Destruction of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Lebanon Bologna by Interaction of Fermentation pH, Heating Temperature, and Time

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**ABSTRACT**

Fermented meats have caused food-borne illness due to enterohemorrhagic *Escherichia coli*. Consumption of Lebanon bologna was epidemiologically associated with a recent outbreak of salmonellosis. The present study was conducted to determine the effects of pH (after the fermentation step), final heating temperature, and time on destruction of *E. coli* O157:H7 and *Salmonella typhimurium* in Lebanon bologna. Raw Lebanon bologna mix was inoculated with either of the pathogens (ca. 10⁸ CFU/g) and fermented for 12 h at 80°F (26.7°C) and then at 100°F (37.8°C) until the pH reached either 5.2 or 4.7. The mix was then heated to 110, 115, or 120°F (43.3, 46.1, or 48.9°C). The bologna was sampled at various times, decimally diluted, and plated on either McConkey sorbitol agar or XLD agar to enumerate *E. coli* O157:H7 and *S. typhimurium*, respectively. Fermentation alone reduced populations of both pathogens by <2 log units and heating alone reduced populations of *E. coli* O157:H7 by <3 log units. A combination of fermenting to either pH 5.2 or 4.7, followed by heating at 110°F (43.3°C) for 20 h, 115°F (46.1°C) for 10 h, or 120°F (48.9°C) for 3 h reduced populations of both pathogens by >7 log units. Overall, *S. typhimurium* cells were either equally or significantly less resistant (*P* < 0.01) than cells of *E. coli* O157:H7. Significant interactions (*P* < 0.01) among the three factors for the destruction of *E. coli* O157:H7 were observed. A process-specific regression equation was developed to predict the destruction of *E. coli* O157:H7 in Lebanon bologna.

Recent outbreaks due to gram-negative food-borne pathogens in fermented meat products have raised questions about the safety of these foods. An outbreak of *Escherichia coli* O157:H7 was linked to consumption of fermented salami in the western United States in 1994 (4, 21). This outbreak affected 20 individuals in Washington and 3 in California and led to hospitalization of two children with hemolytic uremic syndrome (HUS). An Australian outbreak of *E. coli* O111:NM (nonmotile) in 1995 was attributed to the consumption of semi-dry fermented sausage (3). Twenty-three children (aged 4 months to 12 years) who had consumed the implicated sausage were affected by HUS. Lebanon bologna was epidemiologically associated with a 1995 outbreak of salmonellosis in Pennsylvania (22). Twenty-six cases of salmonellosis were reported and *Salmonella typhimurium* was isolated from opened packages of a certain brand of Lebanon bologna taken from refrigerators of several patients. The United States Department of Agriculture’s Food Safety and Inspection Service (USDA-FSIS) developed guidelines for validating a 5-log reduction of *E. coli* O157:H7 in fermented sausage (18). A USDA directive to fermented meat processors, dated August 1995 and subsequently modified in 1996, included options to control *E. coli* O157:H7, such as the application of a validated 5-D process. The directive further stated that, “The use of a validated method by a plant to produce these products is also the type of hazard control procedure that plants will be expected to implement when the HACCP regulations become effective” (1).

