

Survival of *Escherichia coli* O157:H7 during the Manufacture of Pepperoni^{†‡}

DENISE C. R. RIORDAN,^{1*} GERALDINE DUFFY,¹ JAMES J. SHERIDAN,¹ B. SHAWN EBLEN,²
RICHARD C. WHITING,² IAN S. BLAIR,³ AND DAVID A. McDOWELL³

¹The National Food Centre, Teagasc, Dunsinea, Castleknock, Dublin 15, Ireland; ²Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA; and ³University of Ulster, Jordanstown, Newtownabbey, County Antrim, BT37 0QB, Northern Ireland

ABSTRACT

This study investigated the growth and survival of *Escherichia coli* O157:H7 during the manufacture of pepperoni to determine whether a 5-log₁₀-unit decline in numbers, as recommended by the U.S. Food Safety and Inspection Service (FSIS), could be achieved. A range of pepperoni formulations with variations in salt (2.5 to 4.8%) and sodium nitrite (100 to 400 ppm) levels, and with pH (4.4 to 5.6) adjusted by manipulation of dextrose concentrations were prepared. The batters produced were inoculated with *E. coli* O157:H7 380-94 at a level of approximately 6.70 log₁₀ CFU/g; changes in pathogen numbers, pH, titratable acidity, and sodium nitrite concentrations were monitored during fermentation and drying. With the standard commercial formulation (i.e., 2.5% salt, 100 ppm sodium nitrite, pH 4.8) *E. coli* O157:H7 numbers declined by approximately 0.41 log₁₀ CFU/g during fermentation and a further 0.43 log₁₀ CFU/g during subsequent drying (7 days). A regression equation was fitted to the data which showed significantly ($P < 0.001$) greater reductions in pathogen numbers in samples with increased salt and sodium nitrite contents and lowered pH. However declines were in all cases less than the target reduction of 5 log₁₀ CFU/g.

Enterohemorrhagic *Escherichia coli* O157:H7 is recognized as an important foodborne pathogen (23), with 3 to 8 individuals per 100,000 of the population infected annually (20). The number of cases and the frequency of severe accompanying complications are increasing (24). Initial outbreaks of *E. coli* O157:H7 poisoning were traced to ground beef products (21), and subsequent investigations have established that the bacterium is present in the feces and intestines of healthy bovines (25) and can contaminate meat during slaughtering processes (6). An outbreak of *E. coli* O157:H7 in the Western United States associated with salami (7) was particularly significant, as it was the first outbreak of this pathogen in a dried fermented ready-to-eat meat product. Prior to this incident, it was generally believed that conditions in such products, i.e., the low final pH (ca. 4.8), an a_w of <0.80 with high salt (ca. 2.5% added) and high added sodium nitrite (ca. 100 ppm) would preclude pathogen survival. However, studies have indicated that pathogens such as *Salmonella* spp. and *Listeria* spp. can survive in fermented meat products (16, 17). *E. coli* O157:H7 is more acid tolerant than other vegetative foodborne pathogens (12)

and has a low infectious dose (<50 organisms), dry fermented meat products could therefore pose a significant source of infection (24).

Contamination of meat with *E. coli* O157:H7 is usually at very low levels and the organism is unevenly distributed. This makes testing of raw ingredients unreliable. As yet there is no widely accepted rapid method for testing freshly slaughtered meat for the presence of *E. coli* O157:H7, so manufacturers cannot confidently obtain pathogen-free ingredients. Inspection of the plant which produced the salami implicated in the 1994 outbreak concluded that the "production methods complied with federal requirements and industry-developed good manufacturing practices" (24). Recognition of these risks led to a recommendation by the U.S. Food Safety and Inspection Service (FSIS) that the procedures and practices in the production of ready-to-eat fermented products obtain a 5-log₁₀ CFU/g reduction of *E. coli* O157:H7 populations (22). One recently published study outlines heating steps which could be introduced into the manufacturing process after fermentation to achieve the required decline in pathogen numbers (18). However such a processing change is likely to be expensive and could lead to undesirable organoleptic changes in the product. The purpose of this study was to model the effects of a range of concentrations and combinations of salt, sodium nitrite, and pH on the survival of *E. coli* O157:H7 during a standard commercial pepperoni fermentation and drying process to determine whether the necessary reduction could be achieved by these means.

