Validation of Pepperoni Process for Control of Shiga Toxin–Producing Escherichia coli

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ABSTRACT

The objective of this study was to compare the survival of non-O157 Shiga toxin–producing Escherichia coli (STEC) with E. coli O157:H7 during pepperoni production. Pepperoni batter was inoculated with 7 log CFU/g of a seven-strain STEC mixture, including strains of serotypes O26, O45, O103, O111, O121, O145, and O157. Sausages were fermented to pH ≤4.8, heated at 53.3°C for 1 h, and dried for up to 20 days. STEC strains were enumerated at designated intervals on sorbitol MacConkey (SMAC) and Rainbow (RA) agars; enrichments were completed in modified EC (mEC) broth and nonselective tryptic soy broth (TSB). When plated on SMAC, total E. coli populations decreased 2.6 to 3.5 log after the 1-h heating step at 53.3°C, and a 4.9- to 5-log reduction was observed after 7 days of drying. RA was more sensitive in recovering survivors; log reductions on it were 1.9 to 2.6, 3.8 to 4.2, and 4.6 to 5.3 at the end of cook, and at day 7 and day 14 of drying, respectively. When numbers were less than the limit of detection by direct plating on days 14 and 20 of drying (representing a 5-log kill), no more than one of three samples in each experiment was positive by enrichment with mEC broth; however, STEC strains were recovered in TSB enrichment. Freezing the 7-day dried sausage for 2 to 3 weeks generated an additional 1- to 1.5-log kill. Confirmation by PCR revealed that O103 and O157 had the greatest survival during pepperoni productions, but all serotypes except O111 and O121 were occasionally recovered during drying. This study suggests that non-O157 STEC strains have comparable or less ability than E. coli O157 to survive the processing steps involved in the manufacture of pepperoni. Processes suitable for control of E. coli O157 will similarly inactivate the other STEC strains tested in this study.

Shiga toxin–producing Escherichia coli (STEC) strains are often associated with ruminants and have been isolated from cattle carcasses, retail beef, and raw milk (11, 24–26). More than 200 virulent serotypes of STEC have been associated with outbreaks and sporadic human cases of hemolytic uremic syndrome or severe diarrhea. The prototype STEC strain is E. coli O157:H7, whose epidemiology and virulence have been well studied (34, 40). Comparatively little is known about other STEC strains, and although non-O157 STEC strains are thought to cause illness as frequently as E. coli O157:H7 (3, 27, 29), the significance of non-O157 STEC strains in human disease is less clear. From epidemiological data, six serotypes of non-O157 (O26, O103, O111, O45, O121, and O145) comprise a majority of human STEC cases (3, 6–8). Both the incidence and potential severity of human non-O157 STEC infection have led to regulations for the presence of the most common pathogenic serotypes of STEC in raw ground beef or its precursors (1, 42).

Outbreaks involving non-O157 STEC strains have been linked to meat, dairy products, water, both drinking water and pool or lake water, produce, and unpasteurized apple cider (3, 17). Outbreaks involving fermented meats are noteworthy because these products have a lower pH and lower water activity (aw), and they contain curing salts (nitrate and nitrite) that decrease pathogen survival. An E. coli O157:H7 outbreak associated with dry fermented pork and beef salami in late 1994 indicated that pathogenic STEC strains could survive in fermented meats despite multiple antimicrobial hurdles (41). This outbreak was followed by a 1995 mettwurst outbreak in Australia, caused by serotype O111:NM, that resulted in 23 cases of hemolytic uremic syndrome among 88 persons affected (5). In response to these outbreaks, the U.S. Department of Agriculture developed guidelines for manufacturers to validate processes for a 5-log reduction in numbers of E. coli O157:H7 (36). Studies determined that fermentation and drying resulted in a 1- to 2-log reduction in populations of E. coli O157:H7 and that a thermal step was necessary to achieve a 5-log reduction (18–25, 38). Most recently, a fermented, semidry sausage
known as Lebanon bologna was responsible for a 2011 multistate outbreak of *E. coli* O157:H7 that infected 14 persons (10). Previously, STEC serotype O103:H25 in mutton sausage was responsible for an outbreak in Norway in 2006, which included 10 cases of hemolytic uremic syndrome and one death among the 18 cases (39). A Danish outbreak in 2007, due to serotype O26:H11 present in a beef sausage, primarily caused illness in children (18 of 20 cases) with no cases of hemolytic uremic syndrome or death (16).

Little research has been published comparing the relative survival characteristics of different STEC serotypes. Strains O103 and O157 grew better than O111 under conditions of reduced a<sub>v</sub> or high undisassociated lactic acid concentrations in laboratory media, but the effect of competitive microflora or inactivation during heating was not evaluated (30). Currently, manufacturers have validated processes for a 5-log reduction of *E. coli* O157:H7, but it is unknown if these conditions are sufficient for control of non-O157 STEC strains. Considering the outbreaks involving fermented meat products and the paucity of data on the growth and survival of non-O157 STEC strains, the objective of this study was to validate the effects of pepperoni production on the fate of non-O157 STEC strains.

**MATERIALS AND METHODS**

The methodology used in this study was similar to that used in previous validation studies completed to determine methods to control *E. coli* O157:H7 in fermented dry sausage (22).

