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Interventions for the Reduction of *Salmonella* Typhimurium DT 104 and Non-O157:H7 Enterohemorrhagic *Escherichia coli* on Beef Surfaces†

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ABSTRACT

A study was conducted to determine if slaughter interventions currently used by the meat industry are effective against *Salmonella* Typhimurium definitive type 104 (DT 104) and two non-O157:H7 enterohemorrhagic *Escherichia coli* (EHEC). Three separate experiments were conducted by inoculating prerigor beef surfaces with a bovine fecal slurry containing *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 (experiment 1), *E. coli* O157:H7 and *E. coli* O111:H8 (experiment 2), or *E. coli* O157:H7 and *E. coli* O26:H11 (experiment 3) and spray washing with water, hot water (72°C), 2% acetic acid, 2% lactic acid, or 10% trisodium phosphate (15 s, 125 ± 5 psi, 35 ± 2°C). Remaining bacterial populations were determined immediately after treatments (day 0), after 2 days of aerobic storage at 4°C, and after 7, 21, and 35 days of vacuum-packaged storage at 4°C. In addition to enumeration, confirmation of pathogen serotypes was performed for all treatments on all days. Of the interventions investigated, spray treatments with trisodium phosphate were the most effective, resulting in pathogen reductions of >3 $\log_{10}$ CFU/cm^2, followed by 2% lactic acid and 2% acetic acid (>2 $\log_{10}$ CFU/cm^2). Results also indicated that interventions used to reduce *Salmonella* Typhimurium on beef surfaces were equally effective against *Salmonella* Typhimurium DT 104 immediately after treatment and again after long-term, refrigerated, vacuum-packaged storage. Similarly, *E. coli* O111:H8 and *E. coli* O26:H11 associated with beef surfaces were reduced by the interventions to approximately the same extent as *E. coli* O157:H7 immediately after treatment and again after long-term, refrigerated, vacuum-packaged storage. It was also demonstrated that phenotypic characterization may not be sufficient to identify EHECs and that the organisms should be further confirmed with antibody- or genetic-based techniques. Based on these findings, interventions used by the meat industry to reduce *Salmonella* spp. and *E. coli* O157:H7 appear to be effective against DT 104 and other EHEC.

Recognized as a worldwide, emerging foodborne pathogen, *Salmonella* Typhimurium definitive type 104 (*Salmonella* Typhimurium DT 104), exhibits a multiantibiotic resistance pattern to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline *(15)* as well as increasing resistance to trimethoprim *(2, 9)* and ciprofloxacin *(29)*. In the United States, *Salmonella* Typhimurium DT 104 has been isolated from animal sources such as turkey, chicken, swine, cattle, elk, moose, coyote, squirrel, raccoon, chipmunk, dog, emu, cat, and several species of birds *(17)*. First identified in 1984 in the United Kingdom, the number of reported human cases of infection with *Salmonella* Typhimurium DT 104 increased from 259 in 1990 to 4,006 in 1996, and further evidence suggests that the rate of increase is continuing *(17)*. Outbreaks associated with this pathogen include foodborne transmission through unpasteurized dairy products, chicken, pork sausages, or direct contact with infected animals *(17)*. According to a published report by the U.S. Department of Agriculture Food Safety and Inspection Service, further research is needed to address the effectiveness of carcass washing, irradiation, steam pasteurization, etc. in reducing *Salmonella* Typhimurium DT 104 *(17)*.

While most outbreaks of enterohemorrhagic *Escherichia coli* (EHEC) infections have been caused by O157:H7 strains, numerous strains of non-O157 *E. coli* have emerged as important human pathogens. The most common non-O157:H7 serotypes associated with human disease are *E. coli* O26:H11, O103:H2, O111:NM, and O113:H21 *(21)*. These organisms have been associated with sporadic and large outbreaks of human disease worldwide *(21)*. Some non-O157:H7 serotypes are recognized as causes of hemolytic uremic syndrome and hemorrhagic colitis, including O26:H11, O111:H8, O113:H21, O145:NM, O5:NM, and O128:NM *(22, 24)*. In 1995, *E. coli* O111:NM was implicated in an outbreak associated with semidry, fermented sausages *(23)*. *E. coli* O104:H21 was incriminated in a Montana outbreak linked to milk *(1)*. *E. coli* O153:H45 has been associated with outbreaks in Spain and Chile as well as in the United States where meat products from Louisiana and Wisconsin were implicated as the source *(25)*. Results from another study showed that *E. coli* O26:

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TABLE 1. *Organisms, source, and antibiotic concentrations used in this study*

Organism	Strain number	Source	Medium	Antibiotic concentration
Salmonella Typhimurium	14028	ATCC[a]	Rambach	Nalidixic acid 250 μg/ml
Salmonella Typhimurium DT 104	NC 44061	MARC[b]	Rambach	Streptomycin 250 μg/ml
E. coli O157:H7	43895	ATCC	SMAC	Streptomycin 250 μg/ml
E. coli O111:H8	47C3	ECRC[c]	SMAC	Ampicillin 25 μg/ml
E. coli O26:H11	9D (C814-67)	DEC[d]	SMAC	Ampicillin 100 μg/ml

[a] American Type Culture Collection, Manassas, Va.

