks of salmonellosis involving cheese and other dairy products have been caused by faulty pasteurization of milk or post-pasteurization contamination of a product and survival of the pathogen at infectious levels (16).

The factors in cheese that govern the growth and survival of *Salmonella* spp. are incompletely understood. It is likely that increased water activity (a_w) and lower acidity favor survival (reviewed in references 16 and 19). Several previous studies evaluated the survival of *Salmonella* spp. during the preparation and curing of various cheeses (10, 12, 26, 31). These studies demonstrated that when milk became contaminated with *Salmonella* spp. after pasteurization, the pathogens could survive the cheesemaking process and persist for several months in the cheese. However, the survival of *Salmonella* spp. varied considerably in different cheeses and batches, suggesting that the strain and the physiological status of the pathogen may influence survival. In these challenge studies, the *Salmonella* spp. used for inoculation were taken from cultures grown for 18 to 24 h in nutrient broth or Trypticase soy broth (10, 12, 26, 31). The physiology of these salmonellae may not accurately represent that of salmonellae found in dairy plants and the processing environment as the latter organisms may have been exposed to sanitizers or other agents or may have been derived from the feces of human carriers.

Salmonella spp. are known to induce adaptive responses to various stresses, including acids, salts, and temperature, and these adaptive responses may enhance survival in deleterious environments (14). Foster and Hall (8, 9) demonstrated that *Salmonella typhimurium* expresses an acid tolerance response when exposed to external pHs of 5.5 to 6.0 and that the response protects the cells from more severe acid stress. Acid adaptation involves the expression of 18 proteins and serves to provide pH homeostasis and to maintain viability. Acid adaptation and increased resistance to acid stress have also been observed in *Escherichia coli* (11).

Because the microbiological safety of cheese and other fermented dairy products depends in part on acid formation by starter bacteria, we were interested in determining whether acid adaptation would affect the survival of *Salmonella* spp. in these foods. Our results indicate that acid adaptation prolongs the survival of *Salmonella typhimurium* in fermented milk and cheese. These results support the theory that the physiological state of the pathogens used in challenge studies is critical in evaluating the safety of a food and in evaluating Hazard Analysis Critical Control Point programs.

MATERIALS AND METHODS

Chemicals. Chemicals and catalase were purchased from Sigma Chemical Co., St. Louis, Mo. Complex media and ingredients were purchased from Difco Laboratories, Detroit, Mich.

Bacterial strains and media. *S. typhimurium* LT2 (provided by Laszlo Csonka, Purdue University) was used in most experiments. Other *Salmonella* spp., including *Salmonella enteritidis* E40 and *Salmonella choleraesuis* subsp. *choleraesuis* serotype heidelberg SHL 39902 from our laboratory collection and *S. choleraesuis* subsp. *choleraesuis* serotype javiana from the American Type Culture Collection, were also used. Cultures were maintained at 4°C with monthly transfers on tryptose phosphate agar (TPA) plus 0.1% sodium pyruvate (28). TPA consists of the following, per liter: tryptose, 20 g; yeast extract, 2.5 g; glucose, 2.0 g; $Na_2HPO_4 \cdot 7H_2O$, 2.5 g; NaCl, 2.5 g; sodium pyruvate, 1.0 g; and agar, 15 g. Sodium pyruvate was added prior to autoclaving

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(23). *Salmonella* spp. were also cultured in medium E with 0.4% glucose (30), LB medium (5), and bismuth sulfite agar (Difco). Cell growth in broth was monitored by measuring turbidity at 600 nm with a Spectronic 20D instrument (Milton Roy Co., Rochester, N.Y.).

Lyophilized cultures of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus helveticus* (Eurozyme, Paris, France), used as starter cultures in milk fermentation experiments, were kept at −20°C.