**Preparation of bacterial inocula.** Seven STEC strains belonging to serotypes O26, O45, O103, O111, O121, O145, and O157 were used in this study, one strain per each serotype. All strains were clinical isolates obtained from the Centers for Disease Control and Prevention (CDC; via the Wisconsin State Hygiene Laboratory, Madison) with the exception of the O157 isolate, which was part of the Food Research Institute (FRI) stock culture collection. Strain designations include *E. coli* O26:H11, strain H30; *E. coli* O111:H8, strain 00-3142; *E. coli* O103:H2, strain 01-3002; *E. coli* O121:H19, strain 01-3434 (CDC); *E. coli* O45:H2, strain 01-3510; *E. coli* O145:NM, strain 99-3311; *E. coli* O157:H7 FRiKi47 (ATCC 43895; clinical isolate 933 associated from 1983 ground beef outbreak) (37, 43).

Prior to inoculation into pepperoni batter, the cultures were transferred twice in tryptic soy broth (TSB; BD Diagnostic Systems, Franklin Lakes, NJ) supplemented with 1% glucose (37°C, 18 to 20 h; early stationary phase) to acid adapt the strains. Individual strains were washed in 0.1% buffered peptone water, and approximately equal concentrations of cells were pooled to produce a seven-strain mixture, resulting in 7 log CFU/g of pepperoni batter. Individual strains were quantified on Rainbow agar (RA; 37°C, 24 to 48 h; Biolog, Inc., Hayward, CA) before they were mixed, to ensure approximately equal numbers of cells for each strain. The seven-strain mixture was also quantified before and after inoculation in the pepperoni batter.

**Product procurement and pepperoni manufacture.** Pepperoni was manufactured using fresh pork trimmings and fresh lean beef trimmings obtained from a local supplier. The batter (batch weight, 27.2 kg) was formulated to approximately a 30% fat content. The pepperoni formulation consisted of 47.0% pork (80% lean) trimmings, 28.2% beef (80% lean) trimmings, 18.8% pork (50% lean) trimmings, 2.35% salt, 0.94% dextrose, 0.47% sucrose, 1.58% spices (ground black pepper, cayenne pepper, paprika, whole anise seed, and garlic powder; Excalibur Seasoning Co., Ltd., Pekin, IL), 0.38% distilled, deionized water, 0.235% cure (6.25% sodium nitrite and 93.75% salt; equivalent to 156 ppm on a meat block basis), 0.051% sodium erythorbate (equivalent to 547 ppm on a meat block basis), 0.0028% butylated hydroxytoluene, 0.0028% citric acid, and 0.035% lactic acid starter culture (*Pediococcus acidilactici*; Bactoferm HPS, Chr. Hansen, Milwaukee, WI). The pediococcal starter culture was thawed, mixed with the distilled, deionized water, and added to deliver approximately 7 log CFU/g of batter.

Pork and beef trimmings were coarse-ground (Hobart model 84142, Hobart Manufacturing Co., Troy, OH) using a 12.7-mm plate and then placed into a freezer (−20°C) for approximately 4 h, followed by tempering in a cooler (4°C) for up to 12 h until an internal meat temperature of −3.3°C was achieved. The tempered coarse-ground pork and beef was mixed with the cure for 1 min (Buffalo model 2VSS mixer, John E. Smith’s and Sons Co., Buffalo, NY), followed by the addition of the sweeteners, spices, antioxidants and cure accelerator, the salt, and finally the prepared pediococcal starter culture, with an additional 1-min mixing after each addition. For the uninoculated control, 9.07 kg of batter was removed from the mixer and ground through a 4.76-mm plate (Hobart model 84142; Hobart Manufacturing Co.). The remaining 18.16 kg of batter was inoculated with ca. 7 log CFU/g of the seven-strain STEC, mixed for 1.5 min, and then ground using a 4.76-mm plate as described for the control batter. Target stuffing temperature of the batter was −2.2°C. Fibrous pepperoni casings (diameter, 5.1 cm; Vista International Packaging, LLC, Kenosha, WI) were made pliable by soaking in room temperature (ca. 18°C) tap water for 30 min prior to use. Excess water was squeezed manually from the casings prior tostuffing with either the control (uninoculated) or inoculated pepperoni batters using a hand stuffer (Koch Supplies, Inc., Kansas City, MO). The resulting chubs (approximately 450 to 500 g per chub) were sealed using a casing clipper (model SCH 7210, Poly-Clip System GmbH & Co., Frankfurt, Germany) loaded with series 7000-VSCC staples (U.S. Clip Corporation, Mundelein, IL).

Pepperoni chubs were transported on ice to the Alkar-RapidPak Research and Technology Center (Lodi, WI; 30 mi (48 km) from Madison) for processing in a dedicated biosafety level 2 commercial thermal processing oven (Alkar, model 2000, Alkar Engineering Corp., Lodi, WI). The time from batter inoculation to placement of samples in the thermal processing oven was no more than 2.5 h. Thermal processing and drying were completed to replicate procedures outlined by Hinkens et al. (22) (Table 1). Fermentation was conducted at 38.9°C dry bulb and 37.8°C wet bulb until a pH of 4.8 was attained. After fermentation (8 h, 8 min for both trials), the chubs were heated to and held at an internal temperature of 53.3°C for 60 min. Pepperoni chubs were then transported and dried in an environmentally controlled chamber (Biotron facility, University of Wisconsin, Madison) at 13°C dry bulb with 88% relative humidity (RH) for 48 h, 82% RH for 120 h, and 76% RH until pepperoni achieved a moisture/protein (MPR) ratio of 1.61 (ca. 7 days) and through the duration of the 20-day holding interval. Drying was monitored to prevent case hardening (drying too fast) or mold growth (drying too slowly). Sausages were evaluated for drying rates by weight checks throughout drying.