[b] Meat Animal Research Center and was kindly provided by Drs. James Keen and Robert Elder, Animal Health Research Unit, MARC, Clay Center, Nebr.

[c] *E. coli* Reference Center, Pennsylvania State University, University Park, Pa.

[d] Diarrheagenic *E. coli*, University of Maryland Center for Vaccine Development collection and was kindly provided by Dr. Thomas Whittam, Pennsylvania State University, University Park, Pa.

H11 strains from humans and animals with diarrhea were similar, supporting the probability that animals are the source of infections for humans *(14)*. Other sources implicated in outbreaks associated with non-O157 *E. coli* include ground beef and unpasteurized milk, as well as water, person-to-person transfer, or contact with cattle or cattle manure *(7, 14, 28)*.

The following study was conducted to determine if slaughter interventions (water, hot water, organic acid, trisodium phosphate) that are used by the meat industry to reduce *Salmonella* spp. and *E. coli* O157:H7 are effective against *Salmonella* Typhimurium DT 104, *E. coli* O111:H8, and *E. coli* O26:H11.

MATERIALS AND METHODS

Bacterial cultures. Sources and selection of antibiotic-resistant organisms used in this study are depicted in Table 1. *E. coli* O157:H7, *E. coli* O111:H8, *E. coli* O26:H11, *Salmonella* Typhimurium, and *Salmonella* Typhimurium DT 104 were obtained from the Roman L. Hruska U.S. Meat Animal Research Center culture collection and maintained in 75% glycerol at −20°C. An agar overlay diffusion assay was modified from Bauer et al. *(5)* and used to screen cultures for antibiotic resistance *(3)*. Once resistance was determined for a particular antibiotic, selection of antibiotic-resistant strains was performed on Rambach agar (Merck, Darmstadt, Germany) or sorbitol MacConkey agar (SMAC; Difco, Detroit, Mich.), using previously described methods *(13)*. *Salmonella* Typhimurium was selected on agar for nalidixic acid resistance at a concentration of 250 μg/ml (Sigma, St. Louis, Mo.); *Salmonella* Typhimurium DT 104 was selected for streptomycin resistance at a concentration of 250 μg/ml (Sigma). *E. coli* O157:H7, *E. coli* O111:H8, and *E. coli* O26:H11 were selected on SMAC agar for antibiotic resistance at a concentration of 250 μg/ml of streptomycin (Sigma), 25 μg/ml ampicillin (Sigma), and 100 μg/ml ampicillin (Sigma), respectively. Stock cultures of selected antibiotic-resistant isolates were maintained in glycerol at −20°C. The bacterial isolates were propagated for 18 h in trypticase soy broth (Difco) containing the appropriate antibiotic at 37°C prior to the experiment. On the day of the experiment, 18-h cultures containing approximately 9 $\log_{10}$ CFU/ml were diluted 1:100 in sterile physiological saline (pH 7.0) to obtain a viable cell population of approximately 7 $\log_{10}$ CFU/ml. Pathogen cocktails consisting of equal amounts of *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 (experiment 1), *E. coli* O157:H7 and *E. coli* O111:H8 (experiment 2), and *E. coli* O157:H7 and *E. coli* O26:H11 (experiment 3) were made and inoculated into fresh bovine feces for inoculation onto beef surfaces (see below).

Beef tissue, feces, and inoculation procedures. Beef carcass shortplates (experiment 1) or cutaneous trunci (experiments 2 and 3) were obtained within 15 min postexsanguination from beef carcass sides processed at a local slaughterhouse. Shortplates were removed from carcasses between the 5th and the 13th rib and about 25 cm from the vertebrae to within 10 cm of the midline *(12)*. The cutaneous trunci muscle covered the surfaces of the shortplates. Individual shortplates (experiment 1) or pieces of cutaneous trunci used in subsequent experiments (2 and 3) were placed in plastic bags, stored in insulated carriers to prevent rapid cooling, and transported to the Meat Animal Research Center and used within 2 h of slaughter. Areas to be sampled on shortplates were marked with edible ink using a sterile, cotton-tipped swab and a sterile stainless steel, 25-cm^2 template before inoculation with bovine feces. Cutaneous trunci was cut to 15-cm × 15-cm pieces for mounting on the stainless steel boards used in the carcass washer (experiments 2 and 3). Additional cutaneous trunci samples were evaluated for levels of microbial contamination at day 0; no pathogenic microorganisms were isolated from these samples. On each day of an experiment, feces were obtained from three cows or steers fed a corn/silage ration. One hundred grams of each fecal sample were mixed together with 100 ml of pathogen cocktail to obtain a pathogen concentration of approximately 6 $\log_{10}$ CFU/g. Fecal slurries without pathogens were also plated on selective media containing the appropriate antibiotics; no pathogenic microorganisms were isolated from these samples. Shortplates (experiment 1) or prerigor cutaneous trunci (experiments 2 and 3) were paintbrush inoculated with the fecal slurry containing the pathogens of interest and left undisturbed for 15 min. Pathogen populations between 5 and 6 $\log_{10}$ CFU/cm^2 were obtained using this methodology; aerobic plate counts of 6.53, 7.19, and 6.53 $\log_{10}$ CFU/cm^2, respectively, were observed for experiments 1, 2, and 3.