Adaptation of salmonellae to acid and determination of survival. The procedure of Foster and Hall (8) for acid adaptation was used, with minor modifications. *S. typhimurium* LT2 was inoculated into 13 ml of medium E (pH 7.6) with 0.4% glucose and grown statically at 37°C in culture tubes (16 by 125 mm) containing 13 ml of minimal medium E. When the culture reached an A_{600} of ~0.10, it was acidified to pH 5.8 with a small volume of 10 N HCl. The culture was allowed to grow until it had reached an A_{600} of 0.25 to 0.30 and was harvested by centrifugation. The nonadapted control was grown to an A_{600} of 0.25 to 0.30 in nonacidified medium E, and 1 ml was harvested by centrifugation. The acid-adapted and nonadapted cell pellets were washed once in 1 ml of 0.1 M phosphate buffer (pH 7.0) (PB), centrifuged, and resuspended in 100 μl of PB. These cells were used to determine resistance to acids and to inoculate milk. Cells used in the cheese studies were washed in PB and resuspended in 100 μl of 12 mM citrate–27 mM sodium phosphate buffer (pH 5.2).

For acid challenge, acid-adapted or nonadapted cells were added to 4 ml of preacidified E buffer (medium E without the glucose supplementation) to yield a cell concentration of approximately 5×10^7 cells per ml. Various acids (see Results) were added at a final concentration of 125 mM and pH 3.85. The tubes were incubated statically at 25°C, and viability was determined at various times by serial dilution in PB and plating.

Survival of *S. typhimurium* during milk fermentation. Ten milligrams each of freeze-dried cultures of *S. salivarius* subsp. *thermophilus* and *L. helveticus* was added to 100 ml of sterile skim milk (Difco) and incubated for 12 h at 37°C. Five milliliters of the active lactic acid starter culture was added to each of two flasks containing 100 ml of sterile skim milk at 37°C, and fermentation was allowed to proceed until the milk reached a pH of ~5.10. Acid-adapted or nonadapted *S. typhimurium* LT2 was added to a final concentration of approximately 10^4 cells per ml, and viable salmonellae were enumerated at various times by serial dilution in PB and plating on bismuth sulfite agar. Bismuth sulfite agar was chosen over the media selective for *Salmonella* spp., including XLD agar and Hektoen enteric agar, because it was found to promote better recovery of acid-injured cells (data not shown). Supplementation of bismuth sulfite agar with sodium pyruvate (0.1% [wt/vol]) had no effect on the recovery of cells from fermented milk or cheeses (data not shown).

Survival of *S. typhimurium* in cheeses. Swiss and mozzarella cheeses were purchased from a local grocery in Madison, Wis., and mild cheddar cheese was purchased from the Babcock Hall dairy store on the University of Wisconsin—Madison campus. Cheeses were divided into 25-g portions and placed in sterile 18-oz (ca. 532-ml) Whirl-Pak polyethylene bags (Nasco, Ft. Atkinson, Wis.). One hundred microliters of acid-adapted or nonadapted *S. typhimurium* LT2 was spread on the surface of the cheese to yield an initial concentration of ~10^4 salmonellae per g of cheese. Each bag was placed inside a vacuum seal bag and vacuum packaged (15 to 20 in. [ca. 381 to 508 torr] of Hg). The cheese samples were stored under aerobic or anaerobic conditions at 5°C. Both anaerobic and aerobic incubations were done, since these could influence the surface microflora. Aerobic incubation was done under normal atmospheric conditions. For anaerobic incubation, the vacuum-packaged cheese allotments were placed in anaerobic jars (BBL), which were then flushed with N_2 and filled with an atmosphere of 80% N_2–10% CO_2–10% H_2. Individual packages of the cheeses were sampled periodically. The 25-g cheese samples were mixed by manually squeezing the bag containing the cheese. The pH was determined by inserting an Ag-AgCl pH electrode into the cheese slurry. When necessary, a small amount (<1 ml) of sterile distilled water was added to obtain an adequate slurry of the 25-g cheese samples. Two procedures were used for the enumeration of salmonella in cheese. When counts exceeded 10/g, direct plating onto bismuth sulfite agar was used. When counts were below 10/g, a modified version of the Food and Drug Administration isolation procedure (7) combined with a three-tube most-probable-number estimate was used. The 25-g cheese samples were nonselectively enriched in 225 ml of lactose broth (Difco). Ten-milliliter portions of the diluted cheese suspensions were transferred to sterile tubes or further diluted in lactose broth and incubated for 18 to 24 h at 37°C. One-milliliter quantities were then transferred to selenite cystine broth (Difco) and incubated for 18 to 24 h at 37°C. Samples from each most-probable-numbers tube were streaked onto bismuth sulfite agar and incubated for 24 h at 37°C. The presence of salmonellae was confirmed by picking colonies and identifying them with API 20E system identification strips (Analytab Products, Plainview, N.Y.).