Lebanon bologna is a moist, fermented sausage manufactured from lean beef (final moisture-to-protein ratio is ~3.5:1 wt/wt). A commercial Lebanon bologna product contains 65% moisture, 10% fat, and 17% protein (all % wt/wt). Industry sources estimate that the annual production of Lebanon bologna in the United States is over 15 million lb (ca. 6.8 million kg). The traditional process involved the following three steps: (a) aging salted beef at 5°C for 10 days; (b) stuffing and smoking bolognas for 4 days in the smokehouse at 35°C; and (c) mellowing the bolognas at 5°C for 3 days (17). Fermentation was found to occur during the first 2 to 3 days in the smokehouse, with a pH drop of approximately one pH unit. Smith and Palumbo (19) studied the microbiology of the product and found it similar to that of summer sausage and other fermented sausages in that it consisted of a lactic acid fermentation by lactobacilli and production of cured meat color from the reduction of nitrate to nitrite by micrococcii. Smith et al. (20) subsequently evaluated the survival of *Salmonella dublin* and *S. typhimurium* in Lebanon bologna made by the traditional process. In
In most cases, the traditional Lebanon bologna process has been replaced by a more modern process characterized by the use of unaged beef, addition of starter culture(s), and the use of shorter manufacturing times. The modern process involves an initial fermentation step (24 to 30 h) at temperatures between 70 and 100°F (21.1 and 37.8°C) where the pH drops from 6.0 into the range 5.2 to 4.7. The product is then heated further to finishing temperatures ranging from 110 to 120°F (43.3 to 48.9°C) for an additional 11 to 35 h. The final pH of commercial Lebanon bologna is typically 4.5 to 4.3.

Destruction of >5 log units of populations of E. coli O157:H7 in other fermented meats such as pepperoni has been shown to occur at 128°F (53.3°C) after 60 min (9); unfortunately, this process is not a viable option for most Lebanon bologna manufacturers due to undesirable product defects, such as drying and crumbling, that may occur above 120°F (48.9°C). Currently, no literature is available on the effect of various intrinsic factors on the destruction of gram-negative pathogens such as E. coli O157:H7 and Salmonella spp. in the modern Lebanon bologna process. While predictive modeling programs such as the USDA Pathogen Modeling Program (USDA, ARS, Eastern Regional Research Center, Wyndmoor, PA) exist, they cannot predict which combinations of fermentation pH, heating temperature, and time will yield a >5-log population reduction of E. coli O157:H7 in fermented meats. The purpose of the present study was to determine the effects of pH (after the fermentation step), final heating temperature, and time, and the interactions between these factors, on the destruction of E. coli O157:H7 and S. typhimurium in Lebanon bologna produced by the modern process. An additional purpose was to develop an equation that would predict the destruction of E. coli O157:H7 at any combination of the above factors.

**Materials and Methods**

**Inoculum preparation.** The design of this study was based on FSIS challenge-study guidelines for destruction of E. coli O157:H7 in fermented sausage (18). A five-strain cocktail inoculum of E. coli O157:H7 was prepared with the West Coast salami outbreak strain, strains Dec 4E, Dec 4B, Dec 3A (human isolates) and strain 93-0133 (ground beef isolate) obtained from the E. coli Reference Center, Pennsylvania State University, University Park, PA. Similarly a five-strain cocktail inoculum of S. 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-associated strains were typed and confirmed as S. typhimurium by The National Veterinary Services Laboratory, Ames, IA. All cultures were maintained on nutrient agar slants (Difco Laboratories, Inc., Detroit, MI) with monthly transfers. An 18-h culture of each organism was transferred to tryptic soy broth (Difco) containing 1% glucose (Sigma Chemical Co., St. Louis, MO) and grown for 24 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 0.1% peptone water (Difco). Suspensions of the five strains were combined in equal amounts to obtain the 5-strain cocktail inoculum.