Spray-washing procedures. Spray washes with water (pH 7.2), 2% acetic acid (pH 2.9; Sigma), 2% lactic acid (pH 2.4; Sigma), or 10% trisodium phosphate (pH 10.35; Rhone Poulenc, Cranberry, N.J.) were conducted in the insertable pod in a laminar air flow hood *(12)*. The operation parameters for the washer were as follows: spray nozzle oscillation speed, 60 cycles/min; exposure to spray, 15 s; line pressure, 125 ± 5 psi; flow rate, 4.8 liters/min; temperature of spray at nozzle, 35 ± 2°C (model 40605 automatic 10-point temperature scanner, Davis Instruments, Inc., Baltimore, Md.). When using hot water as an intervention, an initial water wash was conducted as described above, followed by

hot water (temperature of spray at nozzle, 72 ± 2°C), 15 s; line pressure, 35 to 40 psi.

Following interventions, one 25-cm^2 sample was excised from the inoculated, treated surface, and bacterial populations were enumerated at day 0 (see below). The remaining tissue was stored aerobically at 4°C. After 2 days of refrigerated aerobic storage, remaining samples were aseptically excised to 25-cm^2 and vacuum packaged (Hollymatic model LV10G, Countryside, Ill.) in a standard vacuum-packaging bag (3.2 mil nylon/copolymer bag with oxygen transmission rate at 23°C of 52 cc/m^2; Hollymatic), and stored at 4°C until enumerated at days 7, 21, and 35.

Bacterial enumeration. On each day of sampling, the 25-cm^2 samples were aseptically removed from the packaging materials and pummeled for 2 min (Stomacher 400, Tekmar, Inc., Cincinnati, Ohio) in a Sterefil Stomacher bag (Spiral Biotech, Bethesda, Md.) with 25 ml of buffered peptone water (pH 7.0; BBL, Cockeysville, Md.) containing 0.1% Tween 20 (Fisher, St. Louis, Mo.). Each stomachate was serially diluted in buffered peptone water and either spiral plated (Autoplate 4000, Spiral Biotech, Bethesda, Md.) in duplicate or spread plated in quadruplicate on the appropriate agar. Remaining populations of antibiotic-resistant *Salmonella* Typhimurium or *Salmonella* Typhimurium DT 104 were plated on Rambach agar containing the appropriate antibiotic. For the detection of antibiotic-resistant *E. coli* O157:H7, *E. coli* O111:H8, or *E. coli* O26:H11, stomachates were spiral plated in duplicate onto SMAC agar containing the appropriate antibiotic. All plates were enumerated manually or with the CASBA IV image analyzer (Spiral Biotech) after incubation for 24 and 48 h at 37°C, respectively, for SMAC and Rambach agar. Only those colonies that exhibited the sorbitol nonfermenting phenotype on SMAC were enumerated to determine remaining bacterial populations. The lowest level of detection of aerobic plate count or pathogenic organisms was 1.30 $\log_{10}$ CFU/cm^2 or CFU/g using spiral-plating procedures; samples that were spread plated in quadruplicate were used to detect total number of CFU/cm^2 or CFU/g.

Confirmation of serotype. After bacterial enumeration on the specified day, three individual colonies from each Rambach plate, exhibiting the desired phenotype, were manually picked from agars. Confirmation of *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 isolates was made using a *Salmonella* spp. latex agglutination assay according to the manufacturer's instructions (Oxoid Reagents, Hampshire, England).

Strain confirmation of *E. coli* O157:H7, *E. coli* O111:H8, and *E. coli* O26:H11 was done using antibodies in an indirect enzyme-linked immunosorbent assay (ELISA) procedure described by He et al. *(16)* with modifications as described below. The monoclonal antibodies (MAbs) utilized for the detection of the corresponding antigens were as follows: 12F5-F6 and 15C4-D5 *(27)*, specific for the O26 and O111 polysaccharide chain, respectively; 13B3 and 2B7, specific for the O157-antigen *(30)* and H7 flagellar antigen *(16)*, respectively. Single colonies, having the characteristic O157 phenotype, were picked at random from SMAC plates and used to inoculate 3 ml of tryptic soy broth. The inoculated cultures were incubated for 18 h at 37°C; the cultures were heat treated in a boiling water bath for 15 min for use in the ELISA assay. Whole cell lysates *(16)* (50 μg/ml of total protein, 100 μl per well) of *E. coli* O26:H11 strain DEC 10A, *E. coli* O111:NM strain 95.0122, and *E. coli* O157:H7 ATCC 43895 (flagella enhanced) were used as positive controls; *E. coli* O78:H11 ATCC 35401 was used as a negative control. Ninety-six-well flexible polyvinyl chloride microtiter plates (Dynatech, Chantilly, Va.) were coated with whole-bacterium antigens (100 μl per well) as described by He et al. *(16)*, and the coated plates were either used immediately or stored for 1 week at −20°C.