At each sampling point, three chubs for each treatment were removed and assayed for microbial populations and proximate analysis as described below. All remaining chubs were removed after 20 days of drying. The production of inoculated pepperoni was replicated twice.

**Chemical analyses of pepperoni.** Triplicate control chubs (without added *E. coli*) were taken for chemical testing at the
Following process steps: batter, postcook, and at 7, 14, and 20 days. Samples were analyzed for moisture (5 h, 100°C, vacuum oven method; AOAC 950.46) (2), protein (Kjeldahl method for total nitrogen, protein factor 6.25; AOAC 991.20), fat (Decagon CX2 a_g meter, Pullman, WA), and fat (Mojonnier, acid hydrolysis method; AOAC 933.05). In addition, the pH was monitored during the fermentation and at each testing interval; a 10-g sample was macerated in 90 ml of deionized water, and the pH was measured on the homogenate using a pH meter (Accumet basic pH meter, Fisher Scientific, Waltham, MA) and electrode (Orion 8163 combination pH electrode, Thermo Scientific, Beverly, MA).

Microbiological analyses of pepperoni. Raw materials of excellent microbiological quality were used in order to keep the background numbers of microorganisms at a minimum. Indigenous microflora, including E. coli, were enumerated on the batter with starter, but prior to inoculation with STEC. Populations were determined by plating serial dilutions onto RA, sorbitol MacConkey agar (SMAC; 37°C, 24 to 48 h; Difco, BD, Sparks, MD), and plate count agar (30°C, 48 h, Difco, BD).

Triplicate inoculated pepperoni chubs were removed and tested for viable O157 and non-O157 STEC strains by direct plating on RA and SMAC at the following sample points: batter prior to stuffing, after fermentation, after reaching target cook temperature of 53.3°C (128°F), after 60-min cook, and following 7, 14, and 20 days of drying at 12.8°C (55°F). When numbers of the pathogen decreased to below the limit of detection by direct plating (<10^2 CFU/g), the presence or absence of the pathogen was determined by enrichment (modified EC [mEC] broth and TSB) and streaking a loopful of enrichment onto RA and SMAC as described below.

Chubs were sampled using a sterile knife to remove the casing and collect a 25-g cross-section from the middle of a chub. The sample was transferred to a sterile polypropylene sampling bag with wire closures (Whirl-Pak bag, Nasco, Fort Atkinson, WI), diluted 1:10 (wt/wt) with buffered peptone water, and the contents were macerated using a stomacher (model 400, Seward Laboratory Systems Inc., Bohemia, NY). The mixture was serially diluted and plated by spreading 0.1 ml of each dilution onto duplicate plates of both RA and SMAC agars. Additional portions (10 ml) of the mixture were transferred to 90 ml of mEC broth (containing 8 mg of novobiocin per liter, (35)) or TSB and incubated for 18 to 24 h at 37°C with shaking. Following overnight incubation for viable O157:H7 and non-O157 STEC cells, enrichments were streaked onto RA and SMAC plates. RA plates were incubated at 37°C for 24 to 48 h, and the numbers of pink, dark blue, or gray–light blue–light purple colonies enumerated. SMAC were also incubated overnight at 37°C and the numbers of sorbitol-negative (white) and -positive (pink-red) colonies enumerated.

On RA, colors of colonies generally represented serotypes, but correlation was not 100% accurate: pink-magenta colonies were typically O26, O103 or O121; gray–light blue–light purple were typically O45, O111, or O157; and dark blue were O145. On SMAC, sorbitol-negative (white) colonies were O157; whereas, all other serotypes and most nonpathogenic E. coli were sorbitol positive (pink-red). Colony color aided in picking representative colonies for confirmation of serotypes.

In addition, the effect of extended hold time without further drying on STEC populations was evaluated. At the end of 1 week of drying (8 days for trial 1, 7 days for trial 2; approximate MPR 1.53 and 1.46, respectively), triplicate chubs from each trial were removed and sampled (25 g) for surviving populations of STEC as described above. The unused portion of each chub was placed in a sterile polyethylene sampling bag with wire closures, sealed, and frozen at −20°C for 3 or 2 weeks for trials 1 and 2, respectively. Samples were thawed at room temperature (~24°C) for 1 h, and then stored at 12.8°C in the sealed bag for two additional weeks without additional drying. The samples were tested microbiologically by direct plating on RA at the time of thawing, and at 7 and 14 days of storage at 12.8°C.

Confirmation of serotypes. Following enumeration on the SMAC plates, five sorbitol-negative colonies were selected at random and tested for the O157 antigen by latex agglutination (Wellcolex E. coli O157 kit, Remel, Lenexa, KS). Sorbitol-positive (red) colonies were not serotyped but reported as surviving STEC strains. Serotypes for isolates from RA plates were confirmed by multiplex PCR. For each sample, approximately 15 colonies per sample comprised equal numbers of dark blue, pink-magenta, and gray–light blue–light purple colonies (if present) were picked and stabbed into vials with tryptic soy agar, and incubated overnight at 37°C. In addition, several atypical, white colonies were tested to determine if they were STEC. Isolates were held at 4°C as stocks for confirmation by multiplex PCR as described below. The numbers of non-O157 STEC and O157 cells recovered by direct plating were calculated from the total numbers of dark blue, pink, and gray–light blue colonies (RA plates) and sorbitol-negative colonies (SMAC plates) by multiplying by the fraction of colonies that were PCR positive for non-O157 serotypes (O26, O45, O111, O103, O121, and O145) or O157 agglutination positive, respectively.

