Survival of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in and on Vacuum Packaged Lebanon Bologna Stored at 3.6 and 13.0°C

NAVEEN CHIKTHIMMAH AND STEPHEN J. KNABEL *

Department of Food Science, The Pennsylvania State University, University Park, Pennsylvania 16802, USA

MS 00-376: Received 25 October 2000/Accepted 9 February 2001

ABSTRACT

*Escherichia coli* O157:H7, *Salmonella* Typhimurium, or *Listeria monocytogenes* was spread onto the surface of Lebanon bologna luncheon slices using sterile glass rods. The inoculated slices were stacked and vacuum packaged. The packages were stored at 3.6 or 13°C. The foodborne pathogens, *E. coli* O157:H7, *Salmonella* Typhimurium, or *L. monocytogenes* were reduced in Lebanon bologna during storage at 3.6 or 13°C. The higher storage temperature (13.0°C) resulted in significantly faster destruction of *E. coli* O157:H7 and *L. monocytogenes*, compared to storage at refrigeration temperature (3.6°C) (P < 0.005). *E. coli* O157:H7 was the most resistant to destruction among the three foodborne pathogens. A linear destruction of *E. coli* O157:H7 occurred only after an initial lag period. Storage temperature did not have a significant effect on the rate of destruction of *Salmonella* Typhimurium. Foodborne pathogens inoculated prior to fermentation did not show any enhanced survival compared to control cells (inoculated after fermentation) during storage of the Lebanon bologna at 3.6°C.

Traditionally, fermented meats have been considered safe due to a combination of hurdle factors such as low pH, low water activity (a_w), presence of competitive microflora, and presence of curing salts. However, recent outbreaks of foodborne illnesses due to gram-negative foodborne pathogens in fermented meats, and *Listeria monocytogenes* in ready-to-eat (RTE) cooked meats, have stimulated additional research on postprocessing contamination of fermented RTE meats. In 1994, an *Escherichia coli* O157:H7 outbreak was epidemiologically linked to the consumption of a dry-cured salami product from delicatessen counters in the western United States (7). The outbreak affected 23 individuals and resulted in the hospitalization of two children with hemolytic uremic syndrome. From early August 1998 through February 1999, at least 50 illnesses caused by a rare strain of *L. monocytogenes* serotype 4b were reported to the Centers for Disease Control and Prevention (CDC) by 11 states (8, 9). Twenty-one deaths were reported, which included 15 adults and 6 stillbirths. The CDC and state and local health departments identified the vehicle for transmission as hot dogs and possibly deli meats produced under many brand names by a single manufacturer.

Lebanon bologna is a moist, smoked, fermented RTE sausage manufactured from lean beef with a final moisture/protein ratio of about 3.5:1 wt/wt. A commercial Lebanon bologna product contains approximately 65% moisture, 10% fat, and 17% protein (30). The final pH of commercial Lebanon bologna is typically between 4.4 and 4.6. Due to undesirable quality changes, Lebanon bologna is often not processed at temperatures above 48.9°C (120°F). In 1995, a salmonellosis outbreak in Pennsylvania was epidemiologically linked to the consumption of Lebanon bologna (31). Ellajosyula et al. (12) demonstrated that a combination of fermentation to pH 4.7 and subsequent gradual heating to 48.9°C (120°F) in 10.5 h destroyed at least 5 log_{10} CFU of *E. coli* O157:H7 and *Salmonella* Typhimurium per g of Lebanon bologna mix during the manufacture of Lebanon bologna. While the above Lebanon bologna manufacturing process ensures at least a 5 log_{10} CFU reduction of *E. coli* O157:H7, *Salmonella* Typhimurium, or *L. monocytogenes*, postprocessing contamination may introduce foodborne pathogens into this RTE product. Numerous studies have demonstrated the growth of foodborne pathogens in unfermented beef and poultry products during storage (5, 6, 17). However, little or no research has been conducted on the survival of foodborne pathogens in fermented RTE meats and Lebanon bologna, respectively, during storage at different temperatures. Survival studies in low pH foods like salami (13), mayonnaise (21), and apple cider (23, 33) demonstrated that *E. coli* O157:H7 survives longer at refrigeration temperatures than at higher temperatures. Therefore, the first objective of this research was to study the survival of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium in Lebanon bologna stored at refrigeration (3.6°C) and abuse temperatures (13.0°C).