The ELISA procedure was performed as follows: the antigen-coated plates were first washed five times with wash buffer (phosphate-buffered saline, 0.1% Tween 80, 0.5% sterile horse serum). One hundred microliters of diluted ascites fluid (1:7,000 and 1:64,000 for O26 and O111 MAbs, respectively) were added to wells. The plates were then incubated for 10 min at 37°C. After five washes with wash buffer, 100 μl of horseradish peroxidase-labeled antibody to mouse IgG and IgM (heavy plus light chains; Kirkegaard & Perry Laboratories [KPL], Gaithersburg, Md.) (diluted 1:1,500 with wash buffer) was added to each well. The plates were incubated for another 10 min at 37°C, washed five times with wash buffer, and 100 μl of a substrate solution (2,2'-azino-di[3-ethyl-benzthiazoline sulfonate] at a concentration of 0.3 g/liter in a glycine/citric acid/0.01% H_2O_2 buffer) (ABTS Peroxidase Substrate; KPL) was added to each well. After 15 min of incubation at room temperature with the substrate solution, the reaction was stopped with 1% sodium dodecyl sulfate and the optical density of the blue-green color reaction was measured with an automated ELISA reader (dual wavelength of 405/490 nm).

Calculations and statistical analyses of population data. After enumeration, bacterial populations from duplicate spiral plates or quadruplicate spread plates were averaged and converted to $\log_{10}$ CFU/cm^2 or CFU/g. Least-squared means of bacterial populations ($\log_{10}$ CFU/cm^2 or CFU/g) for each treatment were calculated from six replications for all meat experiments. Analysis of variance was performed using the General Linear Models procedure of SAS (SAS for Windows, release ver. 6.12, SAS Institute, Inc., Cary, N.C.). Statistical significance was defined as $P \leq 0.05$, unless otherwise noted. Log reductions were calculated as the difference between pathogen population ($\log_{10}$ CFU/cm^2) of control (untreated) surfaces and the remaining population ($\log_{10}$ CFU/cm^2) of treated surfaces.

RESULTS AND DISCUSSION

Experiment 1. Remaining populations of *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 following the various interventions are presented in Table 2. Initial inoculation levels of both organisms were equivalent. Of the treatments examined, 10% trisodium phosphate afforded the greatest $\log_{10}$ reductions (>3.80 $\log_{10}$ CFU/cm^2) of either *Salmonella* spp. observed in this study, regardless of day examined. Similar findings were observed when beef tissue surfaces inoculated with *Salmonella* Typhimurium ATCC 14028 were treated with 12% trisodium phosphate *(11)*. In the present study, *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 were reduced to statistically similar levels, regardless of treatment applied, for populations evaluated on days 0, 2, and 7. However, slight differences were observed between the two strains for water-treated tissues on days 21 and 35. As has been observed previously *(15)*, remaining populations of *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 were lower immediately following treatments with lactic acid, as compared to treatments with acetic acid. After 7 days of vacuum-packaged storage, both *Salmonella* Typhimurium and *Salmonella* Typhimurium DT 104 exhibited population increases (~1 $\log_{10}$ CFU/cm^2) for all treatments. This observation may be attributed to the adaptation of the organisms to the vacuum-packaged atmosphere over time. The

TABLE 2. *Remaining bacterial populations of* Salmonella *Typhimurium and* Salmonella *Typhimurium DT 104 following various interventions and refrigerated, vacuum-packaged storage*

		Treatment[c]					
Day[a]	Bacterial strain[b]	C	W	HW	AA	LA	TSP
0	ST	5.82 A	3.11 A	3.11 A	3.63 A	2.66 A	1.68 A
2[d]		5.18 A	3.77 A	3.10 A	2.40 A	1.22 A	1.11 A
7		5.69 A	4.57 A	4.37 A	3.31 A	2.12 A	1.57 A
21		5.97 A	5.13 A	4.93 A	2.79 A	2.02 A	0.81 A
35		5.77 A	4.95 A	4.00 A	2.77 A	1.47 A	1.36 A
0	ST-DT 104	5.91 A	3.11 A	3.15 A	3.28 A	2.69 A	1.82 A
2[d]		4.98 A	3.39 A	2.84 A	2.22 A	1.20 A	1.09 A
7		5.88 A	4.59 A	3.90 A	3.38 A	2.28 A	1.86 A
21		5.66 A	3.85 B	3.61 A	2.77 A	1.84 A	0.94 A
35		5.66 A	3.46 B	3.10 A	3.27 A	1.25 A	1.02 A

[a] Day of storage.
[b] Bacterial strains: ST, *Salmonella* Typhimurium; ST-DT 104, *Salmonella* Typhimurium DT 104.
[c] C, control; W, water, HW, hot water, 72°C; AA, 2% acetic acid; LA, 2% lactic acid; TSP, 10% trisodium phosphate. Letters in columns denote statistical difference between organisms within a treatment on a given day.
[d] After 2 days of aerobic storage at 4°C, all samples were vacuum packaged and stored at 4°C for the remainder of the experiment.

information obtained from this experiment indicates that treatments effective against *Salmonella* Typhimurium appear to be effective against *Salmonella* Typhimurium DT 104.

