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Viability of *Escherichia coli* O157:H7 in pepperoni during the manufacture of sticks and the subsequent storage of slices at 21, 4 and −20°C under air, vacuum and CO_2

Nancy G. Faith[a], Nelly Parniere[1,a], Trina Larson[a], Timothy D. Lorang[a], John B. Luchansky[a,b,*]

[a]*Department of Food Microbiology and Toxicology, Food Research Institute, 1925 Willow Drive, Madison WI 53706, USA*
[b]*Department of Food Science, University of Wisconsin, Madison WI 53706, USA*

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Abstract

A raw, pepperoni batter (75% pork:25% beef with a fat content of about 32%) was inoculated with a pediococcal starter culture (about 10^8 cfu/g) and a five-strain cocktail of *Escherichia coli* O157:H7 ($\geq 2 \times 10^7$ cfu/g), mixed with non-meat ingredients, and then hand-stuffed into 55 mm fibrous casings to form sticks. The numbers of the pathogen were determined before stuffing, after fermentation, after drying/slicing, and after periods of storage. For storage, slices were packaged under air, vacuum or CO_2 and stored at −20, 4 and 21°C. Sticks were fermented at 36°C and 85% relative humidity (RH) to ≤pH 4.8 and then dried at 13°C and 65% RH to a moisture/protein ratio (M/Pr) of ≤1.6:1. Fermentation and drying resulted in the numbers of the pathogen decreasing by about 2 $\log_{10}$ units. During storage, the temperature rather than the atmosphere had the greater effect on pathogen numbers. The greatest reductions in numbers were observed during storage at 21°C, when numbers decreased to about 2 and 3.8 $\log_{10}$ cfu/g within 14 days in product stored under air and vacuum, respectively, and a 5 $\log_{10}$ reduction was observed for both atmospheres within 28 days. Regardless of the storage atmosphere, numbers did not decrease below 3.6 or 3.7 $\log_{10}$ cfu/g after 90 days of storage at −20 or 4°C, respectively. These data confirm that fermentation and drying are sufficient to eliminate only about 2 $\log_{10}$ cfu/g of *E. coli* O157:H7 from fermented sausage, and that additional strategies, such as storage for at least 2 weeks at ambient temperature in air, are required to achieve a 5 to 6 $\log_{10}$ reduction in the numbers of the pathogen in sliced pepperoni. © 1997 Elsevier Science B.V.

Keywords: *Escherichia coli* O157:H7; Pepperoni; Pathogen; Fermentation; Survival

1. Introduction

There has been much concern over the association of *Escherichia coli* O157:H7 with meats, and considerable research initiated to determine how to control

*Corresponding author. Tel.: +1 608 2637777; fax : +1 608 2631114; e-mail: jbluchan@facstaff.wisc.edu
[1]Present address: The National Institute of Agrifood Industries (INSFA), 65, Rue de Saint-Brieuc, 35042 Rennes, France. Tel.: +33 2 47665221; e-mail: parniere@france-cybermedia.com

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the pathogen in fermented meat products. Shortly after an outbreak of *E. coli* O157:H7 infection which was linked to a dry, fermented pork/beef salami (Centers for Disease Control, 1995a), several laboratories initiated studies on the viability of this pathogen in a variety of fermented meats, to develop manufacturing processes that would reliably reduce pathogen numbers by 5 $\log_{10}$ units. The impetus for these validation-type studies was the regulatory mandate from the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) that manufacturers of dry and semi-dry fermented sausage demonstrate a 5-$\log_{10}$ unit reduction of *E. coli* O157:H7 during processing (Reed, 1995). With the possible exception of beef jerky (Harrison and Harrison, 1996), the few reports published to date have revealed that fermentation and drying are only sufficient to effect a reduction of about 2 $\log_{10}$ units in the numbers of this pathogen during the manufacture of pepperoni (Hinkens et al., 1996), salami (Clavero and Beuchat, 1996; Glass et al., 1992; Nickelson et al., 1996), or summer sausage (Calicioglu et al., 1997). Thus, efforts have intensified to identify additional intervention strategies to obtain greater reductions of the numbers of this pathogen in fermented meats.

