

Effect of pH on Survival, Thermotolerance, and Verotoxin Production of *Escherichia coli* O157:H7 during Simulated Fermentation and Storage†‡

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MS 99-69: Received 17 March 1999/Accepted 13 August 1999

ABSTRACT

Heat treatment is increasingly being introduced to fermented meat processing, since the acid tolerance properties of *Escherichia coli* O157:H7 can permit this organism to survive traditional processing procedures. This study investigated the effect of growth pH and fermentation on the thermotolerance at 55°C of *E. coli* O157:H7 in a model fermented meat system. *E. coli* O157:H7 (strain 380-94) was grown at pH 5.6 or 7.4 (18 h at 37°C), fermented to pH 4.8 or 4.4 in brain heart infusion broth, and stored for 96 h. Cells grown at pH 5.6 had higher *D* values at 55°C (*D*_{55°C}) than cells grown at pH 7.4 (*P* < 0.001). Cells fermented to pH 4.8 had higher *D*_{55°C} than those fermented to pH 4.4 (*P* < 0.001). Cells fermented to pH 4.8 demonstrated an increase in *D*_{55°C} during storage (*P* < 0.001), whereas cells fermented to pH 4.4 showed a decrease in *D*_{55°C} during the same period (*P* < 0.001). The effect of growth pH on verotoxin production by *E. coli* O157:H7 was assessed using the verotoxin assay. Cells grown at pH 5.6 had lower verotoxin production than cells grown at pH 7.4. This effect was not sustained over storage. These results indicate that a lower growth pH can confer cross-protection against heat. This has implications for the production of acidic foods, such as fermented meat, during which a heating step may be used to improve product safety.

Since the early 1980s, when *Escherichia coli* O157:H7 was first implicated in human illness, this organism has become the focus of much research, particularly as it affects meat processing industries. In 1994, *E. coli* O157:H7 was implicated in a foodborne illness outbreak associated with salami (6). The manufacturer in question was deemed to have followed all regulations that existed for production of such foodstuffs at that time (29). As a result of this outbreak, considerable effort has been expended in the investigation of the survival of *E. coli* O157:H7 in fermented meat, with the aim of developing a process that can yield a 5-log₁₀ reduction in pathogen numbers in such products, as recommended by the U.S. Department of Agriculture Food Safety and Inspection Service (24). Various studies have demonstrated that the inclusion of a mild heating step to the fermented meat production process may be successful in achieving the desired reduction in pathogen numbers (5,

12, 15, 25). *E. coli* O157:H7 has a high acid tolerance (9), and growth at pH as low as 4.0 to 4.5 has been recorded in broth studies (3, 9). Acid tolerance has been identified as a major virulence determinant of this organism (13, 30), since it enhances the ability of the cell to survive the acidic conditions of the initial stages of the gastrointestinal tract and go on to colonize the gut (23). Organic acids present in processed foods and pH reductions brought about by increased production of these acids, e.g., during the fermentation of meat, allow *E. coli* O157:H7 to adapt to acidic conditions. The ability to adapt to acidic conditions allows the organism to later tolerate pH levels that would normally inactivate it (2). This property also contributes to the ability of *E. coli* O157:H7 to survive during the processing and storage of fermented meat (12), which generally has a final pH in the range of 4.5 to 5.5 (22). The stress response that gives rise to acid tolerance has been shown to confer some cross-protective effects against heat stresses (7). The possibility of acid tolerance imparting cross-protection against heat would have significant implications for the application of heat as a means of eliminating *E. coli* O157:H7 in fermented meat.

This study aimed to determine if growth pH and simulated fermentation had an impact on the survival and thermotolerance of *E. coli* O157:H7 at 55°C, during and after simulated fermentation and storage. The effects of growth pH and storage at low pH on verotoxin production were also assessed.

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† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

‡ Portions of this research were presented at the Annual Meeting of the Institute of Food Technologists in Orlando, Florida, 14 to 18 June 1997.

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MATERIALS AND METHODS

Organism. *E. coli* O157:H7 (strain 380-94) isolated from a salami outbreak (6) was obtained from The Centers for Disease Control and Prevention, Atlanta, Ga., and maintained on tryptone soy agar (Difco Laboratories, Detroit, Mich.) at 4°C by monthly subculture.