Experiment 2. Populations of *E. coli* O111:H8 and *E. coli* O157:H7 on beef surfaces were statistically equivalent prior to any treatments (Table 3). Interventions with water and hot water were initially more effective against *E. coli* O111:H8, with reductions of 1.85 and 1.84 $\log_{10}$ CFU/cm^2, respectively; whereas *E. coli* O157:H7 was reduced only 0.87 and 0.80 $\log_{10}$ CFU/cm^2 by water and hot water, respectively. However, remaining populations of either pathogen at days 2 or 7 were not statistically different from each other for any of the other treatments investigated. Differences between pathogen populations were observed on days 21 and 35 for water- and hot water-treated surfaces. In these cases, fewer remaining *E. coli* O157:H7 cells were observed, as compared to *E. coli* O111:H8. Regardless of pathogen tested or day of storage, treatments with trisodium phosphate resulted in the lowest remaining populations observed in this experiment. In a previous study, trisodium phosphate, lactic acid, and acetic acid treatments were also found to be effective for reducing *E. coli* O157:H7 on beef surfaces *(10, 11)*. However, immediate or sustained reductions of *E. coli* O157:H7 associated with 2% lactic or 2% acetic acid treatments were not as great (approximately 2 $\log_{10}$ CFU/cm^2) in this study, as compared to previous research (>3.80 $\log_{10}$ CFU/cm^2) *(10, 11, 15)*. These observed differences might be attributed to the individual strains used in the separate studies or the inexact and nonreproducible nature of the design or configuration of these types of decontamination experiments.

TABLE 3. *Remaining bacterial populations of* E. coli *O111:H8 and* E. coli *O157:H7 following various interventions and refrigerated, vacuum-packaged storage*

		Treatment[c]					
Day[a]	Bacterial strain[b]	C	W	HW	AA	LA	TSP
0	O111:H8	5.73 A	3.88 A	3.89 A	3.64 A	3.37 A	0.24 A
2[d]		5.74 A	3.63 A	3.89 A	3.32 A	2.85 A	1.33 A
7		5.83 A	3.83 A	4.26 A	3.97 A	2.82 A	0.92 A
21		6.20 A	4.76 A	5.92 A	3.47 A	3.05 A	1.70 A
35		6.37 A	4.04 A	5.01 A	3.46 A	2.75 A	1.33 A
0	O157:H7	5.83 A	4.96 B	5.03 B	4.06 A	3.83 A	0.86 A
2[d]		6.13 A	4.01 A	4.33 A	3.89 A	3.40 A	2.03 B
7		5.84 A	3.60 A	3.68 A	4.06 A	3.48 A	1.17 A
21		5.11 B	2.99 B	2.59 B	3.59 A	3.24 A	1.65 A
35		5.83 A	2.30 B	1.93 B	3.91 A	2.89 A	1.33 A

[a] Day of storage.
[b] Bacterial strains: *E. coli* strains O157:H7 and O111:H8.
[c] C, control; W, water; HW, hot water, 72°C; AA, 2% acetic acid; LA, 2% lactic acid; TSP, 10% trisodium phosphate. Letters in columns denote statistical difference between organisms within a treatment on a given day.
[d] After 2 days of aerobic storage at 4°C, all samples were vacuum packaged and stored at 4°C for the remainder of the experiment.

TABLE 4. *Remaining bacterial populations of* E. coli *O26:H11 and* E. coli *O157:H7 following various interventions and refrigerated, vacuum-packaged storage*

Day[a]	Bacterial strain[b]	Treatment[c]					
		C	W	HW	AA	LA	TSP
0	O26:H11	5.86 A	4.27 A	3.64 A	3.65 B	2.60 A	0.00 A
2[d]		5.38 A	3.60 A	3.76 A	3.38 A	1.64 A	1.28 A
7		5.32 A	3.57 A	4.15 A	2.88 B	2.30 A	0.00 A
21		5.15 A	2.68 A	2.66 A	1.73 B	0.10 B	0.00 A
35		4.77 A	2.15 A	3.53 A	2.45 A	0.99 A	0.00 A
0	O157:H7	5.88 A	4.34 A	3.99 A	4.18 A	2.88 A	0.00 A
2[d]		5.91 A	4.37 A	4.57 A	3.95 A	0.73 B	0.61 A
7		5.71 A	4.26 A	4.12 A	4.12 A	2.43 A	0.76 A
21		5.53 A	2.88 A	2.49 A	3.17 A	1.14 A	0.15 A
35		5.25 A	1.81 A	1.99 B	3.07 A	1.39 A	0.10 A

[a] Day of storage.
[b] Bacterial strains: *E. coli* strains O157:H7 and O26:H11.
[c] C, control; W, water; HW, hot water, 72°C; AA, 2% acetic acid; LA, 2% lactic acid; TSP, 10% trisodium phosphate. Letters in columns denote statistical difference between organisms within a treatment on a given day.
[d] After 2 days of aerobic storage at 4°C, all samples were vacuum packaged and stored at 4°C for the remainder of the experiment.