* Author for correspondence. Tel: 011 353 1 8383222; Fax: 011 353 1 8383684; E-mail: d.riordan@nfc.teagasc.ie

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MATERIALS AND METHODS

Preparation of bacterial inoculum. *Escherichia coli* O157:H7 380-94, isolated during the 1994 outbreak associated with contaminated salami in the Western United States, was obtained from The Centers for Disease Control in Atlanta, Georgia, and stored at -20°C on Protect beads (Protect Bacterial Preserves, England). An inoculum was prepared by adding one bead to 30 ml of brain heart infusion (BHI) broth (pH 7.4) (Oxoid, Basingstoke, UK) and incubating the culture overnight at 37°C . One milliliter of this culture was resuspended in 30 ml of BHI broth and incubated at 37°C for 16 h, when the culture had entered stationary phase. Cells were recovered by centrifugation at $1,391 \times g$ for 10 min (Kontron T-140 centrifuge), and the pellet was resuspended in 30 ml of BHI broth. Cell numbers were estimated using the direct epifluorescent filter technique acridine orange direct count (11) and adjusted to approximately $9.0 \log_{10}$ CFU/ml.

Pepperoni preparation. Pepperoni was prepared according to the specifications of a local manufacturer (International Meats, The Maudlings, Naas, Co Kildare, Ireland). Beef (plate and flank, forequarter and fat trim) was obtained from the on-site abattoir and pork (leg trim) was obtained from local butcher shops. All meat was minced through a 10-mm die plate (Crypto Peerless mincer, model EB12F) and stored at -20°C . Prior to pepperoni production, a 1-kg meat mixture was prepared containing pork leg trim (40%), beef fat trim (20%), beef forequarter (15%) and beef plate and flank (25%) (all wt/wt). Fat analysis was carried out on each meat sample using an automatic extraction system (JVA Analytical Ltd., Dublin) (4) and the results used to calculate the exact quantity of beef fat to add to produce a 30:70 fat:lean (wt/wt) ratio in the pepperoni batter. The meat mixture was conditioned to 0°C , minced through a 5-mm die plate to produce a homogeneous meat base, and stored overnight at 0°C . Nonmeat ingredients, including sodium ascorbate, spice mix, *Pediococcus* starter culture, sodium nitrite and dextrose were obtained from International Meats. The nonmeat ingredients were added to 1.0 kg of the homogeneous meat base to yield a batter containing sodium ascorbate (0.048%), spice mix (0.302%), *Pediococcus* spp. starter culture (0.028%), salt (2.5%), sodium nitrite (0.1%), mustard flour (1.5%) and dextrose (0.625%) (all wt/wt). Pepperoni batters were also produced with the following formulations: salt (3.3 or 4.8%); sodium nitrite (0.2, 0.3 or 0.4%). Dextrose concentrations of 2.5% and 0.1% were used to give a product with low (4.4) or high pH (5.5), respectively.