RESULTS

Recovery of acid-exposed cells. Since previous studies had demonstrated that acid-injured cells may not be quantitatively recovered on standard culture media (1, 23), we initially evaluated the conditions necessary for the recovery of acid-exposed *S. typhimurium*. *S. typhimurium* was acid adapted with HCl as described in Materials and Methods, and adapted or control cells were exposed to E buffer acidified with lactic acid at a final concentration of 125 mM and pH 3.85. Poor recovery was obtained on LB medium, which has been used by other investigators for the enumeration of acid-treated cells (8, 9). We determined recovery on TPA plus 1% (wt/vol) sodium pyruvate, a medium used in our laboratory for the recovery of heat-stressed cells (18). Recovery was increased markedly on TPA plus pyruvate, possibly because of dismutation of hydrogen peroxide by pyruvate (13). Supplementation with 1.0 or 0.1% sodium pyruvate resulted in equal recoveries of cells on TPA. To further examine rescue by pyruvate, we exposed acid-adapted cells to acidified E buffer containing lactic acid at a final concentration of 125 mM and pH 3.85. Cells were then plated at various times on LB medium or TPA with and without pyruvate supplementation. The addition of 0.1% pyruvate increased recovery ~1,000-fold on both LB medium and TPA after 60 min of exposure. Supplementation of TPA with catalase (75 U/ml) also promoted the recovery of acid-exposed cells ~1,000-fold. The combination of 0.1% pyruvate and catalase (75 U/ml) further increased recovery 10-fold. These results suggest that acid-exposed cells are sensitive to hydrogen peroxide or oxygen radicals and show that the actual number of viable cells may be severely

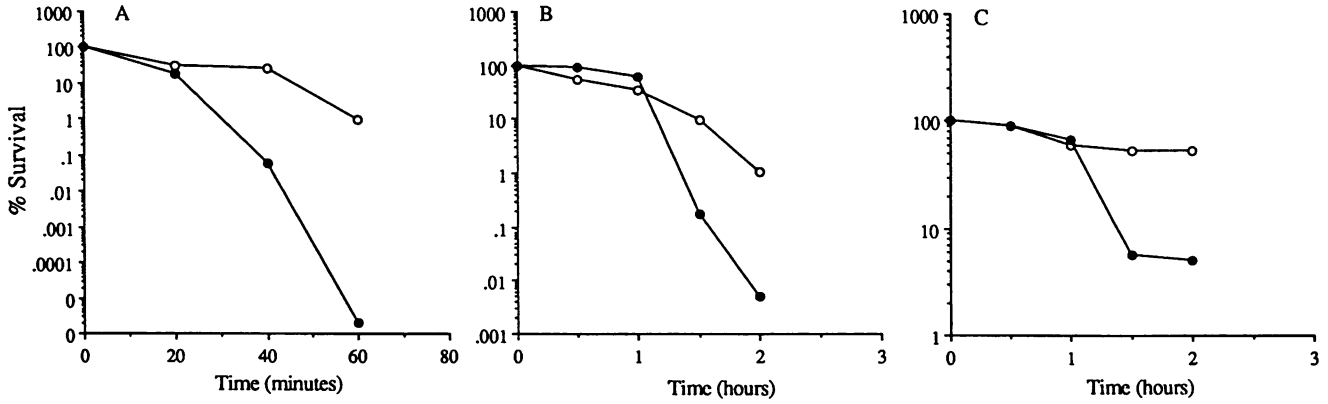

FIG. 1. Survival of acid-adapted (○) and nonadapted (●) *S. typhimurium* cells after exposure to organic acids. Cells were exposed to E buffer acidified to pH 3.85 and containing 125 mM lactic acid (A), 125 mM propionic acid (B), or 125 mM acetic acid (C).

underestimated unless a protective agent is included in the medium.