**Inoculation and processing of the Lebanon bologna mix.** Unfermented raw Lebanon bologna mix containing boneless lean beef (10% fat), salt (3.5%), potassium nitrate (12 ppm), sodium nitrite (200 ppm), a commercial spice formulation, and a commercial starter culture (Pediococcus acidilactici, Lactobacillus plantarum and Micrococcus spp.) was obtained from a major Lebanon bologna manufacturer. This mix was inoculated with either a five-strain cocktail inoculum of E. coli O157:H7 or S. typhimurium (final concentration of pathogen in mix, ca. 10⁸ CFU/g). Initial experiments were conducted by fermenting the mix in 100-ml beakers partially submerged in a constant-temperature water bath. This method was subsequently abandoned, because it resulted in survival of pathogens due to lower temperatures at the top of the beakers. The completely immersed tube method was used to remedy this situation. In this method, a sterile 50-cc plastic syringe was filled with the inoculated mix and delivered through a sterile glass funnel into the bottom of sterile Hungate tubes (27 by 142 mm). The mix was compacted within the tubes with the bottom of a sterile test tube; the tubes were then sealed with rubber stoppers and molten paraffin wax. To precisely control the heating process 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, NH) capable of very uniform temperature increases and having a temperature stability of ±0.1°C. To simulate a commercial process, the tubes were held at 80°F (26.7°C) for the first 12 h and then held at 100°F (37.8°C) until the pH of the mix reached either 5.2 or 4.7 (representing the typical high and low pH levels attained at the end of the fermentation step in the commercial Lebanon bologna process). The mix was then heated gradually to 110°F (43.3°C) (within 0.5 h), or to 115°F (46.1°C) in an additional 5 h, or to 120°F (48.9°C) in 5 more hours and held at these temperatures for predetermined periods of time. The minimum come-up times from the records of the Lebanon bologna manufacturer were used as the come-up times in this study. The bologna mix was sampled immediately after attaining 110, 115, or 120°F (43.3, 46.1, or 48.9°C) and also after various holding times at these temperatures.

To examine the effect of temperature alone on destruction of E. coli O157:H7, the initial fermentation step was eliminated and the mix was heated to 110°F (43.3°C) within 0.5 h, or to 115°F (46.1°C) in an additional 5 h, or to 120°F (48.9°C) in 5 more hours. Following the heating process all samples were blended in a stomacher (Model STO-400; Tekmar, Inc., Cincinnati, OH), decimally diluted in buffered peptone water (Difco) and surface plated onto McConkey sorbitol agar (Difco) to enumerate E. coli O157:H7, and onto XLD agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) to enumerate S. typhimurium.

Colonies suspected to be E. coli O157:H7 or S. typhimurium were confirmed by slide agglutination tests with E. coli O antisum 0157 and Salmonella O antisum group B factors (Difco), respectively. The pH of the bologna mix during the experiment was monitored with a programmable Accumet pH meter (Model 25; Fisher Scientific, Pittsburgh, PA) equipped with a pH electrode designed for monitoring the pH of meat (Mettler Toledo AG, Urndorf, Switzerland). The internal (center) temperature of the mix in the tubes was monitored by using Type-K piercing thermocouple probes (Cole-Parmer Instrument Co., Vernon Hills, IL) connected to a Digi-Sense multi-channel scanning thermometer (Cole-Parmer Instrument Co.).
Statistical analyses. Analysis of variance (ANOVA) and mean separation using Fisher’s protected least significant difference (PLSD) test were conducted on log reduction data of both pathogens with Statview software (Abacus Concepts, Inc., Berkeley, CA). Destruction of E. coli O157:H7 in Lebanon bologna was also studied in the absence of the initial fermentation step; therefore, more advanced analysis was possible with this pathogen. To determine interactions between the factors of pH, temperature, and time, analysis of E. coli O157:H7 log reduction data was conducted using the General Linear Model (GLM) in Statistical Analysis Systems software, 6.12 edition (SAS Institute Inc., Cary, NC). Fermentation pH and heating temperature were treated as factors (pH 4.7, 5.2, and 6.0; temperatures 110, 115, and 120°F [43.3, 46.1, and 48.9°C]) and heating time was treated as a covariable. The general terms included in the analysis were the main effects of fermentation pH, heating temperature, heating time, and the interactions between each of these terms.

To develop an equation that would predict the destruction of E. coli O157:H7, regression analysis in Minitab (Minitab, Inc., State College, PA) was performed on log reduction data of E. coli O157:H7 as a function of pH, temperature, and time. All time points at which viable cells were recovered were used in the analysis along with the first time point at each combination of pH and temperature at which no pathogens were detected. This first time point was represented by the maximum log reduction possible in the experiment based on 8.573 log CFU/g of E. coli O157:H7 in the unfermented mix immediately after inoculation.