As reported by Hinkens et al. (1996), heating pepperoni sticks, after fermentation at 36°C to $\leq$pH 5 and drying, to an internal temperature of 63°C instantaneous or to 53°C for 60 min, resulted in a $\geq$5-$\log_{10}$ unit decrease in numbers without visibly affecting the texture or appearance of the product. Similarly, fermentation at 41°C to pH 4.6 or pH 5 and post-fermentation heating of sticks to an internal temperature of 54°C resulted in a >5 $\log_{10}$ decrease in counts of *E. coli* O157:H7 in summer sausage (Calicioglu et al., 1997). As another example, extended storage or fermentation of sausage sticks at 32°C to pH 4.6 reduced counts of the pathogen by at least 4 $\log_{10}$ cfu/g (Nickelson et al., 1996). Aside from the application of heat, relatively little else has been reported on other intervention strategies to reduce numbers of this pathogen in fermented meats.

There are several categories and varieties of all-beef or beef/pork (55%/45%) fermented sausage that have a moisture/protein (M/Pr) ratio of 1.6:1 and, as such, are called pepperoni (United States Department of Agriculture, Food Safety and Inspection Service, 1986). With about 115 000 000 kg used annually by pizzerias in the USA alone and with about 57% of customers for pizzas in a 2-store survey selecting pepperoni as their favorite topping, pepperoni is arguably the most popular variety of pizza topping in the United States (A. Willman, personal communication). About 90% of the pepperoni destined for use as a topping on frozen pizza is sold in stick form and is typically stored refrigerated under air or CO_2 for up to 30 days, the remainder being pre-sliced and typically stored refrigerated or frozen for up to 30 or 90 days, respectively (T. Lorang, unpublished data). Pepperoni pizza topping targeted for use by pizzerias is sliced in bulk and stored refrigerated or frozen with a shelf life of 30 or 90 days, respectively (T. Lorang, unpublished data). In a previous study (Hinkens et al., 1996), we reported that fermentation/drying of pepperoni sticks reduced the numbers of the pathogen by about 2 $\log_{10}$ units. The present study expanded upon our previous research with pepperoni (Hinkens et al., 1996) and monitored the effect of different storage conditions on the fate of *E. coli* O157:H7 in pepperoni slices intended for use as pizza topping.

2. Materials and methods

2.1. Bacterial strains.

Five strains of *E. coli* O157:H7 were used to inoculate pepperoni. The strains were EC505B, a beef isolate from the University of Wisconsin Food Research Institute; C7927, a human isolate from the Massachusetts apple cider outbreak of 1991 (Besser et al., 1993); F-90, a sausage isolate from the Washington/California dry-cured salami outbreak of 1994 (Centers for Disease Control, 1995a); EC204P, a pork isolate from the University of Wisconsin Food Research Institute; and C9490, a human isolate from the Western States hamburger patty outbreak of 1993 (Centers for Disease Control, 1993). The *E. coli* strains were maintained as recommended by the USDA/FSIS (Reed, 1995). A commercial *Pediococcus acidilactici* starter culture (Lactacel 115; Quest International, Sarasota, FL) was maintained and propagated according to the manufacturer's instructions.

2.2. Preparation of E. coli O157:H7 and P. acidilactici inocula.

Each of the five strains of *E. coli* O157:H7 was grown separately in 250 ml of trypticase soy broth (TSB; Difco Laboratories Inc., Detroit, MI) supplemented with 1% glucose, at 37°C, overnight, with shaking. The five cell suspensions were harvested and combined to achieve a final inoculum of about 1×10^9 cfu/ml containing equal numbers of the strains, as previously described (Hinkens et al., 1996). 6 ml of the thawed pediococcal starter culture were added to 42 ml of sterile dH_2O for addition to 11.33 kg of batter.