Acid adaptation enhances tolerance for organic acids. Acid adaptation has been shown to enhance the resistance of *S. typhimurium* to lethal concentrations of HCl (8), and we were interested in determining whether acid adaptation would also increase the tolerance of *Salmonella* spp. for organic acids found in fermented milk products. We initially tried to induce the adaptive response by lowering the pH of the growth medium to 5.8 by using organic acids such as acetic, lactic, and propionic acids, but they inhibited growth, acid adaptation was therefore induced with HCl. Acid-adapted and control cells were incubated in E buffer acidified to pH 3.85 with 125 mM lactic, acetic, or propionic acid (Fig. 1). With each acid, the adapted cells survived better than their nonadapted counterparts. With each acid, the tolerance was similar between the adapted and nonadapted cells during early exposure, but there was a more rapid inactivation of the nonadapted cells during later exposure. Of the three organic acids tested, lactic acid was the most lethal, resulting in a 10^7- to 10^8-fold reduction in the number of viable nonadapted cells after 60 min of exposure (Fig. 1A). The acid-adapted cells declined in number about 10^2-fold after 60 min, indicating they were about 10^5 times more acid tolerant than the nonadapted cells. Increased resistance of adapted cells was also found with acetic and propionic acids, although the differences in survival were not as marked. The acid-adapted cells survived about 100-fold better than the nonadapted cells after 120 min in the presence of propionic acid and about 10-fold better after 120 min in the presence of acetic acid (Figs. 1B and C). These results show that acid adaptation to HCl also provides increased tolerance for organic acids.

Survival of *S. typhimurium* during milk fermentation. We next examined whether acid adaptation would promote the survival of *S. typhimurium* during active milk fermentation by *S. salivarius* subsp. *thermophilus* and *L. helveticus*. Acid-adapted or nonadapted *S. typhimurium* cells were added to the fermenting milk when the milk had reached pH 5.10 (3.5 to 4.0 h). Cells were added at this stage to avoid the induction of an adaptive response during fermentation in the nonadapted cells. After the addition, fermentation was allowed to continue at 37°C and the numbers of salmonellae were determined. The acid-adapted cells showed increased survival compared with the control cells during the later stages of fermentation (Fig. 2). Three hours after the addition of salmonellae, the pH of the milk was lowered to 4.55 by starter culture fermentation and the numbers of both acid-adapted and nonadapted cells decreased about 10-fold. After this point, however, the nonadapted cells died off very rapidly, whereas the acid-adapted cells died off at a slower rate. After 5 h of fermentation at pH 4.27, a 1,000-fold difference in cell numbers was observed. These results show that acid adaptation enhances survival during active milk fermentation, in which several organic acids and other inhibitors are produced.

Survival of *S. typhimurium* in cheeses. We next evaluated whether acid adaptation would affect survival in cheese. Commercially produced cheeses were surface inoculated with acid-adapted or nonadapted *S. typhimurium* cells at an initial concentration of 10^4/g and kept at 5°C. Cheddar and mozzarella cheeses were chosen because they have been implicated in outbreaks of salmonellosis, and Swiss cheese was used because of the complex secondary fermentation associated with the production of propionic and acetic acids.

The results for the survival of *S. typhimurium* at 5°C in the three cheeses are presented in Fig. 3. In mozzarella samples stored aerobically, a substantial difference (~10-fold) in the numbers of viable salmonellae was noticed after 14 days (Fig. 3A). Acid-adapted samples contained relatively large

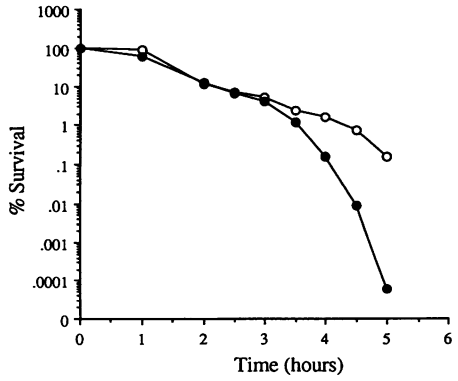

FIG. 2. Survival of *S. typhimurium* during active milk fermentation by *S. salivarius* subsp. *thermophilus* and *L. helveticus*. Symbols: ○, acid adapted; ●, nonadapted. The pH at the time of the addition of cells was 5.15 and further decreased to 4.55 and 4.27 after 3 and 5 h, respectively.