Batter inoculation. The batter (1 kg) was inoculated with 5 ml of the *E. coli* O157:H7 suspension to produce a concentration of approximately $6.7 \log_{10}$ CFU/g and mixed by hand for 1 to 2 min. The inoculated batter was minced again through a 10-mm die plate and stuffed into 30-mm diameter synthetic casings (O'Donnells, Queen St., Dublin) which had previously been soaked in 2% potassium sorbate solution for 30 min. This yielded approximately 12 sausages of 15-cm length. These were hung in incubators at 21°C for 1 h and 32°C for 30 min, and then held in a controlled humidity chamber (Sanyo Gallenkamp) at 38°C , 75% relative humidity (RH) until the pH dropped to 4.8 (ca. 9.5 h). When the desired pH had been reached, the sausages were held at 32°C for 30 min in the humidity cabinet, and then immersed in cold (10°C) water for 20 min to stop the fermentation process. The sausages were subsequently dried by hanging in the controlled humidity chamber at 15°C , 64–70% RH, until a_w was <0.80 (approximately 7 days). Mean initial counts of *E. coli* O157:H7 in the unfermented batter was $6.69 \log_{10}$ CFU/g (standard deviation, 0.20) for each lot

analyzed, indicating that the inoculation method used led to the uniform distribution of the pathogen throughout the product.

Microbiological and chemical analysis of pepperoni. Duplicate samples of aseptically recovered pepperoni were analyzed for level of *E. coli* O157:H7, pH, a_w , titratable acidity, and sodium nitrite concentration. Samples were examined immediately after stuffing, hourly during fermentation (pH only), at the end of fermentation, and every 2 to 3 days during drying (except titratable acidity and sodium nitrite) until the a_w was <0.80 .

Estimation of *E. coli* O157:H7 levels. A 10-g sample was placed in 90 ml of maximum recovery diluent (MRD) (Oxoid) in a stomacher bag with a nylon filter (Seward Medical), homogenized for 1 min in a stomacher (Colworth) and serially diluted (1:10) in 9-ml tubes of MRD. Samples were plated onto tryptone soya agar (TSA) (Oxoid) with a spiral plater (Don Whitley Scientific Ltd., West Yorkshire, England), incubated at 37°C for 2 h to allow recovery of injured cells, and overlaid with sorbitol MacConkey agar (SMAC) (Oxoid) (10). Samples were also plated in duplicate directly onto SMAC. All plates were incubated at 37°C for 24 h. Typical *E. coli* O157:H7 colonies were identified by a commercial latex test (Oxoid, cat. no. DR620) and enumerated with a Seescan Imaging Plate Counter (Seescan PLC, Cambridge, England).

Estimation of pH. Duplicate 2-g samples of pepperoni were homogenized in a laboratory mixer emulsifier (Silverson, Water-side, Chesman, Bucks) in 10 ml of a solution of 5 mM sodium iodoacetate and 150 mM potassium chloride (2). The pH was measured with an Orion 210 pH meter (Orion Research Corp., USA) with a combination electrode.

Estimation of titratable acidity. Titratable acidity was determined by the procedure described by Glass et al. (16). Briefly, 25 g of pepperoni was homogenized in hot deionized water and was made up to 250 ml with distilled water. After separation the fat layer was poured off and the remaining aqueous phase filtered. A 100-ml portion of the filtrate was titrated with NaOH to pH 7.0. Titratable acidity was expressed as percentage of total acid (as lactic acid).

Estimation of a_w . The a_w was measured in triplicate using a cooled dew-point apparatus (model CX-2, Aqualab, Labcell) calibrated with distilled water ($a_w = 1.000 \pm 0.003$) and saturated NaCl solution ($a_w = 0.753 \pm 0.001$ at 25°C).

Estimation of sodium nitrite. Sodium nitrite content was estimated by a modification of the colorimetric method of Fiddler (13). Briefly, hot water was added to the pepperoni homogenate and proteins precipitated with zinc acetate and potassium ferrocyanide. The development of a pink color with addition of sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride to the filtrate and subsequent photometric measurement at 538 nm indicated the level of nitrite present according to a calibration curve.