Multiplex PCR for confirmation of STEC. A multiplex PCR method was used to confirm the inoculation strains, using a modified version of the procedure described by Monday et al. (33). A mixture of eight primer sets was composed of seven serotype-specific primer pairs and one pair of 16S primers for E. coli that was used as an internal control. The concentrations of the primer pairs in the multiplex PCR were described previously (33) with the addition of O45 wzx1(F) and (R) at 300 nM concentration (12). These serotype-specific primers targeted polymorphic regions in the wzx gene of the O-antigen gene cluster, resulting in a unique amplicon size for each serotype (12, 33). Isolates from the process sample points were inoculated into 5 ml of Luria-Bertani medium and incubated at 37°C with shaking (~200 rpm) overnight. These cultures were then heated in a boiling water bath for 30 min and stored at −20°C until they were tested by PCR. The standard
reaction set-up for the multiplex PCR was as follows: 17.5 μl of
2 × PCR master mix (HotStarTaq Master Mix kit, Qiagen,
Valencia, CA), 2.5 μl of 10 × primer mix, 2.5 μl of 50% DMSO,
2.0 μl of boiled culture lysate, and the final volume adjusted to
25 μl with sterile water. The cycling conditions were 95°C for
15 min, 10 cycles of 95°C for 30 s, 59°C to 50°C (~1°C per cycle)
for 20 s, 72°C for 52 s, followed by 35 cycles of 95°C for 30 s,
50°C for 20 s, 72°C for 52 s, and 72°C for 1 min for the final
extension. The resulting fragments from all PCR reactions were
separated in a 2% agarose gel and compared with a 100-bp ladder
(Invitrogen, Carlsbad, CA). Heat-treated control cultures of each
serotype were run with each batch of isolates. Heat-treated extracts
from O111, O45, and O121 control cultures (strains with larger
band sizes) were the most difficult to identify using multiplex PCR
and frequently yielded negative multiplex reactions. Accordingly,
isolates that resulted in only a 16S band (204 bp) but that lacked a
serotype-specific band were tested by singleplex PCR, using the
appropriate serotype primer pair and the following conditions:
12.5 μl of 2 × PCR master mix, 2 μl of 10 μM solutions of forward
and reverse primers, 2 μl of boiled culture lysate, and water to
25 μl. The PCR conditions were 95°C for 15 min, 30 cycles of
95°C for 30 s, annealing at 2°C below the lowest melting
temperature of the primer pair for 15 s, 72°C for 52 s, and finished
with 72°C for 7 min. PCR band sizes were as follows: O157
(133 bp), O26 (268 bp), O103 (320 bp), O145 (418 bp), O45
(527 bp), O121 (651 bp), and O111 (829 bp).

Statistical analysis. Changes in STEC populations (log CFU
per gram) were analyzed using two-way and one-way analysis of
variance to determine significant differences (P < 0.05) at each
sampling interval between trials (A versus B) and between isolation
methods (SMAC versus RA) using Minitab 14.2 statistical software
(Minitab Inc., State College, PA). Comparisons of means were
based on Fisher’s least significant difference to determine the
significant differences between sampling intervals and media type
for each trial with a level of significance (P value) set at 0.05.

RESULTS AND DISCUSSION

Proximate analysis. Pepperoni manufactured in this
study was representative of product found in commerce
today (Table 2). After fermentation and cook, sausage
contained 51.20% ± 1.06% moisture, pH 4.59 ± 0.05,
2.64% ± 0.13% NaCl, 28.65% ± 1.61% fat, 14.60% ±
0.96% protein, and aw 0.959 ± 0.002. Product reached
legal MPR <1.6:1 by day 7 for both trials. The aw of the
sausage at legal MPR was approximately 0.89 to 0.90,
and pH was 4.63 ± 0.05. Acid production in the pepperoni
manufactured in this study was greater than what was
previously reported. The pH decreased to 4.7 within 8.5 h in
this study, compared with pH 4.9 in 9.5 h in a study by
Hinkens et al. (22). These differences may be attributed to
using a different starter culture and including more
fermentable sugar than were used previous studies. This
study used 1% added dextrose, whereas Hinkens et al. (22)
used 0.63% dextrose. Although both studies used a P.
acidi lactici culture, the strains were from different sources.
This study used Chr. Hansen, Bactoferm HPS, with an
optimal fermentation temperature of 38.9°C (102°F),
whereas Hinkens et al. (22) used Quest International
Lactacel 115, with an optimal fermentation temperature
of 35.6°C (96°F).

The drying rate over time of the pepperoni in this study
was substantially faster than in previously published studies
(18, 22) but was within typical ranges of contemporary
practices, where 7 to 14 days are commonly seen (31). The
MPR reached the legal maximum <1:6:1 after 7 days of
drying (Table 2) compared with 18 to 20 days of drying in
previous studies. Rapid drying was attributed to optimal
airflow and relative humidity control in the drying chamber.
Drying rates over time for both trials were nearly the same,
suggesting uniformity in drying between trials (Fig. 1).