Prior exposure to mild acid conditions has been shown to enhance the survival of *L. monocytogenes* (16), *E. coli* O157:H7 (28), and *Salmonella* Typhimurium (27) in acidic environments. Therefore, the second objective of this study was to examine whether exposure to slowly decreasing pH during Lebanon bologna fermentation enhances survival of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Typhimurium during storage of the finished product at 3.6°C.
MATERIALS AND METHODS

_L. monocytogenes_ inoculum preparation_. A three-strain cocktail of _L. monocytogenes_ was prepared using the 1998 hot dog outbreak strain from the CDC, Atlanta, Ga., type strain ATCC 43256 (isolated from Mexican-style cheese) from the American Type Culture Collection, Rockville, Md., and strain Scott A from The Pennsylvania State University Department of Food Science culture collection (University Park, Pa.). All cultures were maintained on tryptic soy agar with 0.6% yeast extract (Difco Laboratories, Detroit, Mich.) with monthly transfers. A 20-h culture of each organism was transferred to tryptic soy broth with 0.6% yeast extract (Difco) and grown for 20 h at 37°C. Cells were harvested by centrifugation at 9,000 × g for 10 min at 4°C. The cell pellets from individual strains were washed and resuspended in 0.85% saline. Suspensions of the three strains were combined in equal amounts to obtain the three-strain cocktail inoculum.

_E. coli_ O157:H7 inoculum preparation_. A five-strain cocktail of _E. coli_ O157:H7 was prepared with the West Coast salami outbreak strain, strains Dec 4E, Dec 4B, and Dec 3A (human isolates), and strain 93-0133 (ground beef isolate) obtained from the _E. coli_ Reference Center, The Pennsylvania State University. All cultures were maintained on tryptic soy agar with 0.6% yeast extract (Difco) with monthly transfers. A 20-h culture of each organism was transferred to tryptic soy broth with 0.6% yeast extract (Difco) and grown for 20 h at 37°C. Cells were harvested by centrifugation at 9,000 × g for 10 min at 4°C. The cell pellets from individual strains were washed and resuspended in 0.85% saline. Suspensions of the five strains were combined in equal amounts to obtain the five-strain cocktail inoculum.

_Salmonella Typhimurium_ inoculum preparation_. A five-strain cocktail of _Salmonella Typhimurium_ was prepared using type strain ATCC 13600 (American Type Culture Collection, Rockville, Md.), and Lebanon bologna outbreak-associated strains 9089, 8687, 8390, and 8347 (food isolates; Division of Clinical Microbiology, Pennsylvania Department of Health). These Lebanon bologna outbreak-associated strains were typed and confirmed as _Salmonella Typhimurium_ by the National Veterinary Services Laboratory, Ames, Ia. The cultures were maintained on tryptic soy agar with 0.6% yeast extract (Difco) with monthly transfers. A 20-h culture of each organism was transferred to tryptic soy broth with 0.6% yeast extract (Difco) and grown for 20 h at 37°C. Cells were harvested by centrifugation at 9,000 × g for 10 min at 4°C. The cell pellets from individual strains were washed and resuspended in 0.85% saline. The concentration of cells in the 0.85% saline was determined by measuring the optical density at 625 nm in a Milton Roy Spectronic 20D spectrophotometer (Spectronic Instruments, Rochester, N.Y.) and comparing this reading with a standard curve of _OD_625 versus CFU/ml. Suspensions of the five strains were combined in equal amounts to obtain the five-strain cocktail inoculum.