Experiment 3. Equivalent populations of *E. coli* O26:H11 and *E. coli* O157:H7 were observed prior to any interventions (Table 4). With the exception of 2% acetic acid, all remaining pathogen populations were statistically similar to each other following immediate application of water, hot water, 2% lactic acid, and 10% trisodium phosphate. After 2 days of refrigerated storage following treatments with 2% lactic acid, remaining *E. coli* O157:H7 populations were statistically different from populations of *E. coli* O26:H11. After 7 and 21 days of refrigerated, vacuum-packaged storage, *E. coli* O26:H11 levels were significantly different from *E. coli* O157:H7 on 2% acetic acid-treated surfaces. By day 35, pathogen populations were statistically similar for all treatments except hot water. As was observed in experiments 1 and 2, 10% trisodium phosphate was the most effective intervention investigated in this experiment. Populations of *E. coli* O157:H7 and *E. coli* O26:H11 subjected to 10% trisodium phosphate treatments were reduced immediately (>5 $\log_{10}$ CFU/cm^2) and remained virtually undetectable (<1.28 $\log_{10}$ CFU/cm^2) for the 35 days of refrigerated, vacuum-packaged storage. Treatments with 2% acetic acid and 2% lactic acid did reduce the number of these shiga toxin-producing *E. coli* (STEC); however, immediate reductions were not greater than 2 or 3 $\log_{10}$ CFU/cm^2, respectively. By day 35, it appears that 2% lactic acid treatments resulted in greater reductions against *E. coli* O26:H11 and *E. coli* O157:H7 than did 2% acetic acid. Similar studies have demonstrated the greater effectiveness of lactic acid versus acetic acid for reducing the levels of *E. coli* O157:H7 *(8, 15)*. Hence, the enumeration data suggest that *E. coli* O26:H11 was reduced to an equal extent as *E. coli* O157:H7 following interventions and long-term, refrigerated, vacuum-packaged storage.

Confirmation of serotypes. In addition to enumeration data, individual colonies exhibiting the typical pathogen phenotype from each of the treatments and days were picked from Rambach or SMAC plates containing appropriate antibiotics. A commercially available latex agglutination assay (*Salmonella* spp.) or MAbs (*E. coli*) were used to confirm serotypes.

From each intervention and day evaluated in experiment 1 of this study, one to three pink colonies were picked at random from each Rambach agar plate containing the appropriate antibiotic (a total of 12 plates per treatment) and assayed for agglutination with *Salmonella* spp. antigens. All pink colonies tested in this manner exhibited agglutination (data not presented). Blue colonies picked from Rambach agar were indicative of *E. coli* contamination (according to the manufacturer's information) and did not agglutinate in this assay. Based on these findings, organisms exhibiting the appropriate antibiotic resistance on Rambach agar were isolated following the various interventions and confirmed by an agglutination assay to be *Salmonella* spp. No further testing was performed on these colonies.

Confirmation of the *E. coli* serotypes in experiments 2 and 3 was determined for each of the treatments and time periods using MAbs specific for the O26-, O111-, and O157-polysaccharide antigens as well as flagella H7 antigen. Before each experimental procedure, pure cultures and fecal slurry containing the pathogens of interest were plated on the appropriate antibiotic-selective SMAC plates, and single colonies were then assayed with all the MAbs used in this study for serotype confirmation. Interestingly, the colonies of all three STEC strains appeared colorless on SMAC; i.e., they were unable to ferment sorbitol, which is a well-described phenotypic characteristic of the prototype O157:H7 EHEC strain *(19)*. However, one study found that 67% of the STEC strains isolated from hemolytic uremic syndrome patients in Chile, including O26, O111, and O55 serotypes were sorbitol nonfermenting, but no methylumbelliferyl-β-glucuronide reaction was performed on these strains *(22)*. The researchers also reported that the sorbitol nonfermenting phenotype was observed in 5% of the *E. coli* strains isolated from human normal flora. The findings of

these investigators support our observations on the phenotypic characteristics of *E. coli* O26 and O111 strains used in this study. Our findings and the data reported by Ojeda et al. *(22)* also suggest that phenotypic screening for sorbitol nonfermenting coliforms to detect *E. coli* O157:H7 in animals, food products, and environmental samples may be impractical because of the possibility that non-O157:H7 EHEC and other nonpathogenic *E. coli* strains may also be present. Therefore, the use of direct confirmation of colonies on agar plates with either nucleic acid probes or antibodies for detecting EHEC or other STEC organisms is warranted *(18, 22, 24, 27, 30)*. After each sampling period (experiments 2 and 3), a single colorless, sorbitol nonfermenting colony was selected at random from each corresponding antibiotic selective SMAC plate (n = 12, from a total of 12 plates per treatment). All the colonies tested for *E. coli* O157:H7 gave a positive ELISA reaction following all treatments and time periods assayed (data not shown). In contrast, not all of the *E. coli* O26:H11 and O111:H8 exhibiting the sorbitol nonfermenting phenotype gave a positive ELISA reaction (data not shown). Approximately 12% of the colonies with the presumptive sorbitol nonfermenting phenotype tested by ELISA were not the pathogenic strains of interest, despite the use of an antibiotic-selective media to reduce the growth of other competing organisms. This observation is not surprising because Ojeda at al. *(22)* demonstrated a small percentage of *E. coli* strains with sorbitol nonfermenting phenotype in normal flora from clinical sources. In summary, the data suggest that other *E. coli* contaminants are present that exhibit the sorbitol nonfermenting phenotype in spite of the care taken in enumerating and selecting the appropriate colonies. Considering these observations and the ELISA assays conducted for each of the strains examined, *E. coli* O111:H8 appeared to be more susceptible to the intervention treatments after 7 days, whereas strain O26:H11 showed susceptibility only after 21 days (data not shown).