2.3. Manufacture of pepperoni.

A flow diagram for pepperoni manufacture and storage is provided in Fig. 1. The raw meat supplied by a commercial manufacturer was maintained at about 4°C during batter preparation which was conducted essentially as described (Hinkens et al., 1996). Raw batters were prepared in 46 kg batches, which were composed of 75% pork and 25% beef with a fat content of about 32% and contained 0.63% dextrose (A. E. Staley, Decatur, IL), 3% spice mix (Doskocil house blend; Doskocil Company, Inc., Jefferson, WI), and 2% cure mixture (Doskocil house blend). The batter was inoculated with the cocktail of *E. coli* O157:H7 to obtain numbers of $\geq 2 \times 10^7$ cfu/g of batter, and with the thawed pediococcal starter culture to achieve about 10^8 cfu/g of batter. A control batter without *E. coli* O157:H7 was similarly prepared. The batter was ground using a commercial grinder (model 84142; Hobart Manufacturing Co., Troy, OH) through a 1/8" plate and then stuffed using a hand stuffer (Koch Supplies, Inc., Kansas City, MO) into 55 mm fibrous casings (TeePak, Inc., Westchester, IL), to a final length of 47 cm to give sticks which each weighed about 700 g. The sticks were transferred to a smokehouse (model 1000; Vortron, Inc., Beloit, WI) and fermentation was conducted as previously described (Hinkens et al., 1996) to a final pH of < pH 4.8, which was achieved after 16 to 20 h.

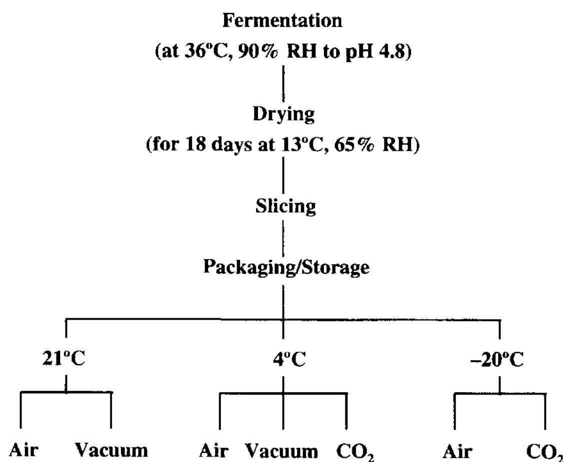

Fig. 1. Pepperoni manufacture. Flow diagram depicting the pepperoni processes evaluated.

2.4. Drying, slicing and storage of pepperoni.

The sticks were dried at 13°C and 65% RH for about 18 days to a M/Pr of ≤1.6:1 in an environmentally controlled chamber (Biotron facility; University of Wisconsin, Madison, WI). The dried sticks were cut into slices of about 1.9 g each using a Globe slicing machine (Model 500; Stamford, CT). Portions, each of 55 slices, were placed into clear, polyethylene bags (Curlon® Grade 863; 18 × 23 cm; O_2 < 1.0 CC per 100 $inch^2$ per 24 h at 23°C and 9% RH with MVTR 0.5 gm H_2O per 100 $inch^2$ per 24 h at 38°C and 90% RH; Curwood Inc., Oshkosh, WI) for packaging under air, CO_2 (≤ 1% residual O_2), or vacuum. The latter two atmospheres were generated using a Multivac vacuum-packaging machine (Sepp Haggenmüller KG, Germany), and the percent residual oxygen present was determined on representative bags packaged under CO_2 using a headspace analyzer (Model HS-750: Modern Controls, Inc., Minneapolis, MN). For slices stored under air, the bags were sealed about 0.5 cm from the top using an impulse heat sealer (model TISH-300; Electric Heating Equipment Co., Ltd., Taiwan, R.O.C.) to achieve an unoccupied volume of about 650 ml. Packages were stored at −20, 4, or 21°C for 1, 4, 7, 14, 28, 60, or 90 days. At each sampling interval, three packages were removed from storage and a 25 g portion of pepperoni from each package was analyzed microbiologically.

2.5. *Microbiological analyses of pepperoni batters, sticks, and slices.*

Microbiological testing was conducted for each of three trials using triplicate samples of meat and duplicate platings of each dilution. Batter, sticks, or slices were tested for viable *E. coli* O157:H7 by direct plating of macerated and diluted meat samples prior to stuffing, after fermentation, after drying/slicing, and after storage at different temperatures and atmospheres. At each sampling interval, three 25-g portions of the batter, or a 25-g cross section from the middle of each of three sticks, or a 25-g composite of slices were aseptically transferred to Stomacher bags (Seward Medical, London, UK) containing 225 ml of 0.1% peptone and processed and plated onto MacConkey sorbitol agar (MSA; Difco) as described previously (Hinkens et al., 1996). When numbers of the pathogen decreased below 10^1 cfu/g, the presence/absence of the pathogen was determined by enrichment as previously described (Hinkens et al., 1996). The raw meat was tested for background levels of *E. coli* O157:H7 and other non-sorbitol-fermenting bacteria by spread plating macerated and diluted meat samples onto MSA plates and for total aerobic bacterial numbers by spread plating macerated and diluted meat samples onto trypticase soy agar (TSA; Difco) plates. Immediately after stuffing, the sticks were also tested for viable pediococci and lactic acid bacteria (LAB) by spread plating macerated and diluted meat samples onto MRS (Difco) agar plates. Plates were incubated at 42°C for 24 h to recover *E. coli* O157:H7 and at 37°C for 48 h to recover pediococci/LAB. Representative isolates of each type were confirmed as previously described (Hinkens et al., 1996).