Inoculated finished product study: preparation of inoculated Lebanon bologna luncheon slices. Luncheon slices of Lebanon bologna were obtained from a commercial Lebanon bologna manufacturer. A 0.2-ml inoculum of _L. monocytogenes_ cocktail (about 10⁶ cells) was added onto the surface of each Lebanon bologna luncheon slice. The cells were spread on the surface of the Lebanon bologna luncheon slices using a sterile glass rod. Five inoculated slices (100 ± 2 g) were stacked and placed in 8- by 10-in. pouches made from 3-Mil packaging film (0.75-gauge nylon with E.V.O.H. and 2.25-gauge polyethylene) with an O₂ transmission rate of 0.6 cc/100 in²/24 h at 32°F and water vapor transmission rate of 0.6 g/100 in²/24 h at 100°F and 100% relative humidity (Koch Supplies, Kansas City, Mo.). Packages were then vacuum packaged at 97% vacuum using an Ultravac 250 vacuum packager (Koch). Each gram of product contained approximately 6.7 log₁₀ CFU of _L. monocytogenes_. Twenty such packages were prepared. The same procedure was repeated separately for _E. coli_ O157:H7 and _Salmonella Typhimurium_ cocktails. Each gram of product contained approximately 7 log₁₀ CFU of _E. coli_ O157:H7 or approximately 8 log₁₀ CFU of _Salmonella Typhimurium_.

Storage of Lebanon bologna and enumeration of inoculated pathogens. The packages were stored at 3.6 or 13°C to simulate refrigerated and temperature-abuse conditions, respectively. Samples were collected at specific intervals, and pathogens were enumerated for up to 4 months of storage time.

Inoculated raw mix study: preparation of prefermentation-inoculated treatment samples. Unfermented raw Lebanon bologna batter containing boneless lean beef (10% fat), a commercial spice formulation, and a commercial starter culture ( _Pediococcus acidilactici, Lactobacillus plantarum_, and _Micrococcus halobius_ ) was obtained from a commercial Lebanon bologna manufacturer. The product was inoculated with foodborne pathogens to yield 6.3 log₁₀ CFU of _L. monocytogenes_, 7.5 log₁₀ CFU of _E. coli_ O157:H7, or 7.5 log₁₀ CFU of _Salmonella Typhimurium_ per g. The inoculated batter was then stuffed into sterile hungate tubes (27 by 142 mm) (Bellco Glass, Vineland, N.J.) using a sausage stuffer (The Sausage Maker, Buffalo, N.Y.). The tubes were then closed with rubber stoppers and sealed with molten paraffin wax as described previously (12). To control the heating process precisely and to ensure a consistent come-up time, the sealed tubes were immersed in a programmable microprocessor-controlled temperature water bath (model 221; Neslab Instruments, Portsmouth, N.H.) capable of very uniform temperature increases and having a temperature stability of ±0.01°C. The tubes were held at 26.7°C for 12 h and then at 37.7°C to ferment the product to pH 5.0. The fermented product was then removed from the tubes, vacuum packaged as described above, and stored at 3.6°C. Samples were collected at specific intervals, and pathogens were enumerated for up to 3 months of storage time.

Preparation of postfermentation-inoculated control samples. The raw Lebanon bologna mix without any inoculated pathogens was fermented in tubes held at 26.7°C for 12 h and then at 37.7°C to ferment the Lebanon bologna to pH 5.0 as in the process described above. The products from the tubes were then removed and tested to confirm the absence of pathogens, and the remaining product was transferred to vacuum packages. Foodborne pathogens were then inoculated directly onto the fermented Lebanon bologna to yield 7.3 log₁₀ CFU of _L. monocytogenes_, 8.6 log₁₀ CFU of _E. coli_ O157:H7, or 8.5 log₁₀ CFU of _Salmonella Typhimurium_ per g. The cells were thoroughly distributed throughout the cut pieces of Lebanon bologna by massaging the package by hand for 2 min. The packages were then vacuum packaged as described above. The vacuum packages were stored at 3.6°C, samples were collected at specific intervals, and pathogens were enumerated for up to 3 months of storage time.