Analysis of results. Pepperoni formulation combinations were produced according to a fractional factorial design. Twenty-one experimental conditions including 31 replicates were prepared. Analysis was carried out on 19 sets of experimental conditions (29 replicates) as fermentation did not occur in two formulations (see Results). Least-squares regression analysis was carried out using the RS1 statistics package (BBN Software Products Corporation, Bolt Beranek and Newman Inc., Cambridge, MA). A multiple regression was carried out on the decline in *E. coli* O157:H7 numbers in relation to differences in salt content, pH, and nitrite

content. The model included linear, first- and second-order terms as follows:

$$TD = a + b.S + c.N + d.pH + e.S^2 + f.N^2 + g. pH^2 + h.S.N + i.S.pH + j.N.pH,$$

where TD = total decline in numbers of *E. coli* O157:H7 (determined on TSA overlaid with SMAC), S = salt content, and N = nitrite content.

RESULTS

Chemical analyses of pepperoni. In pepperoni prepared to the standard formulation (Table 1, no. 1), the pH declined from 6.13 to 4.80 during fermentation (up to 10 h), with limited further decline (<0.1 pH unit) during the subsequent drying period (158 h) (Fig. 1). The a_w of the product fell from approximately 0.96 to 0.93 during fermentation and subsequently declined to the target value of <0.80 after a further 7 days. Fermentation and drying times were generally extended with nonstandard formulations (Fig. 2).

The final titratable acidity levels increased during drying. Titratable acidity measurements were significantly higher ($P < 0.001$) in those product formulations which reached lower final pH values, probably due to the higher levels of available dextrose (results not shown).

The inclusion of ascorbate in the batter mixture led to rapid destruction of the sodium nitrite present in the meat batter (14). Nitrite levels were negligible after 1 day of drying (results not shown). The level of nitrite added, rather than that measured, was used in all calculations.

Survival of *E. coli* O157:H7 in standard pepperoni formulation. In the standard formulation (no. 1) declines in total *E. coli* O157:H7 numbers (injured plus noninjured

cells) (TSA overlaid with SMAC) were 0.41 log₁₀ CFU/g during fermentation and 0.43 log₁₀ CFU/g during the drying period (Fig. 3). Estimates of uninjured cell numbers obtained from the SMAC counts (0.37 log₁₀ CFU/g reduction during fermentation and 0.76 log₁₀ CFU/g reduction during drying) were similar to the total cell counts, suggesting that there were not significant numbers of sublethally injured *E. coli* O157:H7 cells in the standard formulation pepperoni.

Survival of *E. coli* O157:H7 in nonstandard pepperoni formulation. In all formulations *E. coli* O157:H7 numbers declined during the fermentation phase and during the drying phase (Table 1) often with significant levels of sublethal cell damage. Fig. 4 shows the changes in *E. coli* O157:H7 numbers in a high-salt, high-sodium nitrite, low-final pH pepperoni formulation (no. 13). There were greater declines in total *E. coli* O157:H7 counts during fermentation (3.36 log₁₀ CFU/g) and drying (1.43 log₁₀ CFU/g) than the respective declines with the standard formulation. During the fermentation phase (up to 24 h), no sublethally injured cells were detected, although significant overall reductions in total *E. coli* O157:H7 numbers were recorded. During the drying phase (24 to 216 h), there were substantial differences between total (TSA with SMAC overlay) and noninjured (SMAC) counts. The pattern of cell death and of the development of sublethal injury during the drying process was triphasic. In the early stages of the drying phase, 24 to 72 h, there was a significant decline in noninjured counts, suggesting that considerable sublethal cell damage was occurring although net cell death was limited. Between 72 and 168 h there was little change in total count or noninjured count, suggesting that no further cell death or injury was occurring. After 168 h, both total and noninjured counts

TABLE 1. Decline of *E. coli* O157:H7 in pepperoni produced to standard and modified formulations