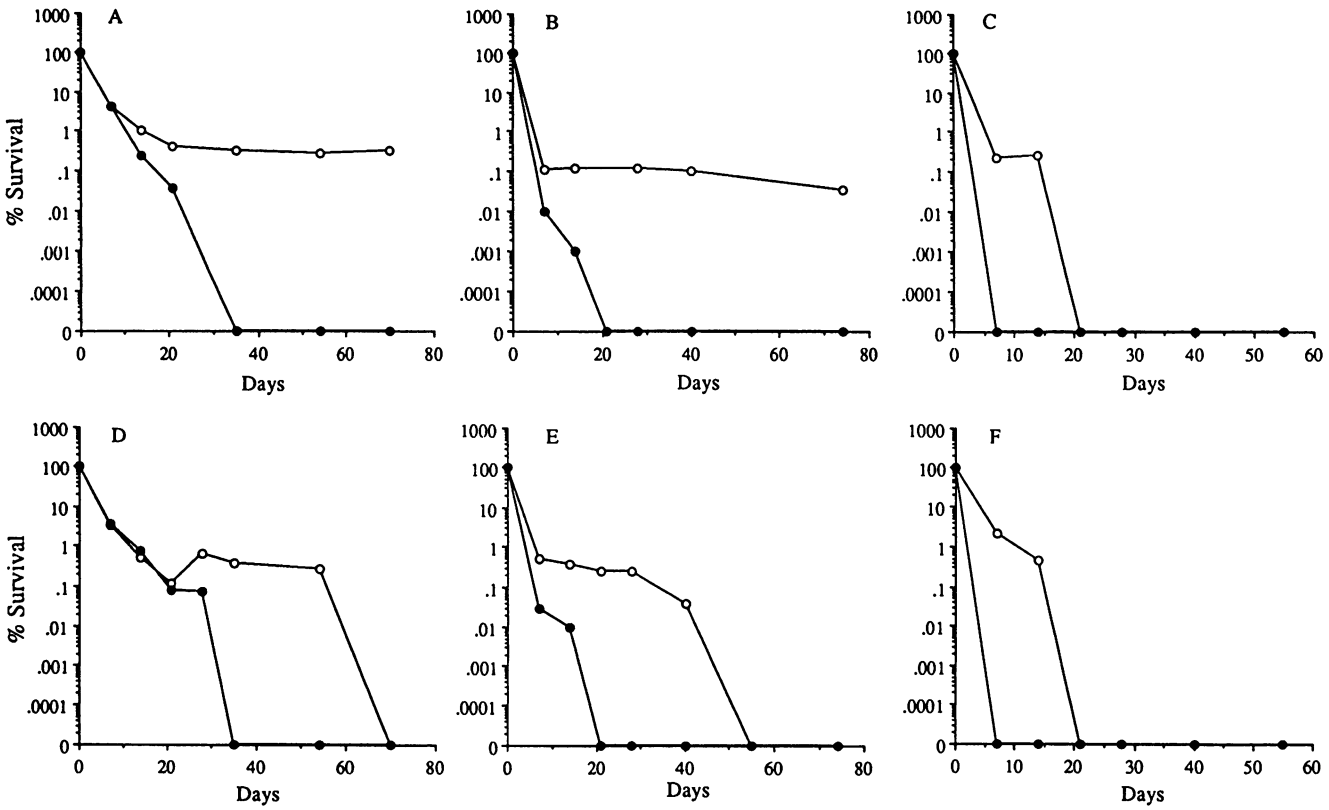

FIG. 3. Survival of *S. typhimurium* in cheeses during storage at 5°C under aerobic (A, B, and C) or anaerobic (D, E, and F) conditions. Symbols: ○, acid adapted; ●, nonadapted. The cheeses used were mozzarella (A and D), cheddar (B and E), and Swiss (C and F).

numbers of salmonellae after 74 days, whereas salmonellae were not recovered after 21 days from nonadapted samples. In mozzarella cheese stored anaerobically, acid-adapted salmonellae were detected through at least 54 days, whereas non-adapted salmonellae died off after 28 days (Fig. 3D).

The same pattern of survival was found with cheddar cheese kept at 5°C. Acid-adapted salmonellae were still present through 74 days of aerobic storage, whereas non-adapted salmonellae stored under identical conditions were not detected after 14 days (Fig. 3B). Cells incubated under anaerobic conditions died off at a faster rate than those incubated aerobically (Fig. 3E). Nonadapted cells were not detected after 14 days, and acid-adapted cells were not detected after 40 days.