Microbiological results. Total microbial populations
in the uninoculated batter enumerated on plate count agar,
SMAC, and RA were ca. 7.5, 3.7, and 3.2 log CFU/g,
including atypical colonies on the selective agars. Most of
the colonies on SMAC were red (non-O157) and smaller
than typical E. coli colonies even after an additional 24-h
incubation; none of the white colonies were O157 (based on
latex agglutination confirmation). Atypical white colonies,
but no typical STEC colonies, were isolated from the
uninoculated batter on RA (minimum detection 1.7 log
CFU/g). Atypical colonies were infrequently observed in
inoculated samples during fermentation, cooking, and

<p>| TABLE 2. Proximate analysis of pepperoni during various stages of manufacture |</p>
<table>
<thead>
<tr>
<th>Sampling interval</th>
<th>% moisture</th>
<th>aw</th>
<th>pH</th>
<th>% NaCl</th>
<th>% fat</th>
<th>% protein</th>
</tr>
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<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batter</td>
<td>49.9 ± 1.53</td>
<td>0.963 ± 0.001</td>
<td>—</td>
<td>2.73 ± 0.18</td>
<td>25.4 ± 0.5</td>
<td>14.6 ± 0.9</td>
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<tr>
<td>Postcook</td>
<td>51.3 ± 0.79</td>
<td>0.960 ± 0.000</td>
<td>4.58 ± 0.04</td>
<td>2.51 ± 0.10</td>
<td>27.6 ± 2.3</td>
<td>15.7 ± 0.1</td>
</tr>
<tr>
<td>D-7</td>
<td>31.8 ± 1.60</td>
<td>0.897 ± 0.002</td>
<td>4.66 ± 0.04</td>
<td>3.42 ± 0.28</td>
<td>39.0 ± 2.3</td>
<td>20.8 ± 0.8</td>
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<tr>
<td>D-14</td>
<td>28.6 ± 0.42</td>
<td>0.865 ± 0.008</td>
<td>4.64 ± 0.05</td>
<td>3.89 ± 0.21</td>
<td>Not tested</td>
<td>23.0 ± 0.1</td>
</tr>
<tr>
<td>D-20</td>
<td>25.2 ± 0.35</td>
<td>0.838 ± 0.002</td>
<td>4.66 ± 0.02</td>
<td>4.36 ± 0.14</td>
<td>39.0 ± 0.05</td>
<td>25.1 ± 0.5</td>
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<tr>
<td><strong>Trial 2</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Batter</td>
<td>53.8 ± 1.8</td>
<td>0.960 ± 0.001</td>
<td>—</td>
<td>2.51 ± 0.04</td>
<td>29.3 ± 0.0</td>
<td>13.6 ± 0.0</td>
</tr>
<tr>
<td>Postcook</td>
<td>50.5 ± 1.5</td>
<td>0.960 ± 0.001</td>
<td>4.61 ± 0.06</td>
<td>2.77 ± 0.05</td>
<td>29.9 ± 0.6</td>
<td>14.6 ± 0.2</td>
</tr>
<tr>
<td>D-7</td>
<td>29.5 ± 0.9</td>
<td>0.887 ± 0.002</td>
<td>4.65 ± 0.04</td>
<td>3.40 ± 0.08</td>
<td>38.1 ± 1.3</td>
<td>20.2 ± 0.2</td>
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<tr>
<td>D-14</td>
<td>25.7 ± 0.5</td>
<td>0.848 ± 0.003</td>
<td>4.74 ± 0.02</td>
<td>Not tested</td>
<td>Not tested</td>
<td>22.3 ± 0.3</td>
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<tr>
<td>D-20</td>
<td>21.9 ± 0.3</td>
<td>0.829 ± 0.002</td>
<td>4.67 ± 0.02</td>
<td>4.37 ± 0.14</td>
<td>41.8 ± 0.8</td>
<td>22.6 ± 0.1</td>
</tr>
</tbody>
</table>

*a* — not tested. For comparison, the average pH value of the batter for two preliminary runs was 6.16 ± 0.02 (n = 4).
drying and were not any of the seven serotypes tested, based on PCR confirmation.

Technical information for RA acknowledges that microorganisms other than STEC can grow on this agar (Biolog, Inc.). Enterococcus faecalis will appear as white colonies and nontoxigenic E. coli can appear as pink, magenta, purple, or blue colonies. This background microflora may account for the several colonies isolated during the duration of the study that were not typeable by the multiplex PCR.

Batters were inoculated with 7 log CFU of STEC per g of batter with approximately equal populations of each serotype added (Table 3). Changes in the numbers of STEC bacteria in inoculated product during processing are summarized in Table 4, and a summary of log reduction of STEC is found in Table 5. Differences in inactivation rates were observed between the two trials, but they were not directly correlated to analytical values.