No.	Formulation			Enumerated on TSA with SMAC overlay (log ₁₀ CFU/g)		Enumerated on SMAC (log ₁₀ CFU/g)		Total pH change
	Salt (%)	NaNO ₂ (ppm)	pH ^a	Decline after fermentation	Total decline after drying	Decline after fermentation	Total decline after drying	
1	2.5	100	std.	0.41	0.84	0.37	1.13	1.33
2	2.5	100	low	0.38	1.71	0.68	2.51	1.44
3	2.5	100	high	0.11	1.85	0.07	2.14	0.65
4	2.5	200	high	0.22	0.67	0.03	0.83	0.58
5	2.5	300	low	0.86	1.98	0.71	2.14	1.53
6	2.5	300	high	0.44	1.54	0.26	1.95	0.53
7	3.3	100	std.	0.16	0.79	-0.04	0.80	1.32
8	3.3	100	low	0.52	1.10	0.48	1.30	1.09
9	3.3	100	high	0.08	0.32	0.13	0.38	0.63
10	3.3	200	std.	0.44	1.83	-0.26	1.74	1.19
11	3.3	200	low	1.82	2.97	1.61	2.88	1.29
12	3.3	200	high	0.36	0.89	0.18	0.80	0.52
13	3.3	300	low	3.36	4.79	4.00	7.02	1.61
14	3.3	300	high	0.63	1.39	0.44	1.50	0.78
15	3.3	400	std.	0.69	1.80	0.77	2.13	1.11
16	4.8	100	std.	0.66	0.94	0.72	1.49	1.13
17	4.8	100	low	0.47	1.87	0.94	2.07	1.45
18	4.8	100	high	0.69	0.99	0.96	1.43	0.53
19	4.8	300	high	1.74	2.49	2.16	4.30	0.57

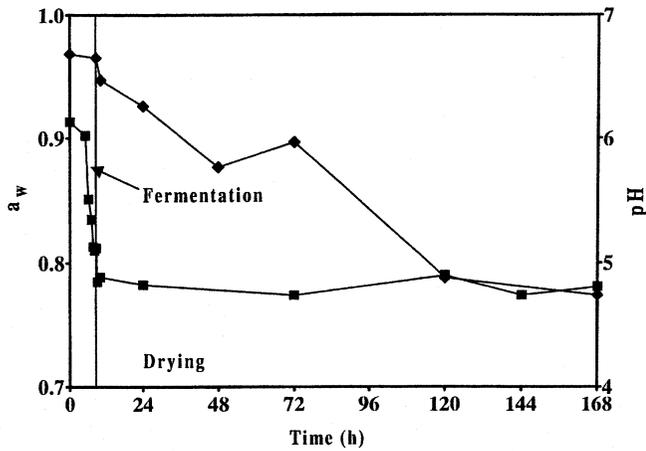


FIGURE 1. Changes in pH and a_w during standard pepperoni production. Formulation: 2.5% salt, 100 ppm sodium nitrite, pH 4.80. ■, pH; ◆, a_w .

declined rapidly, so that by 216 h the residual population was composed entirely of injured cells.

Greater reductions in pathogen numbers were observed in formulations which combined higher salt and sodium nitrite levels with lowered pH ($P < 0.001$). Two formulations which combined high salt levels (4.8%) with low pH and high sodium nitrite levels did not ferment. In formulations with high salt levels in which fermentation did occur, salt did not appear to significantly influence total or noninjured counts. However, it has been suggested that high salt concentrations may retard starter culture activity, leading to extended fermentation and drying periods with such formulations (26).

No growth of *E. coli* O157:H7 was observed with any formulation, probably due to the inhibitory effects of low pH and a_w . Only formulation no. 13, with a total decline in numbers of 4.79 \log_{10} CFU/g, approached the FSIS target of a 5- \log_{10} -unit reduction in pathogen numbers in a fermented product; however, the level of nitrite added is above that permitted in commercial meats (5).