Broths. Standard brain heart infusion (BHI) broth was prepared by dissolving BHI powder (37 g·liter⁻¹) in 1 liter of distilled water. Buffered BHI (BBHI) broths were prepared by adding BHI powder (37 g·liter⁻¹) to 1-liter volumes of citrate phosphate-buffered solutions adjusted to pH 5.7, 5.4, 5.1, 4.8, and 4.4, by the method of McIlvaine (19) and were denoted F_{pH5.7}, F_{pH5.4}, F_{pH5.1}, F_{pH4.8}, and F_{pH4.4}, respectively. An additional broth was prepared, adjusted to pH 5.6, and used as a growth medium. BBHI broths supplemented with salt were prepared by the addition of 33 g·liter⁻¹ NaCl to 1-liter volumes of the pH-adjusted BBHI broths (excluding BBHI at pH 5.6) to achieve a final NaCl content of 3.8% (wt/vol) (BHI broth powder contains 0.5% [wt/vol] salt). These broths are denoted NaCl_{3.8%}.

All broths were dispensed in 9-ml volumes in test tubes and sterilized by autoclaving at 121°C for 15 min. The pH of the broths was measured using an Orion 210 pH meter (Orion Research, Boston, Mass.). The pH of all broths changed less than 0.1 pH unit during sterilization. The citrate phosphate-buffering system maintained all BBHI broth pH values within ±0.1 pH unit during incubation periods. The pH values of *E. coli* O157:H7 cultures incubated in unbuffered BHI declined from 7.4 to approximately 6.0 within 18 h.

Production of acid-adapted and non-acid-adapted inocula. Ten-milliliter volumes of BBHI (pH 5.6, i.e., the pH of raw meat (17)) and BHI (pH 7.4) (unbuffered) were inoculated with a loop full of cells from the tryptone soy agar maintenance culture and incubated for 18 h at 37°C. Preliminary growth studies in BHI indicated that such cultures entered stationary phase within 15 h under these conditions (data not shown). These cultures were denoted G_{pH5.6} (acid-adapted cells) and G_{pH7.4} (non-acid-adapted cells), respectively.

Simulated fermentation and storage procedure. The acid-adapted and non-acid-adapted cultures were subjected to simulated fermentation and storage processes, as outlined in Figure 1. A total of eight *E. coli* O157:H7 cultures (G_{pH5.6} or G_{pH7.4}) were prepared (i.e., incubated at 37°C for 18 h) for each simulated fermentation and storage procedure. At the end of the 18-h growth period, 1-ml aliquots of *E. coli* O157:H7 inocula (either G_{pH5.6} or G_{pH7.4}) were sequentially transferred through a series of BBHI broths (9-ml tubes) with successively lower pH values, i.e., 5.7 to 5.4 to 5.1. The cells were held for 130 min at each pH. One-milliliter aliquots were then treated in one of the following ways. (i) They were transferred into BBHI (F_{pH4.8}) and held for 50 min at 37°C before storage at 15°C. This process simulated the final pH values attained during typical pepperoni production. (ii) They were transferred into BBHI (F_{pH4.8}), held for 130 min, further transferred into BBHI (F_{pH4.4}), and held for 50 min at 37°C before storage at 15°C. This process simulated the final pH values attained during the production of low pH pepperoni (26).

The time scale for simulated fermentation was based on previous studies carried out in pepperoni (27). Following the simulated fermentation processes, cells were stored for up to 96 h at 15°C (the drying temperature of fermented meat) (26). This was performed to observe if any additional adaptation to the acidic storage conditions occurred. Samples were removed after 0, 24,

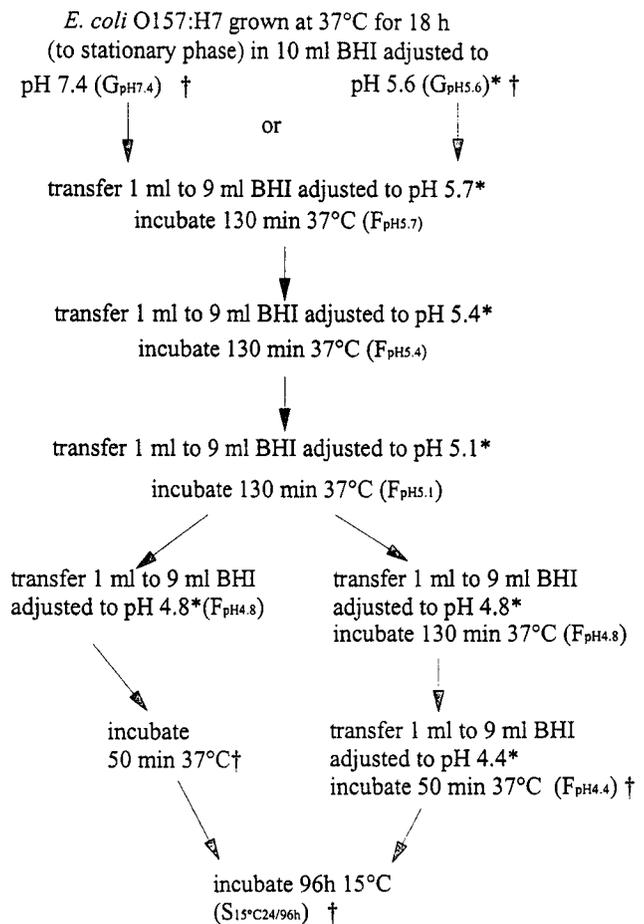


FIGURE 1. Simulated fermentation and storage of *E. coli* O157:H7 in BHI.

and 96 h of storage and are denoted S_{15°C0h}, S_{15°C24h}, and S_{15°C96h}, respectively.