The rates of inactivation of O157 and other STEC bacteria observed in this study were similar to that previously reported when using the same isolation methodology (SMAC and mEC enrichment) (18–20, 22). Data revealed a 5-log reduction of sorbitol-negative E. coli (O157:H7) in fermented sausage cooked at 53.3°C for 1 h and dried for 7 days. Log reductions of sorbitol-positive STEC bacteria were >5.0 and 4.8 at day 7 of drying for trials 1 and 2, respectively, and >5 log reduction for all samples assayed at days 14 and 20. No STEC bacteria were isolated from any of the trial 1 samples on days 7, 14, or 20, using mEC enrichment to SMAC. For trial 2, both sorbitol-positive and -negative colonies were isolated from all three enriched samples for day 7 of drying; no STEC bacteria were isolated at day 14, but sorbitol-positive colonies were recovered from one of the three samples assayed at day 20.

When using different culture media, including RA and nonselective TSB enrichment, STEC bacteria were recovered at greater frequency from the inoculated products (P < 0.05). Total STEC populations were reduced by 3.8 to 4.2, 4.6 to 5.3, and >5 log CFU/g after 7, 14, and 20 days of drying, respectively (Table 4). When populations were reduced to levels undetectable by direct plating (representing at least a 5-log reduction), all samples were positive for STEC bacteria by TSB enrichments through the end of drying and were not any of the seven serotypes tested, based on PCR confirmation.

Technical information for RA acknowledges that microorganisms other than STEC can grow on this agar (Biolog, Inc.). Enterococcus faecalis will appear as white colonies and nontoxigenic E. coli can appear as pink, magenta, purple, or blue colonies. This background microflora may account for the several colonies isolated during the duration of the study that were not typeable by the multiplex PCR.

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The rates of inactivation of O157 and other STEC bacteria observed in this study were similar to that previously reported when using the same isolation methodology (SMAC and mEC enrichment) (18–20, 22). Data revealed a 5-log reduction of sorbitol-negative E. coli (O157:H7) in fermented sausage cooked at 53.3°C for 1 h and dried for 7 days. Log reductions of sorbitol-positive STEC bacteria were >5.0 and 4.8 at day 7 of drying for trials 1 and 2, respectively, and >5 log reduction for all samples assayed at days 14 and 20. No STEC bacteria were isolated from any of the trial 1 samples on days 7, 14, or 20, using mEC enrichment to SMAC. For trial 2, both sorbitol-positive and -negative colonies were isolated from all three enriched samples for day 7 of drying; no STEC bacteria were isolated at day 14, but sorbitol-positive colonies were recovered from one of the three samples assayed at day 20.

When using different culture media, including RA and nonselective TSB enrichment, STEC bacteria were recovered at greater frequency from the inoculated products (P < 0.05). Total STEC populations were reduced by 3.8 to 4.2, 4.6 to 5.3, and >5 log CFU/g after 7, 14, and 20 days of drying, respectively (Table 4). When populations were reduced to levels undetectable by direct plating (representing at least a 5-log reduction), all samples were positive for STEC bacteria by TSB enrichments through the end of drying and were not any of the seven serotypes tested, based on PCR confirmation.

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![FIGURE 1. Drying rates reported as % weight loss for pepperoni over time (days).](http://meridian.allenpress.com/doi/pdf/10.4315/0362-028X.JFP-11-486)

**TABLE 3. Calculated populations of each E. coli strain (log CFU per gram of batter) for each trial based on enumeration of washed cells on Rainbow agar prior to inoculation and total recovered from inoculated batter at time 0**

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Log CFU/g of batter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
</tr>
<tr>
<td>O26</td>
<td>6.40</td>
</tr>
<tr>
<td>O45</td>
<td>6.28</td>
</tr>
<tr>
<td>O103</td>
<td>6.56</td>
</tr>
<tr>
<td>O111</td>
<td>6.35</td>
</tr>
<tr>
<td>O121</td>
<td>6.51</td>
</tr>
<tr>
<td>O145</td>
<td>6.45</td>
</tr>
<tr>
<td>O157</td>
<td>6.21</td>
</tr>
<tr>
<td>Inoculum mix</td>
<td>7.18</td>
</tr>
<tr>
<td>Populations recovered from batter</td>
<td>6.99</td>
</tr>
</tbody>
</table>

An experiment was conducted to determine if additional holding time without drying would impart additional reduction of STEC. Chubs from trials 1 and 2 that had been pulled for testing at 1-week of drying were frozen at −20°C for 2 to 3 weeks. Retained chubs were thawed for 1 h at 24°C, sampled for STEC populations, then placed in moisture-impermeable pouches and stored at 12.8°C (55°F) for an additional 3 weeks. Data revealed that the freeze-thaw treatment resulted in 1- to 1.5-log reduction in STEC populations for all samples when measured using the more sensitive RA (Table 6). Four of the six samples tested were positive using TSB enrichment immediately after thawing, but only 1 of 6 and 0 of 6 were STEC positive after an additional 7 and 14 days of hold time at 12.8°C (without additional drying). The additional kill was attributed to prolonged exposure of damaged cells to the low pH–low moisture conditions.

**Confirmation of serotypes.** Colony color differences on RA for the various serotypes were helpful in selecting colonies for confirmation, but correlation between colony color and serotypes was not consistent throughout the study. Dark blue, purple, or black colonies tended to be O145, gray or light blue–purple tended to be O45, O111, or O157, and pink-magenta tended to be O121, O26, or O103.

PCR confirmed that serotypes O103 and O157 had the greatest survival rates, but all serotypes except O121 were occasionally recovered after the 1-h cook (Table 7). Serotypes O103, O145, and O157 survived most consistently during cooking; serotypes O103 and O157 survived longest during drying, but were only resuscitated by nonselective TSB enrichment at day 20.