RESULTS

Different results were observed depending on whether the parameters (fermentation pH, heating temperature, and time) were tested alone or in combination. Fermentation alone to pH 5.2 or 4.7 without any heat treatment resulted in <2-log-unit reduction of E. coli O157:H7 or S. typhimurium (Fig. 1). Heating Lebanon bologna without the initial fermentation step resulted in <3-log-unit reduction of E. coli O157:H7 for all heating temperature and time combinations tested (Figs. 2 to 4). However, the combination of fermentation and heating significantly increased destruction.

Fermentation to either pH 5.2 or 4.7 and heating at 110°F (43.3°C) for 20 h yielded a >7-log-unit reduction of both pathogens (Fig. 2). Also heating at 115°F (46.1°C) for 10 h or longer after fermentation resulted in a >7 log reduction of both pathogens (Fig. 3). Greater than 7 log reduction of S. typhimurium occurred by gradual heating to 120°F (48.9°C) in 10.5 h, after fermentation to either pH 5.2 or 4.7. A similar reduction of E. coli O157:H7 occurred at 120°F (48.9°C) after fermenting to pH 4.7, but not pH 5.2 (Fig. 4). Overall, S. typhimurium was either equally or significantly less resistant than E. coli O157:H7 to various treatments (Fig. 1 to 4). Figure 5 illustrates the effect of fermentation pH and come-up time to final heating temperature on the destruction.
of *E. coli* O157:H7. Large log reductions occurred when the mix was fermented to pH 5.2, 4.7 and heated to 120°F.

Analysis of variance of the *E. coli* log reduction data using the GLM showed significant interactions (*P* < 0.01) between pH and heating temperature, pH and heating time, and heating temperature and heating time (Table 1). A regression equation (*r*² = 0.93) was developed that predicted destruction of *E. coli* O157:H7 in the Lebanon bologna process studied in this experiment. The terms pH, temperature, time; interactions of pH/temperature, pH/time, temperature/time; pH², temperature², and time² were treated as predictors of the response variable, log reduction of *E. coli* O157:H7.

The equation for predicting log reductions of *E. coli* O157:H7 in the Lebanon bologna process described in this study is as follows:

\[
y = 5.55137 + 0.656406*(T_f - 115) + 0.372561*(t - 10)
- 4.36832*(pH - 5.35)
+ 0.0211477*(T_f - 115)*(t - 10)
- 0.456395*(T_f - 115)*(pH - 5.35)
- 0.232276*(t - 10)*(pH - 5.35)
+ 0.0409055*(T_f - 115)^2
- 0.00125562*(t - 10)^2 - 0.870784*(pH - 5.35)^2,
\]

where *y* is the log reduction of *E. coli* O157:H7, *pH* indicates the fermentation pH, *T_f* indicates the final heating temperature in °F, and *t* time in hours at the final heating temperature. The correlation *r*² = 0.93, residual SD = 0.786, replicate SD = 0.412.

**DISCUSSION**

*E. coli* O157:H7 can survive in a variety of acidic foods such as apple cider (16, 25), mayonnaise (5, 23, 24), and fermented meats (7, 12). Only slight reductions of populations of this organism were reported by fermentation alone in fermented meat products (7, 9). Lin et al. (14) demonstrated the existence of three acid-resistant systems in *E. coli* O157:H7 strains which allowed the organisms to survive extreme acid exposure (pH 3.0). Similarly, various acid-resistant systems have been reported in *S. typhimurium* (6, 10). These systems appear to afford cross-protection against other stresses such as salt and heat (11). *Salmonella typhimurium* has a minimum growth pH of 4.0, a characteristic not possessed by other enteric bacteria such as *E. coli* K-12 and MC 4100, *Enterobacter cloacae*, and *Shigella sonnei* (13). Buchanan and Edelson (2) demonstrated that when grown in tryptic soy broth containing >0.75% glucose, the cultures of *E. coli* O157:H7 reached and maintained a pH that fostered maximal acid tolerance. In our study, all cultures were grown in tryptic soy broth containing 1% glucose before being inoculated into raw Lebanon bologna mix and challenged by fermentation and heat. Given the above mecha-
nisms of acid tolerance and the level of glucose in TSB, it is not surprising that little reduction of populations of these pathogens occurred due to fermentation alone in this study (Fig. 1).