The survival of acid-adapted and control cells was also evaluated in Swiss cheese. Although this cheese had a relatively high pH (5.6), salmonellae survived very poorly (Fig. 3C and F). Acid-adapted cells were detected after 14 days but were not detected in subsequent samplings. Non-adapted cells were not detected in the first sampling of 7 days. Swiss cheese is unique among these cheeses, in that propionic acid bacteria produce a variety of end products, including propionate and acetate, which may be inhibitory to salmonellae.

The decrease in the numbers of salmonellae during storage of the various cheeses was probably not caused by changes in pH. The initial pHs of mozzarella, cheddar, and Swiss cheeses were 5.3, 5.2, and 5.6, respectively. The pHs of all three cheeses changed ≤0.1 pH unit during incubation at 5°C.

Acid adaptation of other *Salmonella* spp. Acid adaptation was observed in three other *Salmonella* spp. tested: *S. enteritidis* E40, *S. choleraesuis* subsp. *choleraesuis* serotype heidelberg SHL 39902, and *S. choleraesuis* subsp. *choleraesuis* serotype javiana (Fig. 4). Each of these species displayed increased acid tolerance for lactic acid after adaptation, with *S. choleraesuis* subsp. *choleraesuis* serotype javiana and *S. enteritidis* showing the most marked adaptive responses. Acid-adapted *S. choleraesuis* subsp. *choleraesuis* serotype javiana and *S. enteritidis* survived 1,000- and 10,000-fold better than their nonadapted counterparts after 60 min of exposure to the acidic buffer. *S. heidelberg* showed a less dramatic response, and acid-adapted cells survived 100-fold better than nonadapted cells after 60 min of exposure.

DISCUSSION

Salmonellosis continues to persist as a problem in the food industry, and dairy products have been responsible for several large outbreaks (2, 4). The largest detected outbreak of food poisoning in recent years occurred in the United States and was probably caused by improperly pasteurized milk (4). Fermented dairy products, including natural cheese, have maintained a fairly good record of safety (15, 16). The factors involved in cheese manufacturing and ripening that provide protection are incompletely understood but probably include acidity, water activity (a_w), and inhibitory factors produced by starter organisms and ripening flora (25). Previous studies supported the theory that acid production by the starter culture is the most important factor controlling *Salmonella* spp. during cheddar cheese manufac-

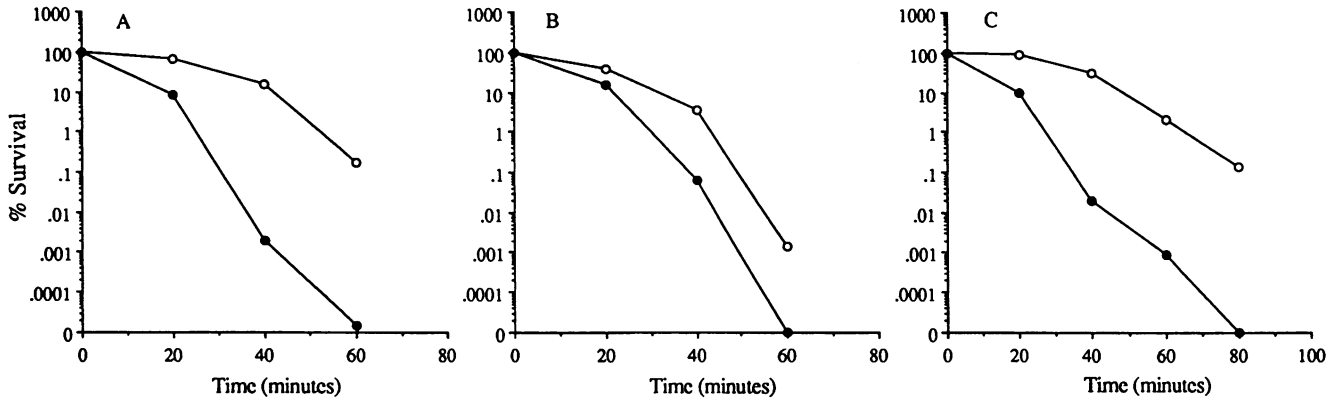

FIG. 4. Acid adaptation and tolerance of various *Salmonella* spp. Cells were exposed to acidified E buffer containing 125 mM lactic acid at pH 3.85. Symbols: ○, acid adapted; ●, nonadapted. (A) *S. enteritidis* E40. (B) *S. choleraesuis* subsp. *choleraesuis* serotype heidelberg SHL 39902. (C) *S. choleraesuis* subsp. *choleraesuis* serotype javiana.