Multiple regression analysis showed that the synergistic combination of sodium nitrite levels, total pH change, and

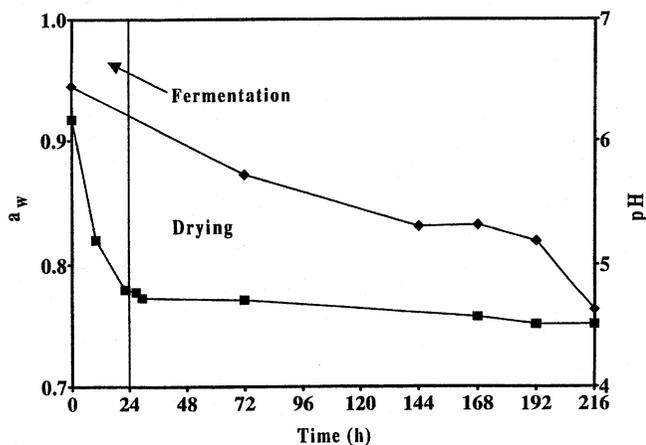


FIGURE 2. Changes in pH and a_w during nonstandard pepperoni production. Formulation: 3.3% salt, 300 ppm sodium nitrite, pH 4.56. ■, pH; ◆, a_w .

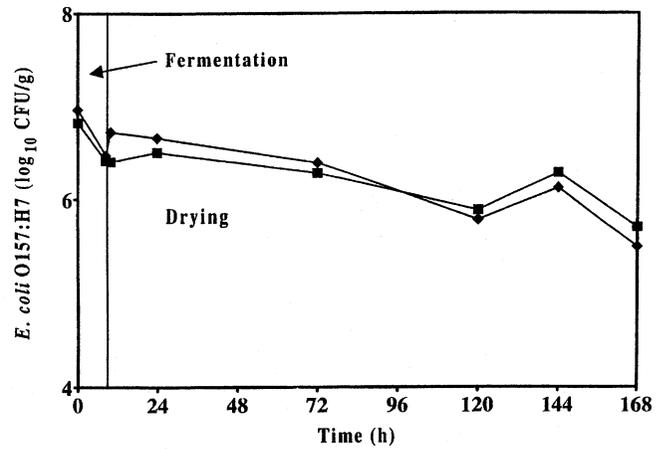


FIGURE 3. Decline in *E. coli* O157:H7 during standard pepperoni production. Formulation: 2.5% salt, 100 ppm sodium nitrite, pH 4.80. ■, total cell population; ◆, noninjured population only.

salt levels had significant effects on *E. coli* O157:H7 survival during both fermentation ($P < 0.001$) and drying ($P < 0.001$). The pH had a significant effect on total *E. coli* O157:H7 population decline ($P < 0.05$), as did the synergistic effects of salt and sodium nitrite ($P < 0.01$), and of pH and sodium nitrite ($P < 0.05$) (Table 2). The regression equation fitted well to the data ($r^2 = 0.73$). The F value for the equation for 9 and 19 degrees of freedom was 5.60 ($P < 0.001$).

DISCUSSION

This study examines the survival of *E. coli* O157:H7 during fermentation and drying of pepperoni. Previous studies of this organism in culture broth have shown the effects of a number of stresses, including pH, water activity, and sodium nitrite on the growth of this pathogen (3, 8, 15). However, the pathogen may behave differently when growing in a semisolid meat product, where additional stresses apply. Components present in commercial meat product formulations but not in laboratory media result in changes in the environment of the organism and could significantly