2.6. *Chemical analyses of pepperoni sticks and organoleptic observations of slices.*

At each sampling time (before stuffing, after fermentation, after drying, and after storage), three control sticks were removed for chemical analyses. The control sticks were either transported on ice directly to a commercial testing laboratory, or were held at −20°C for up to 7 days and then delivered for testing. Chemical analyses were performed on a composite from each of these three sticks following Association of Official Analytical Chemists (AOAC) procedures. Each composite was tested for fat (AOAC procedure 960.39), moisture (AOAC procedure 950.46), protein (AOAC procedure 928.06), and salt (AOAC procedure 935.47) as reported for meat products by McNeal (1990). The water activity (a_w) was determined using a Rotronic water activity meter (model DT; Huntington, NY). The pH and titratable acidity (TA) were determined as previously described (Luchansky et al., 1992). The TA was expressed as the percent lactic acid. Pepperoni slices were also evaluated for any changes in color or texture, or for the appearance of yeast/molds, by visual inspection and palpation of slices.

2.7. *Statistical analyses.*

Data were analyzed using version 5 of the Statistical Analysis System user's guide (SAS Institute, Cary, NC).

3. Results

3.1. *Testing of raw meat.*

Analyses of the raw meat before inoculation with the serotype O157:H7 cocktail and pediococcal starter culture revealed that none of the raw meat tested contained indigenous *E. coli* O157:H7 by direct plating (data not shown). The raw meat produced total aerobic plate counts ranging from 1.8×10^3 to 5×10^5 cfu/g of meat (average = 1.7×10^5 cfu/g). The pediococci/LAB count of the raw meat ranged from 3×10^2 to 2.7×10^3 cfu/g of meat (average = 1.8×10^3 cfu/g). The pediococci/LAB count after the addition of the starter culture to the batter ranged from 5×10^7 to 1.2×10^8 cfu/g of meat (average = 7.8×10^7 cfu/g). The proximate composition of the pepperoni also displayed the expected levels for pH, a_w, salt, protein, moisture, and fat (Table 1).

3.2. *Microbiological testing of pepperoni sticks and slices during manufacture and storage.*

The raw batter contained 7.8±0.52 $\log_{10}$ cfu/g of *E. coli* O157:H7. Thereafter, counts of the pathogen decreased to 6.8±0.40 $\log_{10}$ cfu/g after fermentation

Table 1
Chemical composition of pepperoni during manufacture and storage.

Manufacturing step	Chemical Analysis (value ± s.d; n = 3)							
	pH	TA (%)[a]	M/Pr[b]	a_w	Salt (%)	Moisture (%)	Protein (%)	Fat (%)
Batter	5.91±0.05	0.35±0.04	3.60±0.09	0.96±0.00	3.28±0.06	47.93±0.80	13.32±0.14	33.29±0.25
After fermentation[c]	4.82±0.01	0.60±0.06	3.54±0.08	0.96±0.00	3.24±0.09	46.80±1.59	13.26±0.69	35.09±1.68
After drying/slicing[c]	4.77±0.01	0.78±0.09	1.63±0.33	0.90±0.03	3.97±0.70	29.03±4.03	17.89±1.04	46.23±2.49
After storage[d] *(Temp., Atmos.)*								
21°C Air	5.10	0.89	1.41	.883	4.46	27.65	19.56	48.04
21°C Vacuum	4.76	0.95	1.40	.881	4.43	26.77	19.06	48.83
4°C Air	4.77	0.96	1.43	.885	4.55	27.66	19.34	48.19
4°C Vacuum	4.78	0.96	1.43	.882	4.46	27.11	18.98	48.88
4°C CO_2	4.68	0.99	1.48	.881	4.35	27.83	18.83	48.06
−20°C CO_2	4.67	0.92	1.48	.885	4.62	27.61	18.65	46.78
−20°C Air	4.70	1.38	1.89	.900	4.12	31.82	16.86	45.13