Enumeration and confirmation of foodborne pathogens. Cells of _L. monocytogenes_ were enumerated using modified Oxford agar (MOX agar; BBL, Cockeysville, Md.). Cells of _E. coli_ O157:H7 were enumerated using MacConkey sorbitol agar (MSA; Difco), and cells of _Salmonella Typhimurium_ were enumerated using xylose lysine deoxycholate agar (XLD agar; Difco). Colonies suspected to be _L. monocytogenes_ were confirmed using the following tests: Gram stain reaction, catalase, tumbling motility, and _Micrococcus halobius_.
at 25°C, carbohydrate fermentation (rhamnose, but not xylose), and weak β-hemolysis on columbia horse blood bilayer plates (Remel, Lenexa, Kans.). Colonies suspected to be E. coli O157:H7 and Salmonella Typhimurium were confirmed by slide agglutination tests with E. coli O antisera O157 (Difco) and Salmonella O antisera group B factors (Difco), respectively.

**Statistical analyses.** All experiments were replicated once for a total of two trials for each experiment. Analysis of variance was conducted on mean log$_{10}$ reduction data for L. monocytogenes, E. coli O157:H7, or Salmonella Typhimurium using Minitab version 12 for Windows (Minitab, State College, Pa.). D-values (days per log reduction) for each storage temperature–pathogen combination were calculated from plots of log$_{10}$ CFU/g versus storage time. Analysis of variance and means separation of D-values were also conducted using Minitab version 12 for Windows.

**RESULTS**

L. monocytogenes, E. coli O157:H7, and Salmonella Typhimurium inoculated onto slices of commercial Lebanon bologna luncheon slices were reduced to undetectable levels (<10 CFU/g) during the storage period at either 3.6 or 13°C. The higher storage temperature (13°C) significantly increased the rate of destruction of L. monocytogenes ($P < 0.05$) and E. coli O157:H7 ($P < 0.005$) compared to storage at 3.6°C. L. monocytogenes had a $D_{3.6°C}$ value of 5.02 days (slope = −0.1912, $r^2 = 0.9677$) and a $D_{13.0°C}$ value of 2.97 days (slope = −0.6654, $r^2 = 0.9937$) (Fig. 1A). For E. coli O157:H7, $D_{3.6°C}$ was 21.65 days, and $D_{13.0°C}$ was 12.05 days (Fig. 1B). Storage temperature did not have a significant effect on the rate of destruction of Salmonella Typhimurium ($P > 0.05$); $D_{3.6°C}$ was 4.99 days, and $D_{13.0°C}$ was 4.89 days (Fig. 1C).

The survival (during storage) of L. monocytogenes, E. coli O157:H7, and Salmonella Typhimurium inoculated into raw Lebanon bologna batter prior to fermentation is presented in Figure 2A through 2C. When compared with the controls (inoculation after fermentation; Fig. 1A through 1C), inoculation prior to fermentation (Fig. 2A through 2C) did not significantly enhance the survival of the foodborne pathogens during the storage of Lebanon bologna at 3.6°C ($P > 0.05$).

**DISCUSSION**

Low pH is a critical hurdle factor foodborne pathogens encounter in a fermented sausage product (26). Glass and Doyle (17) demonstrated that while significant multiplication of L. monocytogenes occurred in unfermented meat products (pH > 5.4), the organism survived but did not grow in summer sausage (pH 4.8) during storage at 4.4°C for 12 weeks. While L. monocytogenes increased from $5 \times 10^2$/g to $2.1 \times 10^5$/g during storage of frankfurters (unfermented meat product) at 4°C for 20 days, populations of deviations are marked with vertical bars. (C) Survival of Salmonella Typhimurium on sliced vacuum packaged Lebanon bologna stored at 3.6°C (squares) and 13.0°C (circles). Standard deviations are marked with vertical bars.
the foodborne pathogen decreased from $8 \times 10^6$ to 1.1 \times 10^4 during the production of fermented sausages and remained constant during 20 days of storage (5). In another study, a commercial sausage batter inoculated with $4.8 \times 10^4$ CFU of \textit{E. coli} O157:H7 per g was fermented to pH 4.8 and dried until the moisture/protein ratio was less than or equal to 1.9:1 (18). The sausage chubs were then vacuum packaged and stored at 4°C for 2 months. \textit{E. coli} O157:H7 survived, but did not grow, during fermentation, drying, or subsequent storage at 4°C, and it decreased by about 2 log_{10} CFU/g by the end of storage. Lebanon bologna has a pH of approximately 4.4 to 4.6. Given its low pH, it is not surprising that foodborne pathogens in Lebanon bologna decreased during storage at refrigeration or abuse temperatures. The destruction of \textit{E. coli} O157:H7 in Lebanon bologna observed in this study is consistent with the findings of Glass et al. (18).