Using multiplex PCR to confirm serotypes O45, O111, and O121 provided inconsistent results and typically required singleplex PCR for confirmation. Although these isolates were initially problematic to confirm, all colonies except one that were picked for PCR confirmation and were positive for 16S (E. coli) were confirmed for a specific serotype. Therefore, we are confident that the last surviving
### TABLE 4. Populations of Shiga toxin–producing E. coli and recovery by enrichment during manufacture of pepperoni

<table>
<thead>
<tr>
<th>Sampling interval</th>
<th>Direct plating (log CFU/g)</th>
<th>Presence by enrichment (no. positive/no. of chubs tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total SMAC</td>
<td>Sorbitol negative (O157)</td>
</tr>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batter</td>
<td>6.79</td>
<td>1.7 H</td>
</tr>
<tr>
<td>pH 4.8</td>
<td>5.93 b</td>
<td>1.7 H</td>
</tr>
<tr>
<td>0 h, 53.3°C</td>
<td>5.31 cD</td>
<td>1.7 H</td>
</tr>
<tr>
<td>1 h, 53.3°C</td>
<td>4.14 E</td>
<td>1.7 H</td>
</tr>
<tr>
<td>Drying, 7 days</td>
<td>&lt;1.7 H</td>
<td>&lt;1.7 H</td>
</tr>
<tr>
<td>Drying, 14 days</td>
<td>1.70 H</td>
<td>&lt;1.7 H</td>
</tr>
<tr>
<td>Drying, 20 days</td>
<td>&lt;1.7 H</td>
<td>&lt;1.7 H</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batter</td>
<td>6.87</td>
<td>1.7 H</td>
</tr>
<tr>
<td>pH 4.8</td>
<td>5.14 ± 0.72 b</td>
<td>1.7 H</td>
</tr>
<tr>
<td>0 h, 53.3°C</td>
<td>5.31 b</td>
<td>1.7 H</td>
</tr>
<tr>
<td>1 h, 53.3°C</td>
<td>3.93 ± 0.32 d</td>
<td>1.7 H</td>
</tr>
<tr>
<td>Drying, 7 days</td>
<td>2.00 E</td>
<td>&lt;1.7 H</td>
</tr>
<tr>
<td>Drying, 14 days</td>
<td>&lt;1.7 H</td>
<td>&lt;1.7 H</td>
</tr>
<tr>
<td>Drying, 20 days</td>
<td>&lt;1.7 H</td>
<td>&lt;1.7 H</td>
</tr>
</tbody>
</table>

- *n* = 3 at each sampling interval. Standard deviations < 0.3 log CFU/g, unless otherwise stated. SMAC, sorbitol MacConkey agar; mEC, modified EC enrichment broth; —, no enrichment completed at this sampling interval.
- Means within a trial with different letters are significantly different (*P* > 0.05).
- Survivors confirmed as O103, O145, O157.
- Survivors confirmed as O26 (single colony), O145, O157.

### TABLE 5. Summary of moisture/protein ratio and log reduction of Shiga toxin–producing E. coli during manufacture of pepperoni

<table>
<thead>
<tr>
<th>Change (log CFU/g) compared with inoculated batter (time 0)</th>
<th>Sorbitol MacConkey agar</th>
<th>Rainbow agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture/protein ratio</td>
<td>Total SMAC</td>
<td>Sorbitol negative (O157)</td>
</tr>
<tr>
<td><strong>Trial 1 batter</strong></td>
<td>3.41</td>
<td>-- b</td>
</tr>
<tr>
<td>pH 4.8</td>
<td>-- b</td>
<td>-- b</td>
</tr>
<tr>
<td>IT, 53.3°C</td>
<td>-- b</td>
<td>-- b</td>
</tr>
<tr>
<td>1 h, 53.3°C</td>
<td>3.28</td>
<td>-- b</td>
</tr>
<tr>
<td>Drying, 7 days</td>
<td>1.53</td>
<td>&gt; -5</td>
</tr>
<tr>
<td>Drying, 14 days</td>
<td>1.25</td>
<td>&gt; -5</td>
</tr>
<tr>
<td>Drying, 20 days</td>
<td>1.01</td>
<td>&gt; -5</td>
</tr>
<tr>
<td><strong>Trial 2 batter</strong></td>
<td>3.95</td>
<td>-- b</td>
</tr>
<tr>
<td>pH 4.8</td>
<td>-- b</td>
<td>-- b</td>
</tr>
<tr>
<td>IT, 53.3°C</td>
<td>-- b</td>
<td>-- b</td>
</tr>
<tr>
<td>1 h, 53.3°C</td>
<td>3.47</td>
<td>-- b</td>
</tr>
<tr>
<td>Drying, 7 days</td>
<td>1.46</td>
<td>&gt; -5</td>
</tr>
<tr>
<td>Drying, 14 days</td>
<td>1.15</td>
<td>&gt; -5</td>
</tr>
<tr>
<td>Drying, 20 days</td>
<td>0.97</td>
<td>&gt; -5</td>
</tr>
</tbody>
</table>

- Includes all other serotypes, plus indigenous *E. coli*.
- --, not tested at this sampling interval; IT, internal temperature.
TABLE 6. Populations of Shiga toxin–producing E. coli in pepperoni after frozen storage (−20°C) and nondrying storage at 12.8°C for 2 to 3 additional weeksa