turing and ripening (12). Hargrove et al. (12) showed that the survival of salmonellae ranged from 2 to 9 months in 65 lots of cheddar cheese. The variables that significantly affected survival were pH and type and amount of the starter culture. Cheeses that had abnormally high pHs caused by starter culture failure (dead vats) had little inhibitory activity against salmonellae. It appeared that a pH of 5.2 to 5.3 was sufficient to cause the death of salmonellae. In cheeses prepared with milk preacidified with lactic and acetic acids, the starter culture grew slowly, the cheese became gassy within 3 months, and salmonellae declined slowly during storage. These studies indicated the value of good starter culture activity and acidity for inhibiting pathogen survival.

Although several investigators have evaluated the survival of salmonellae in various cheeses, these studies all used as inocula overnight cultures grown in rich media (10, 12, 26, 31). The use of nonstressed cultures may not accurately represent the physiological state of salmonellae in the food environment or from human carriers, since adaptive responses to acidity and osmolarity may promote growth and survival in environments hostile to nonadapted cells. Acid adaptation in *Salmonella* spp. has been shown to be triggered by external pHs of 5.5 to 6.0. This response maintains the intracellular pH above 5.0 to 5.5 and promotes survival (9).

In the present study, we examined whether acid adaptation affected the sensitivity of *Salmonella* spp. to organic acids encountered in cheese and persistence in fermented milk and cheese. To adequately quantitate survival, we found it necessary to evaluate methods for recovery. It was previously shown that the incorporation of catalase in the recovery medium enhances the recovery of injured cells (20, 23). Pyruvate, which nonenzymatically destroys H_2O_2 (13), also enhances recovery (23). In a previous study (8, 9), LB agar without protecting agents was used for the recovery of cells given a challenge pH of 3.3. Our results indicate that recovery may be enhanced more than 1,000-fold by the addition of 0.1% pyruvate to the recovery medium.

We found that acid-adapted cells had increased resistance to organic acids found in fermented dairy products. Adapted cells also survived better than nonadapted cells during fermentation of milk and in cheeses stored at 5°C. Increased survival in the cheeses may not have been caused solely by acid adaptation but could also have involved adaptive responses to other stresses, including starvation, heat shock, oxidative stress, or osmotic stress. A certain degree of cross-protection exists among these responses (21). Matin (21) suggested that resistance to environmental stresses in *Salmonella* spp. and *E. coli* is influenced by nutrition, growth phase, and preexposure to the stresses.

Many foods depend on acidity for the elimination of salmonellae and some other sensitive pathogens. The acid ingredients of mayonnaise can eliminate salmonellae over a period of hours to days (29). In yogurt, lactic acid was reported to be the main inhibitory factor active against *S. typhimurium* (27). *Salmonella* spp. are at a disadvantage to cope with a low pH, compared with some other bacteria, including the lactic acid bacteria used for cheese manufacturing. Certain lactic acid bacteria possess metabolic systems for the generation of basic end products from arginine, enabling them to continue intermediary metabolism when glycolysis stops because of a low pH (3). Some lactic acid bacteria not only are resistant to acid but require a low pH to grow (17, 22). Lactobacilli can lower their internal cytoplasm to 4.4 to 4.8 and survive well at an external pH of 3.5 (17, 22). The low internal growth-limiting pH probably contributes to the ability of lactobacilli to lower the external pH to values lethal to *Salmonella* spp. and related pathogens during food fermentations. These beneficial organisms have a clear advantage for survival in these products, and their metabolism contributes to the elimination of pathogens.

In summary, we have shown that acid adaptation promotes the persistence of *Salmonella* spp. in cheeses. Acid adaptation is probably an important survival mechanism in the food plant environment, and it would be useful to use adapted cells in the evaluation of sanitation procedures and Hazard Analysis Critical Control Point programs. This work could allow for the development of biomarkers, such as some of the acid response proteins, as tests of the conditions of specific food ecosystems. A better understanding of adaptations to stress may increase our understanding of food safety systems.

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