[a] TA, titratable acidity as % lactic acid.
[b] M/Pr, moisture to protein ratio.
[c] values are the averages of 3 trials.
[d] values for 14 days of storage from trial #2.

and to about 5.9±1.10 $\log_{10}$ cfu/g after drying. Within 14 days counts of the pathogen were reduced by about an additional 2 (vacuum) and 4 (air) $\log_{10}$ during storage at 21°C compared to a 1 to 2 $\log_{10}$ decrease at 4°C and about a 1.5 $\log_{10}$ decrease at −20°C. Whereas pathogen numbers decreased to below detection by direct plating within 28 (air) and 60 (vacuum) days during storage at 21°C, the greatest reductions during storage at 4°C and −20°C, regardless of atmosphere, were approximately 4 $\log_{10}$ after 90 days. However, it was still possible to recover the pathogen by enrichment during storage at 21°C on days 28 (air), 60 (vacuum), and 90 (vacuum). Differences among treatments were also observed that were attributed to the storage atmosphere, but such differences were not as significant as the differences observed with storage temperature. In general, storage under air was more detrimental to the serotype O157:H7 cocktail than storage under vacuum or under CO_2 (Table 2). At day 14, numbers of the pathogen after storage under air at 21°C were significantly ($P < 0.05$) lower than observed for other treatments at day 14.

There was no appreciable difference in the proximate composition of sticks/slices related to storage temperature or atmosphere (Table 1). However, after

Table 2
E. coli O157:H7 counts in pepperoni slices during storage at different temperatures under different atmospheres.

Temperature	Atmosphere	Time (days) of storage ($\log_{10}$ cfu g^{-1} ± s.d.; $n = 3$)						
		1	4	7	14	28	60	90
21°C	Air	5.51±0.56	4.71±0.67 (a)[a]	3.90±0.96 (a)	< 1.88±1.55 (a)	< 1.0±0.0 (a)[b]	N.T.[c]	N.T.
	Vacuum	5.59±0.67	4.93±0.63 (a)	4.39±0.78 (a)	3.77±0.88 (b)	1.90±0.51 (a)	< 1.0 (a)[b,d]	< 1.0 (a)[b,d]
4°C	Air	5.85±0.60	5.75±0.80 (b)	5.63±1.07 (b)	5.12±1.06 (c)	4.76±0.79 (b)	4.40±0.45 (b)	3.71±1.61 (b)
	Vacuum	5.95±0.76	6.20±1.01 (b)	5.56±1.07 (b)	5.29±1.03 (c)	5.07±1.12 (b)	4.77±0.78 (b)	4.29±1.26 (b)
	CO_2	5.83±0.81	5.87±0.96 (b)	5.54±0.74 (b)	5.81±0.87 (c)	5.32±0.90 (b)	4.65±0.81 (b)	4.13±1.46 (b)
−20°C	Air[e]	5.23±0.36	5.02±0.08 (a)	4.38±0.17 (a)	4.41±0.31 (b)	3.43±1.04 (c)	4.13±0.71 (b)	3.58±0.64 (b)
	CO_2	5.08±0.65	4.91±0.85 (a)	4.16±0.14 (a)	4.45±0.96 (b)	3.74±0.71 (c)	3.91±0.34 (b)	3.91±1.90 (b)

[a] Values in a column that are not followed by the same letter are significantly different ($P \leq 0.05$).
[b] Positive for *E. coli* O157:H7 by enrichment.
[c] N.T. = not tested due to mold contamination.
[d] Values from trial #3.
[e] Values from trials #2 and #3.

storage of slices in air at 21°C for 28 days the product changed from bright orange to grayish brown and yeast and mold growth was observed on slices from several packages (data not shown). Storage of slices at lower temperatures and/or at other atmospheres did not result in any visible changes in color and did not support mold or yeast growth (data not shown).