<table>
<thead>
<tr>
<th>Direct plating (log CFU/g)</th>
<th>Presence by enrichment (no. positive/no. of chubs tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mEC to SMAC, sorbitol negative (O157)</td>
</tr>
<tr>
<td>Total Rainbow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.17</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td></td>
</tr>
<tr>
<td>Initial populations of 7-day drying samples</td>
<td></td>
</tr>
<tr>
<td>0 day at 12.8°C (thawed)b</td>
<td></td>
</tr>
<tr>
<td>7 days at 12.8°C</td>
<td></td>
</tr>
<tr>
<td>14 days at 12.8°C</td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td></td>
</tr>
<tr>
<td>Initial populations of 7-day drying samples</td>
<td></td>
</tr>
<tr>
<td>0 day at 12.8°C (thawed)c</td>
<td></td>
</tr>
<tr>
<td>7 days at 12.8°C</td>
<td></td>
</tr>
<tr>
<td>14 days at 12.8°C</td>
<td></td>
</tr>
</tbody>
</table>

a Standard deviations for direct plate counts were <0.3 log CFU/g. mEC, modified EC enrichment broth; SMAC, sorbitol MacConkey agar.
b Three weeks of storage at −20°C prior to thawing.
c Survivors confirmed as O103, O157.
d Survivors confirmed as O103.
e Two weeks of storage at −20°C prior to thawing.

serotypes were O103 and O157, with a single O26 isolate recovered from a day 20 enrichment from trial 2.

Among the 470 isolates run with PCR confirmation, only seven were untypeable. Collectively, six pink colonies isolated on RA from trail 1 batter, trial 2 batter, and trial 2 day 14 were negative for 16S and negative for all the seven serotypes. One gray isolate from trail 1 postcook was positive for 16S, but was not typeable as one of the target serotypes after several repeated attempts.

Several important differences between this and previous studies should be noted. First, we observed only a 3.2- to 4.2-log reduction of sorbitol-negative E. coli (confirmed as O157 by latex agglutination) after the 1-h cook at 53.3°C (128°F), compared with a 5-log kill of E. coli O157:H7 observed after thermal treatment in previous studies (18–20, 22).

The pepperoni in this study dried at a substantially faster rate than in a previous study. Sausages reached the legal MPR of 1.6:1 within 7 to 14 days, compared with 18 to 20 days for other published studies (18–20, 22). At the time when the pepperoni was sufficiently dried according to regulatory labeling standards, a 5-log reduction of all STEC bacteria was achieved, using older measuring methods (SMAC and mEC broth enrichments). When more sensitive recovery procedures were used (RA and TSB enrichments), the apparent log reduction of both O157 and O103 was less than 5 log until 14 to 20 days of drying. However, a follow-up experiment suggests that additional holding time under conditions of low pH and low aw may be sufficient to eliminate surviving pathogens and generate the required 5-log reduction of E. coli. Further work should be done to confirm the ability of low pH and low aw to inactivate the pathogen during additional hold time where drying conditions do not exist.

Our results are in agreement with recently published data showing that certain non-O157 STEC strains may react similarly to E. coli O157:H7 under food-relevant conditions, including temperature, pH, aw, and salt (14, 28, 32). For example, Enache et al. (15) compared the thermal resistance...
parameters (D-values) in apple juice of six non-O157 STEC serotypes with those of O157 (15). They concluded that the interventions commonly used for reducing E. coli O157:H7 in apple juice would be similarly effective against non-O157 STEC serotypes. The researchers found that E. coli O157:H7 was more heat resistant at 56 °C than were non-O157 serotypes, but the difference at higher temperatures diminished to the point of statistical insignificance (P > 0.05). In the current study, additional lethality was observed when pepperoni chubs were stored at 12.8 °C for 7 and 14 days (without additional drying). The additional bacterial destruction may be attributed to prolonged exposure of injured cells to the low pH–low moisture conditions and to higher than refrigeration temperature; it is well recognized that bacterial pathogens survive better in products stored at refrigeration temperature than at ambient temperature (20 to 30 °C) (14, 28).

It is interesting to note that Enache et al. (15) found that some strains of serotype O103 and O145 were among the most heat resistant, but both serotypes have been implicated in several outbreaks related to consumption of uncooked food products, including punch, lamb sausage, ice cream, and lettuce (3, 9, 13, 39); of all serotypes tested, E. coli O26 strains were the least heat resistant in apple juice. In our study, confirmation by PCR revealed that O103 and O157 had the greatest survival rates, but other serotypes (i.e., O145 and O26) were occasionally recovered during drying. However, O26 might not be among the most resistant strains used in this study because the recovered organism at day 20 was a single isolate from only one enrichment sample, and no recovery was observed after drying at days 7 and 14.

This study was designed to compare the survival rate of non-O157 STEC strains with that of E. coli O157:H7 during the manufacture of pepperoni, following processing conditions from previous validated studies. Data from our experiments confirmed previous studies that demonstrated a 5-log kill of E. coli O157:H7, as well as six other STEC serotypes, when the product reaches a MPF of ≥1.6:1 and isolates are recovered using SMAC and mEC broth enrichments. These data suggest that current treatments used to eliminate E. coli O157 in fermented dried sausages should be sufficient to kill other non-O157 STEC strains prior to the time at which the sausage will be released for sale.

ACKNOWLEDGMENTS

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