The foodborne pathogens in Lebanon bologna were reduced to undetectable levels after 10 weeks during the storage period. Cutter and Siragusa (10) studied the efficacy of acetic, lactic, and citric acid rinses for controlling \textit{E. coli} O157:H7 attached to beef carcass tissue. The type of acid was not a significant factor in survival. The reduction of the surface pH from 6.5 to as low as 3.5 upon application of acids was suggested to be a factor contributing to inactivation of \textit{E. coli} O157:H7. Lebanon bologna contains about 1% lactic acid (30). The reduced pH (4.4 to 4.6) and extended exposure times are probably main factors promoting the destruction of foodborne pathogens in Lebanon bologna. A study by Glass et al. (18) demonstrated growth of \textit{E. coli} O157:H7 in tryptic soy broth acidified with lactic acid to pH 4.6. Therefore, the observed destruction of the pathogens in Lebanon bologna may be due to factors other than the low pH of Lebanon bologna. The effect of individual hurdles and interactions among hurdle factors, low pH, low water activity, presence of curing salts, and the antagonistic action of the active starter cultures could explain the destruction of foodborne pathogens in Lebanon bologna. Ellajosyula et al. (12) reported that a combination of fermentation to pH 4.7 and subsequent gradual heating to 48.9°C in 10.5 h destroyed at least 5 log_{10} units of \textit{E. coli} O157:H7. They further reported a significant interaction between fermentation pH, heating temperature, and heating time on the destruction of \textit{E. coli} O157:H7 during the manufacture of Lebanon bologna. Other studies (4, 20)

**FIGURE 2.** (A) Effect of prefermentation inoculation on survival of \textit{L. monocytogenes} during storage of vacuum packaged Lebanon bologna at 3.6°C. Inoculation into mix prior to fermentation (circles); inoculation onto finished product (squares). Standard deviations are marked with vertical bars. (B) Effect of prefermentation inoculation on survival of \textit{E. coli} O157:H7 during storage of vacuum packaged Lebanon bologna at 3.6°C. Inoculation into mix prior to fermentation (circles); inoculation onto finished product (squares). Standard deviations are marked with vertical bars. (C) Effect of prefermentation inoculation on survival of \textit{Salmonella Typhimurium} during storage of vacuum packaged Lebanon bologna at 3.6°C. Inoculation into mix prior to fermentation (circles); inoculation onto finished product (squares). Standard deviations are marked with vertical bars.
have demonstrated that interactions among fermented meat hurdle factors such as low pH, presence of curing salts, packaging type, and storage temperature increased the destruction of both L. monocytogenes and E. coli O157:H7. The antagonistic action of lactobacilli on E. coli O157:H7, Salmonella spp., Staphylococcus aureus, and Pseudomonas spp. has been demonstrated. This antagonism is due to the production of lactic acid and the formation of hydrogen peroxide by the lactobacilli (3, 11, 22). Antagonism may also be due to the production of bacteriocins. During the manufacture of a fermented semidry sausage, a pediocin-producing strain of Pediococcus caused approximately a 2 log$_{10}$ CFU reduction of L. monocytogenes and P. acidilactici, L. plantarum, and M. halobius. Biswas et al. (2) reported that lower pH favors an increased level of pediocin production. Thus, the concentration of pediocin in a low pH product like Lebanon bologna might be further increased.