Heat-treated samples. Decimal reduction times (*D* values) at 55°C (*D*_{55°C}) were determined on samples withdrawn (i) from the stationary phase (18 h) inocula, i.e., G_{pH5.6}/G_{pH7.4}; (ii) immediately after the simulated fermentation process, i.e., G_{pH5.6}/G_{pH7.4}:F_{pH4.8}:S_{15°C0h} and G_{pH5.6}/G_{pH7.4}:F_{pH4.4}:S_{15°C0h}; (iii) 24 h after the simulated fermentation process, i.e., G_{pH5.6}/G_{pH7.4}:F_{pH4.8}/F_{pH4.4}:S_{15°C24h}; and (iv) 96 h after the simulated fermentation process, i.e., G_{pH5.6}/G_{pH7.4}:F_{pH4.8}/F_{pH4.4}:S_{15°C96h}.

Heat treatment procedure. Samples were added to the submerged coil apparatus using two systems, as follows. (i) Cells were suspended in the BHI in which they had been grown or stored. Samples G_{pH7.4} or G_{pH5.6} were at pH 6.0 and 5.6, respectively, following growth overnight at 37°C. Cells that had undergone simulated fermentation were at pH 4.8 or 4.4. These samples were, therefore, heated in an acidic environment and are denoted H_{pH6.0}, H_{pH5.6}, H_{pH4.8}, and H_{pH4.4}, respectively. (ii) One-milliliter aliquots of cells were suspended in 9 ml of Butterfield's phosphate-buffered dilution water (1) (pH 7.0—neutral pH environment). These samples are denoted H_{pH7.0}.

In this way, any effect of the environment pH at the time of heating on the thermotolerance of the organism could be observed.

Samples were heat treated using a submerged heating coil system comprised of a stainless steel coil submerged in a thermostatically controlled water bath, equilibrated to 55°C, by the method of Cole and Jones (8). Samples (9.5 ml) were loaded into

TABLE 1. $D_{55^{\circ}\text{C}}$ values (min) of *Escherichia coli* O157:H7 heated in neutral or acidic environments, following inoculum preparation, simulated fermentation, and storage^a

Initial inoculum ^c	After simulated fermentation to:	$D_{55^{\circ}\text{C}}$ values (min)											
		Heated in neutral pH ^b				Heated in acidic pH				Grown at pH 7.4 ^d			
		Grown at pH 5.6	Grown at pH 4.4	pH 4.8	pH 4.4	Grown at pH 7.4	Grown at pH 4.4 ^f	pH 4.8	pH 4.8 ^d	Grown at pH 5.6 ^e	Heated in acidic pH	Grown at pH 4.4 ^f	Grown at pH 7.4 ^d
NaCl	0.5%	50.28 (8.56)	4.04 (0.60)	6.60 (0.91)	2.60 (0.64)	17.73 (2.13)	5.66 (0.66)	5.27 (0.48)	37.24 (0.14)	6.39 (2.59)	4.25 (0.80)	19.46 (6.14)	5.63 (0.67)
NaCl	3.8%	—	—	—	1.71 (0.0)	—	2.31 (1.50)	—	—	—	—	—	—
NaCl	0.5%	—	4.01 (1.77)*	7.94 (1.76)	2.46 (0.35)	—	7.93 (0.50)	4.70 (1.66)	—	12.16 (2.54)	3.25 (0.42)	—	6.87 (0.60)
NaCl	3.8%	—	—	—	0.58 (0.14)	—	1.17 (0.43)	—	—	—	—	—	—
NaCl	0.5%	—	4.48 (0.54)	9.72 (0.68)	2.72 (0.22)*	—	8.40 (0.53)	5.03 (0.96)	—	7.93 (1.78)	4.01 (1.04)	—	7.05 (0.14)
NaCl	3.8%	—	—	—	0.73 (0.12)*	—	1.97 (0.49)	—	—	—	—	—	—

^a All values are the mean of three replicates with the exception of those indicated by *, which are the mean of two replicates only. Standard deviations are in parentheses.