4. Discussion

Escherichia coli O157:H7 has caused considerable illness and death during the 1990s (Griffin, 1995). Notable examples include foodborne outbreaks associated with undercooked hamburger patties from a fast food restaurant chain (Centers for Disease Control, 1993), apple cider prepared using fallen apples contaminated with deer feces (Besser et al., 1993), unpasteurized apple juice or juice mixtures containing apple juice (Centers for Disease Control, 1996), and fermented sausage with an unconfirmed source of contamination (Centers for Disease Control, 1995a). There was also an outbreak in Japan with an unconfirmed source involving >9,000 individuals (Anonymous, 1996) and an outbreak in Australia from uncooked, semidry fermented sausage contaminated with another enterohemorrhagic serotype, *E. coli* O111:NM (Centers for Disease Control, 1995b). In hindsight, the relative tolerance of *E. coli* O157:H7 to both acid (Arnold and Kaspar, 1995; Benjamin and Datta, 1995; Miller and Kaspar, 1994) and heat (Jackson et al., 1995) may partly explain the ability of this pathogen to persist and cause illness in certain foods previously regarded as low risk. There may also be significant variations in the degree of acid and/or heat tolerance from strain-to-strain, and a subpopulation of cells of a given strain of O157:H7 may also be more or less resistant to heat and/or acid than are the majority of cells within the same population (Todd et al., 1993; C. W. Kaspar and J. B. Luchansky, unpublished data). Largely as a result of the Washington/California salami outbreak of 1994 (Centers for Disease Control, 1995a), the USDA/FSIS developed guidelines for sausage manufacturers to validate processes to ensure a 5 log reduction in counts of *E. coli* O157:H7 in fermented meats (Reed, 1995).

Several factors in the formulation and preparation of pepperoni may affect viability of serotype O157:H7 strains of *E. coli*. Previous studies indicated that indigenous bacteria, the spice/cure mix, and smoking do not appreciably influence numbers of *E. coli* O157:H7 in pepperoni (Hinkens et al., 1996). Amongst the other factors that could influence the viability of serotype O157:H7 strains in a fermented meat, the combined effects of time, temperature, and pH/acid type influence the survival of the pathogen. For example, extended time at ≤pH 4.6 and/or ≥32°C as well as extensive drying at elevated temperature, such as 60°C for 10 h, resulted in an appreciable decrease in pathogen numbers (Harrison and Harrison, 1996; Nickelson et al., 1996). However, it is unlikely that these parameters could be adjusted sufficiently to deliver a 5-D kill of *E. coli* O157:H7 in pepperoni. Similar results were also observed in synthetic media. For example, *E. coli* O157:H7 was more sensitive to acid conditions at abusive than at refrigeration temperatures (Abdul-Raouf et al., 1993; Besser et al., 1993; Miller and Kaspar, 1994). Although post-fermentation heating of pepperoni sticks and/or manufacture of sticks at reduced pH levels delivered the mandated reduction of 5 log cfu g^{-1} in counts of *E. coli* O157:H7 (Hinkens et al., 1996; Nickelson et al., 1996; Calicioglu et al., 1997), some sausage varieties cannot be so treated without causing undesirable sensory or textural changes in the product and/or without substantially increasing processing costs. Heating may also cause 'greasing out' and may yield a product more prone to cupping when used as a pizza topping (S. Seideman, personal communication). If heating regimes appropriate to the product cannot be identified, then storage of pepperoni slices in air at ambient temperature may provide a practicable safety measure.

In the present study, pathogen numbers were monitored during storage of pepperoni slices at different temperatures and under different atmospheres. During storage, the numbers of the pathogen were reduced to a greater extent at ambient temperature than at chiller or freezing temperatures. The data revealed that storing pepperoni slices at 21°C under air or vacuum were sufficient to deliver a 5 log reduction in pathogen numbers. The data also revealed that refrigerated or frozen storage resulted in an additional about 1.5 to 2.5 log reduction over the about 2 log reduction achieved via fermentation and

drying. Although storage at ambient temperature resulted in the greatest reduction in pathogen numbers, this treatment produced some undesirable changes in product appearance. Studies are underway to quantify the effect of cooking pizza containing pepperoni slices spiked with *E. coli* O157:H7 on pathogen viability and to evaluate other cost-effective intervention strategies such as food grade (bio)preservatives and both pre- and post-fermentation pasteurization which might be used to reduce the likelihood of foodborne illness due to *E. coli* O157:H7.

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