In addition, sorbitol nonfermenting colonies that were selected from SMAC/ampicillin plates (selective for O26:H11 strains; experiment 3) and corresponding to day 35 acetic acid and lactic acid treatments gave a positive reaction on the ELISA assay with MAbs 13B3 and 2B7 that are specific for O157 and H7 antigen, respectively. *E. coli* O157:H7 ATCC 43895 used initially to inoculate the feces was ampicillin sensitive because no growth was observed on any of the SMAC plates containing either 25 or 100 μg/ml ampicillin. The colonies isolated from the SMAC plates selective for O26:H8 strain were small and clear, suggesting the presence of ampicillin-resistant *E. coli* O157:H7. Further testing of these isolates for streptomycin susceptibility demonstrated resistance (data not presented). Therefore, it appears that ampicillin-resistant *E. coli* O157:H7 strains were present initially, either on the meat or in feces and possibly grew to detectable levels over time. These observations suggest that naturally occurring ampicillin-resistant *E. coli* O157:H7 not only exhibit resistance to spray treatments with organic acids but also are capable of survival and growth on beef tissue under refrigerated, vacuum-packaged storage.

It is known that bacteria can develop antibiotic resistance using various mechanisms. Transformation of *E. coli* and other bacteria in different food matrices occurs under conditions commonly found in processing and storage of food *(4, 6)*. Bauer et al. *(4)* found that *E. coli* had a higher rate of transformation ($\sim 10^{-7}$) in milk, soy drink, tomato and orange juice at temperatures below 5°C. Based on the findings of Bauer et al. *(4)*, it is possible that transformation of *E. coli* could occur on meat surfaces. This observation may explain how *E. coli* O157:H7 acquired ampicillin resistance in this study. Other common mechanisms of resistance in gram-negative bacteria include production of β-lactamase *(26)* or emergence of antibiotic-resistant strains in livestock fed subtherapeutic levels of antibiotics *(20)*. It is possible that long-term use of antibiotics may allow for the selection of antibiotic-resistant pathogens via the mechanisms mentioned above or through currently unknown mechanisms.

CONCLUSIONS

Spray treatments with water, hot water, organic acids, and trisodium phosphate on beef surfaces appear to reduce effectively populations of the pathogens tested in this study. More importantly, interventions examined to reduce *Salmonella* Typhimurium on beef surfaces appear to be equally effective against *Salmonella* Typhimurium DT 104. Similarly, *E. coli* O111:H8 and *E. coli* O26:H11 associated with beef surfaces were reduced by the interventions to approximately the same extent as *E. coli* O157:H7. Based on these findings, interventions used by the meat industry to reduce *Salmonella* spp. and *E. coli* O157:H7 appear to be effective against *Salmonella* Typhimurium DT 104 and non-O157:H7 STEC. It should be noted that these results are based on experiments conducted with laboratory strains under controlled conditions and may not represent the results of other EHEC or STEC strains that may be encountered during the slaughter process. Therefore, intervention studies using feces containing naturally occurring EHEC are warranted. Furthermore, it was demonstrated that non-O157:H7 STEC exhibit similar phenotypes as *E. coli* O157:H7 on SMAC agar plates, and subsequent confirmation of STEC colonies with either MAbs or nucleic acid probes is justified to identify accurately the organism(s) of interest.

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REFERENCES

1. Anonymous. 1995. Outbreak of acute gastroenteritis attributable to *Escherichia coli* serotype O104:H21-Helena, Montana, 1994. Morbid. Mortal. Weekly Rep. 44:502–503.
2. Baggesen, D. L., and F. M. Aarestrup. 1998. Characterisation of recently emerged multiple antibiotic-resistant *Salmonella enterica* serovar typhimurium DT 104 and other multiresistant phage types from Danish pig herds. Vet. Rec. 143:95–97.
3. Barry, A. L., F. Garcia, and L. D. Thrupp. 1970. An improved single disk method for testing the antibiotic susceptibility for rapidly growing pathogens. Am. J. Clin. Pathol. 53:149–158.