*Escherichia coli* O157:H7 and *S. typhimurium* do not possess any unusual heat resistance. The D value for *E. coli* O157:H7 in ground beef at 125°F is 115.5 min (15) and for *Salmonella* spp. is 61 min (8). A ≥5-log-unit reduction of populations of *E. coli* O157:H7 in pepperoni was shown to occur at 128°F or higher (9). Smith et al. (20) indicated that a 2-day fermentation to pH 4.7 without starter culture and heating to 125°F in 42 min was necessary to destroy 4 log units of *S. typhimurium* cells in Lebanon bologna. Their study was based on the traditional Lebanon bologna manufacturing process and had a lower initial inoculum level of *S. typhimurium* (10⁴ CFU/g) than in the present study (10⁸ CFU/g). Major changes in the Lebanon bologna process since then, the lack of sufficient inoculum to demonstrate a 5-log reduction, and the absence of *E. coli* O157:H7 in the experimental design makes this study (20) potentially inaccurate for evaluating the safety of the modern Lebanon bologna process.

Compared to *E. coli* O157:H7, *S. typhimurium* was either significantly less resistant or equally resistant to various treatments; therefore, more focus was placed on *E. coli* O157:H7 in the present study. We report here a significant interaction between fermentation pH, heating temperature, and time for the destruction of *E. coli* O157:H7 in the modern Lebanon bologna process (Table 1). Neither fermentation to pH 4.7 alone (Fig. 1) nor gradual heating to 120°F alone (Fig. 4) destroyed >2 log units of *E. coli* O157:H7 cells. However, a combination of fermentation to pH 4.7 and subsequent gradual heating to 120°F in 10.5 h destroyed at least 7 log units of populations of both *E. coli* O157:H7 and *S. typhimurium* (Fig. 4). These results demonstrate the importance of combining adequate fermentation (pH decrease), final heating temperature, and time in destroying *S. typhimurium* and *E. coli* O157:H7 in Lebanon bologna.

This study has identified both critical control points (fermentation pH, final heating temperature, heating time) and critical limits (e.g., pH 4.7, 120°F, 0 h) that can be integrated into a hazard analysis critical control point (HACCP) plan to ensure destruction of *S. typhimurium* and *E. coli* O157:H7 in Lebanon bologna without causing loss of product quality. It is important that manufacturers validate the ability of the above critical limits in destroying 5 log units of populations of these pathogens in their own specific fermented meat products.

The prediction equation may have many commercial uses. It would allow the prediction of log reduction of *E. coli* O157:H7 for any given combination of fermentation pH, final heating temperature, and heating time. In addition, the processor may be able to accurately predict the additional temperature and/or time needed to produce a 5-log reduction at a given fermentation pH.

It is important to realize that the prediction equation is only valid for the process described in this study. A microprocessor-controlled-temperature water bath was utilized to accurately simulate the manufacturer’s minimum come-up times to 110, 115, and 120°F. These constant come-up times permitted the development of the prediction equation based on final heating temperature. Since come-up time to final heating temperature contributed significantly to pathogen destruction (Fig. 5), shorter come-up times would result in lower log reductions than predicted by the equation. However, different processors of fermented meats can use this same approach to develop their own prediction equations to enhance the safety of their processes. Application of similar prediction equations would allow fermented meat processors to accurately monitor and control the safety of their processes.

In order to validate the commercial utility of the predictive equation for the process described in this study, further research on destruction of *E. coli* O157:H7 in Lebanon bologna will be conducted by fermenting and heating the Lebanon bologna mix in casings using a pilot-scale commercial smokehouse located in the Department of Food Science at The Pennsylvania State University.

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