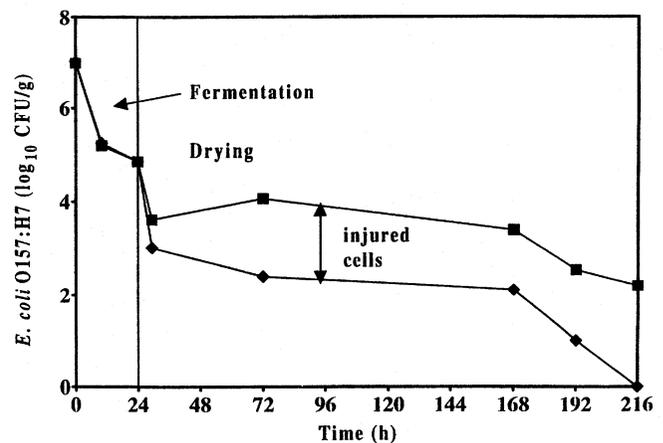


FIGURE 4. Decline in *E. coli* O157:H7 during nonstandard pepperoni production. Formulation: 3.3% salt, 300 ppm sodium nitrite, pH 4.56. ■, total cell population; ◆, noninjured population only.

TABLE 2. Least squares regression coefficients of percent added salt, added sodium nitrite, and total pH change on the decline in total *E. coli* O157:H7 log CFU/g

Term	Coefficient	Significance level
constant	8.674	0.066
salt	-2.117	0.293
nitrite	-0.016	0.195
pH	-8.466	0.025
salt ²	0.097	0.674
nitrite ²	0.000	0.132
pH ²	2.748	0.067
salt × nitrite	0.006	0.007
salt × pH	0.721	0.129
nitrite × pH	0.011	0.025

affect the extent to which the environment will support or suppress growth. For example, sodium nitrite, stable in a laboratory broth, is rapidly destroyed by reaction with ascorbate, which is present in many commercial meat products. Similarly, data from studies in which this pathogen was inoculated into a fermented and matured (dried) product (8, 9) may not reflect the pattern of challenges faced by organisms which are present in the product throughout the normal production process. Organisms inoculated into mature products will not, for example, have been subject to, or conditioned by, the stresses of changing pH encountered in a fermenting product. This may be particularly relevant in products like pepperoni, where this study has shown complex and varying patterns of cell damage and cell death during development and maturation. The fact that this organism can survive commercial processing, albeit with sublethal damage, is a cause for concern. A number of factors may contribute to its survival in such products. It has been reported that *E. coli* O157:H7 can grow at a pH as low as 4.5, although its exact tolerance is influenced by the specific acid present (19). Increased acid tolerance offers such an organism ecological advantages by allowing the pathogen to survive in the gastric system and to establish infection in the colon and other organs (1). In this study the acid tolerance of the organism may have been enhanced by the metabolic state of the inoculum, in that the cells were in stationary phase. Stationary-phase cells have a higher acid tolerance (3), enhancing their survival during fermentation and drying.

This study shows that *E. coli* O157:H7 can survive in pepperoni formulations which have higher salt and sodium nitrite concentrations than current commercial products. This finding confirms previous reports from studies using broth cultures which suggested that this organism had higher salt and nitrite tolerances than other enteric organisms, including *Salmonella* spp. (19). The ability of *E. coli* O157:H7 to survive such adverse conditions, and recognition of its low infectious dose, means that its presence in fermented meat products and their ingredients is particularly undesirable.

This findings of this study indicate that a strategy based simply on increasing the concentrations of inhibitory ingredients currently used is unlikely to prove successful, as the necessary additive levels would result in unacceptable

changes in the organoleptic qualities of the product. A more successful approach may be developed by introducing a heating step after fermentation. This approach has been recommended by other researchers (18) who reported heating steps that resulted in the 5-log₁₀ kill recommended by FSIS. However, heating may have additional undesirable organoleptic effects on the final product. A composite, multiple hurdle strategy including some minor changes in product formulation, e.g., lowered pH, 3.3% salt, 100 ppm sodium nitrite, and possibly the addition of sodium lactate together with a mild heating step (40°C) could be effective.

In conclusion, it is clear that the current commercial formulations and processing protocols used are insufficient to adequately suppress the presence of *E. coli* O157:H7 in fermented meat products. Further work is required to establish effective means of producing pepperoni and related products which satisfy the organoleptic requirements of consumers and the food safety criteria of statutory agencies.

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