4. Bauer, F., C. Hertel, and W. P. Hammes. 1999. Transformation of *Escherichia coli* in foodstuffs. System. Appl. Microbiol. 22:161–168.
5. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45:493–496.
6. Bräutigam, M., C. Hertel, and W. P. Hammes. 1997. Evidence for natural transformation of *Bacillus subtilis* in foodstuff. FEMS Microbiol. Lett. 155:93–98.
7. Cray, W. C., Jr., L. A. Thomas, R. A. Schneider, and H. W. Moon. 1996. Virulence attributes of *Escherichia coli* isolated from dairy heifer feces. Vet. Microbiol. 53:369–374.
8. Cutter, C. N., and G. R. Siragusa. 1994. Efficacy of organic acids against *Escherichia coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. J. Food Prot. 57:97–103.
9. Daly, M., J. Buckley, E. Power, C. O'Hare, M. Cormican, B. Cryan, P. G. Wall, and S. Fanning. 2000. Molecular characterization of Irish *Salmonella enterica* serotype Typhimurium: detection of class I integrons and assessment of genetic relationships by DNA amplification fingerprinting. Appl. Environ. Microbiol. 66:614–619.
10. Dorsa, W. J., C. N. Cutter, and G. R. Siragusa. 1997. Effects of acetic acid, lactic acid and trisodium phosphate on the microflora of refrigerated beef carcass surface tissue inoculated with *Escherichia coli* O157:H7, *Listeria innocua,* and *Clostridium sporogenes.* J. Food Prot. 60:619–624.
11. Dorsa, W. J., C. N. Cutter, and G. R. Siragusa. 1998. Bacterial profile of ground beef made from carcass tissue experimentally contaminated with pathogenic and spoilage bacteria before being washed with hot water, alkaline solution, or organic acid then stored at 4 or 12°C. J. Food Prot. 61:1109–1118.
12. Dorsa, W. J., C. N. Cutter, G. R. Siragusa, and M. Koohmaraie. 1996. Microbial decontamination of beef and sheep carcasses by steam, hot water spray washes, and a steam-vacuum sanitizer. J. Food Prot. 59:127–135.
13. Eisenstadt, E., B. C. Carton, and B. J. Brown. 1994. Gene mutation, p. 304–306. *In* P. Gerhardt (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
14. Griffin, P. M., and R. V. Tauxe. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli,* and associated hemolytic syndrome. Epidemiol. Rev. 13:60–98.
15. Hardin, M. D., G. R. Acuff, L. M. Lucia, J. S. Oman, and J. W. Savell. 1995. Comparison of methods for decontamination from beef carcass surfaces. J. Food Prot. 58:368–374.
16. He, Y., J. E. Keen, R. B. Westerman, E. T. Littledike, and J. Kwang. 1996. Monoclonal antibodies for detection of the H7 antigen of *Escherichia coli.* Appl. Environ. Microbiol. 62:3325–3332.
17. Hogue, A., J. Akkina, F. Angulo, R. Johnson, K. Peterson, P. Aini, and W. Schlosser. 1997. Situation assessment: *Salmonella* Typhimurium DT 104. U.S. Department of Agriculture, Food Safety and Inspection Service; http://www.fsis.usda.gov/ophs/stdt104.htm.
18. Ingram, D. T., C. M. Lamichhane, D. M. Rollins, L. E. Carr, E. T. Mallinson, and S. W. Joseph. 1998. Development of a colony lift immunoassay to facilitate rapid detection and quantification of *Escherichia coli* O157:H7 from agar plates and filter monitor membranes. Clin. Diagn. Lab. Immunol. 5:567–673.
19. March, S. B., and S. Ratnam. 1986. Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. J. Clin. Microbiol. 23:869–872.
20. Meng, J., S. Zhao, M. P. Doyle, and S. W. Joseph. 1998. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM from animals, food, and humans. J. Food Prot. 61:1511–1514.
21. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli.* Clin. Microbiol. Rev. 11:142–201.
22. Ojeda, A., V. Prado, J. Martínez, C. Arellano, A. Borczyk, W. Johnson, H. Lior, and M. M. Levine. 1995. Sorbitol-negative phenotype among enterohemorrhagic *Escherichia coli* strains of different serotypes and from different sources. J. Clin. Microbiol. 33:2199–2201.
23. Paton, A. W., R. M. Ratcliff, R. M. Doyle, J. Seymour-Murray, D. Davos, J. A. Lanser, and J. C. Paton. 1996. Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli.* J. Clin. Microbiol. 34:1622–1627.
24. Paton, J. C., and A. W. Paton. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11:450–479.
25. Reed, C. A. 1996. S. O. S. . . HELP prevent *E. coli* O157:H7. . . et al.! J. Am. Vet. Med. Assoc. 209:1213.
26. Reguera, J. A., F. Baquero, J. C. Pérez-Díaz, and J. L. Martínez. 1991. Factors determining resistance to β-lactam combined with β-lactamase inhibitors in *Escherichia coli.* J. Antimicrob. Chemother. 27:569–575.
27. Rivera-Betancourt, M., and J. E. Keen. 1999. Identification and detection of *Escherichia coli* O26 and O111 using monoclonal antibodies, p. 197, C-450. Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999. American Society for Microbiology, Washington, D.C.
28. Tarr, P. I., and M. A. Neill. 1996. Perspective: the problem of non-O157:H7 shiga-toxin (verotoxin)-producing *Escherichia coli.* J. Infect. Dis. 174:1136–1139.
29. Threlfall, E. J., F. J. Angulo, and P. G. Wall. 1998. Ciprofloxacin-resistant *Salmonella typhimurium* DT104. Vet. Rec. 142:255.
30. Westerman, R. B., Y. He, J. E. Keen, E. T. Littledike, and J. Kwang. 1997. Production and characterization of monoclonal antibodies specific for the lipopolysaccharide of *Escherichia coli* O157. J. Clin. Microbiol. 35:679–684.