^b Heated in Butterfield's phosphate-buffered dilution water at pH 7.0 ($H_{\text{pH}7.0}$).

^c Heated in BBHI at pH 5.6 ($H_{\text{pH}5.6}$).

^d Heated in BHI at pH 6.0 ($H_{\text{pH}6.0}$).

^e 18-h growth at 37°C.

^f Heated in BBHI at pH 4.4 ($H_{\text{pH}4.4}$).

^g Values in this column indicate length of time stored at 15°C (h).

fermented meat environment (5, 12). The possibility of sublethal acid-induced injury of *E. coli* O157:H7 sensitizing cells to subsequent heat treatment would have significant implications for food manufacturers using hurdle technology to ensure the safety of their fermented meat product.

To assess the thermotolerance of *E. coli* O157:H7, the organism was heated in either a neutral (Butterfield's phosphate-buffered dilution water) or acidic (BHI) pH environment. The derived $D_{55^\circ\text{C}}$ values were not significantly affected by the pH of the heating environment in this study. Non-spore-forming bacteria generally display maximum thermotolerance at neutral pH (14). The above atypical observations may be related to the unusual acid tolerant properties of this organism, in that the mildly acidic heating environments had no significant effects on the thermotolerance of the organism.

Increased salt content led to a reduction in the heat resistance of *E. coli* O157:H7 in the simulated fermentation system. The $D_{55^\circ\text{C}}$ for cells subjected to simulated fermentation and storage in the presence of 3.8% salt were 1.5 to 6.8 times lower than those determined for cells subjected to simulated fermentation and storage in the presence of 0.5% salt. The use of high or low salt concentrations (within the range of 2.5 to 4.8% NaCl) did not significantly affect the survival of *E. coli* O157:H7 in pepperoni batter in previous studies (26). This difference between the results obtained using a broth-based system and those obtained using a meat-based system underline the necessity for caution in the extrapolation of results obtained in a simple broth-based system in the accurate prediction of thermotolerance of *E. coli* O157:H7 in a complex food environment, such as fermented meat. The behavior of this or any pathogen should be assessed in a representative food environment before adoption of intervention strategies such as heating.

Cell populations remained constant during $S_{15^\circ\text{C}-96\text{h}}$, with cells stored in broth with 0.5% salt exhibiting a small measure of growth (0.5 to 1.0 log₁₀) during storage. Cell numbers declined throughout storage in broth containing 3.8% salt ($G_{\text{pH}7.4}:\text{NaCl}_{3.8\%}:\text{F}_{\text{pH}4.4}:\text{S}_{15^\circ\text{C}-96\text{h}}:\text{H}_{\text{pH}7.0}$), indicating that these cells had not adapted to their environment in the same way as cells stored in broth with 0.5% salt. This observation is in agreement with the findings of Rowbury (27), who stated that *E. coli* transferred from low to high-salt environments were more acid sensitive than cells that were not introduced to the high-salt environment.

The findings of this study suggest that *E. coli* O157:H7 that has been adapted to mildly acidic conditions during growth may subsequently demonstrate enhanced resistance to heat in an acidic environment. The results also showed that acid-adapted cells produced less toxin than non-acid-adapted cells. In this study, the stresses induced by the acidic growth environment initially reduced the production of toxin by acid-adapted cells ($G_{\text{pH}5.6}$) in comparison with toxin production by non-acid-adapted cells ($G_{\text{pH}7.4}$). However, this effect appeared to be temporary, with cells $G_{\text{pH}5.6}$ regaining the ability to produce toxin following adaptation to the acidic surroundings during storage in BBHI (pH 4.8). Buncic and Avery (4) found that the rate of verotoxin production by *E. coli* O157:H7 in the gastrointestinal tract may

be affected if the organism encountered low-pH conditions before ingestion. Sampling was not continued past 48 h in the current study. Further research is required to identify the profile of toxin production by acid-adapted and non-acid-adapted cells during extended storage. Such research would be useful in assessing the potential of *E. coli* O157:H7 to produce toxin in the gastrointestinal tract.

Fermented meat is a complex food medium, containing multiple hurdles to ensure product safety (18). This study has shown that exposure to stress from one processing hurdle (i.e., acid) can confer greater resistance on the organism to another, previously unencountered, stress, i.e., heat. The extent of acquired heat resistance can, however, be reduced by extended fermentation. It is important that the possibility of cross-protection be recognized when designing schedules and protocols for the production of fermented meat products.

ACKNOWLEDGMENTS

We acknowledge the assistance of members of staff at the Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, Pa.; Peter Cooke, Shawn Eblen, Marsha Golden, and Patricia Klein for valuable technical assistance; John Phillips for statistical analysis of results; and Pina Fratamico for helpful discussions.

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