The gram-negative pathogens E. coli O157:H7 and Salmonella Typhimurium were the most resistant to destruction in Lebanon bologna, while the gram-positive pathogen, L. monocytogenes, was the least resistant. The enhanced survival of the gram-negative organisms in Lebanon bologna might be due to their acid tolerance response (ATR) mechanisms. Enteric microorganisms have evolved several mechanisms for handling acid stress. E. coli O157:H7 has three known acid resistance systems: oxidative, glutamate dependent, and arginine dependent (29). While the oxidative system is a general protection mechanism, the other two are dependent on the production of arginine decarboxylase and glutamate decarboxylase, respectively. Once activated, these systems persist for prolonged periods of cold storage even at acidic pH ranges (29). E. coli O157:H7 in Lebanon bologna had an initial lag period of survival before a linear destruction trend during storage when the product was stored either at 3.6 or 13°C. This initial lag period of enhanced survival might be attributed to its acid tolerance mechanisms. Salmonella Typhimurium studies have revealed two stationary-phase ATR systems: one that is acid inducible and RpoS independent and one that is unresponsive to pH but RpoS dependent (24, 25). The ATR systems of Salmonella Typhimurium appear to afford protection against other stresses such as salt and heat (27). L. monocytogenes is regarded as a poor survivor at acidic pH values. Gray and Killinger (19) reported that L. monocytogenes usually dies at pH levels below 5.6. The ATR mechanisms of the enteric bacteria and the low pH sensitivity of L. monocytogenes may explain the enhanced survival of E. coli O157:H7 and Salmonella Typhimurium in Lebanon bologna.

Storage temperature is an important factor determining survival of foodborne pathogens in low pH foods. E. coli O157:H7 cells die more rapidly in low pH foods like apple cider (33), commercial mayonnaise (21), and pepperoni (14) stored at higher temperatures (20 to 25°C) than at refrigeration temperatures. The present study with a low pH meat product, Lebanon bologna, is consistent with the studies mentioned above. Interaction between low pH and high temperature, as reported by Ellajosyula et al. (12), could explain the higher destruction rates of foodborne pathogens in acidic food products stored at abuse temperatures. The sausage storage chart of the Food Safety and Inspection Service (32) recommends a refrigerated storage time of 3 months for unopened packages and 3 weeks for opened packages of semidry sausage, the category under which Lebanon bologna is generally classified. The information brochure also concludes: “It is too early to suggest changes to basic handling recommendation for consumers since a complete scientific evaluation is not yet available.” This study clearly demonstrates that storage of Lebanon bologna above refrigeration temperatures actually enhances destruction of foodborne pathogens in these products. Therefore, the Food Safety and Inspection Service might want to reconsider storage recommendations for fermented vacuum packaged RTE meats.

Acid adaptation by exposure to mild acid conditions has been shown to enhance the survival of E. coli O157: H7 (28), Salmonella Typhimurium (27), and L. monocytogenes (16) in acidic foods. Acid adaptation by exposure to pH 5.5 for 1 h enhanced the survival of L. monocytogenes in acidified dairy products (16). In a study by Leyer et al. (28), E. coli O157:H7 was adapted to acid by culturing for one to two doublings at pH 5.0. Acid-adapted cells had an increased resistance to lactic acid, survived better than non-adapted cells during a sausage fermentation, and showed enhanced survival in shredded dry salami (pH 5.0) and apple cider (pH 3.4). Therefore, it was hypothesized in the present study that a slow decrease in pH during Lebanon bologna fermentation might enhance the survival of foodborne pathogens during storage of the finished product. However, prior acid exposure during fermentation of Lebanon bologna did not enhance survival of L. monocytogenes during storage. These findings are consistent with the observations of Gahan et al. (16). While acid-adapted L. monocytogenes cultures demonstrated increased survival during active milk fermentation by a lactic acid culture, nonadapted Listeria cultures failed to become fully adapted to acidic conditions during fermentation, even when added at an inducing pH (pH 5.5) (16). In Salmonella spp., acid tolerance to pH 3.3 is induced by a short-term acid shock (pH 4.3 for 10 to 20 min). However, continuing acid shock beyond 30 min eliminated subsequent survival at pH 3.3 (15). Further research on ATRs under slowly decreasing pH conditions may help explain the lack of enhanced survival due to prior exposure to fermentation observed in the present study.

ACKNOWLEDGMENTS

We thank Seltzer’s Lebanon Bologna Company for financial assistance; Robert Guyer for technical assistance; Nicole Schleef, Justin Comes, and Srilatha Pandrangi for experimental help; and Bethany Oaks and Carey Brickner for media preparation.

REFERENCES

Pediococcus during the manufacture of fermented semidyrry sausage. J. Food Prot. 53:194–197.


