Meat and Muscle Biology™

Characterization of *Salmonella* Strains and Environmental Microorganisms Isolated From a Meat Plant With *Salmonella* Recurrence

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Abstract: *Salmonella enterica* is a leading cause of foodborne illness in the United States. In this study, 3 *S. enterica* strains (serovars Cerro, Montevideo, and Typhimurium) were isolated from a beef processing plant with a history of *Salmonella* recurrence. Floor drains of the hotbox area and storage cooler of the same plant were sampled for environmental microorganisms. Biofilm formation on stainless steel (SS) by the microorganisms recovered from the drains and surface colonization by the isolated *S. enterica* strains were assessed. Pathogen survival and community compositions after sanitization (quaternary ammonium compound [QAC]) were examined. Alone the Cerro strain exhibited greater ($P < 0.05$) surface attachment (5.4 $\log_{10}$ CFU/SS chip) than the Montevideo or Typhimurium strains (4.6 and 4.8 $\log_{10}$ CFU/SS chip, respectively). The Typhimurium strain was the most QAC tolerant (0.3 log reduction), whereas the Cerro and Montevideo strains exhibited similar sensitivities (1.6 log and 1.1 log reduction, respectively). The environmental microorganisms from the drain samples all developed biofilms ranging from 5.7 to 6.0 $\log_{10}$ CFU/SS chip. All *S. enterica* strains were efficiently integrated into the drain biofilms where the Montevideo and Typhimurium strains ranged from 3.8 to 4.7 $\log_{10}$ CFU/chip and the Cerro strain 4.0 to 5.8 $\log_{10}$ CFU/chip. Enhanced QAC tolerance was only exhibited by the Montevideo strain when integrated into the drain biofilms. Metagenomic analysis of the drain biofilms showed that the most abundant genus was *Pseudomonas* (57.7%), followed by *Brochothrix* (28.6%), *Serratia* (7.2%), *Lactococcus* (4.2%), and *Carnobacterium* (1.1%), with all varying significantly among the different biofilm samples and their response to QAC treatment. Thus, various factors underlie the survival advantages of the *S. enterica* strains, allowing them to tolerate stress as well as outcompete and coexist with environmental companion bacteria for protection, which might lead to pathogen prevalence and recurrent product contamination.

Key words: *Salmonella enterica*, meat plants, biofilm formation, sanitization, environmental microorganisms
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Introduction

Salmonella enterica is the second leading cause of bacterial foodborne illnesses in the United States, resulting in approximately 1.03 million infections, 19,300 hospitalizations, and 400 deaths annually (Scallan et al., 2011). Most human salmonellosis cases (> 95%) are related to the consumption of contaminated foods, of which beef products are associated with approximately 9% of the cases (Scallan et al., 2015). Importantly, although more than 2,600 *S. enterica* serovars have been identified, only 20 major serovars are responsible for over 70% of human infections (CDC, 2019). Previous studies suggested that certain *S. enterica* serovars may be better at

adapting to their environment, including meat processing plants, with stronger survival ability and, subsequently, a higher chance of causing food contamination (Wang et al., 2017; Bosilevac et al., 2019).

It is well known that biofilm formation increases bacterial stress tolerance, and certain *S. enterica* strains were found better adapted to the meat processing environment via biofilm formation, allowing them to evade cleaning and sanitization procedures (Wang et al., 2017). Available results have also demonstrated the critical impact of environmental microorganisms on pathogen tolerance and survival via mixed biofilm formation as the synergistic and/or antagonistic interactions within the multispecies community can either promote or inhibit the growth and colonization of specific pathogens (Fox et al., 2014). A recent study (Chitlapilly Dass et al., 2020) reported that *Escherichia coli* O157:H7 cells in mixed biofilms formed by microorganisms in certain drain samples collected from a plant with high *E. coli* (EC) O157:H7 prevalence displayed significantly stronger sanitizer tolerance. The unique composition of the environmental microbiota and their interactions with the pathogen may play critical roles in EC O157:H7 tolerance and survival and could be partially responsible for its increased prevalence rate in meat products at the particular processing plant.

In a beef processing plant environment, the areas where biofilm formation may impact contamination of finished products are the hotbox and the storage cooler, where the sides of beef spend significant time before being processed. Split carcasses (sides of beef) exit the harvest floor and enter the "hotbox", a refrigerated room where rapid chilling is applied to "hot" carcasses. The air temperature in a hotbox is near 0°C, and periodic sprays of cold water are applied to the carcasses to chill them. Carcasses are held in the hot box for approximately 16 h and then transferred to storage coolers where they are graded and sorted before being fabricated. Coolers maintain an air temperature of ~5°C, and carcasses are held in a cooler for 8 to 48 h depending on the processing plant and product needs. Lastly, carcasses exit the coolers and enter the cutting room where the air temperature aims to be approximately 7°C or lower for processing into whole muscle cuts and trimmings and are packaged for distribution. All these areas of the processing plant environment have wastewater drains that collect the spray chill, cleaning, or other liquid runoff from the sanitization of processing equipment. Samples collected from floor drains, therefore, can serve as convenient representatives of the microorganisms in the local environment.

The present study analyzed the environmental microbial communities in floor drains at a beef processing plant with self-reported *Salmonella* recurrence, which is defined as the recurring detection of *Salmonella* in finished products above an acceptable sporadic level without identifiable aberrations in the food safety system while, in the meantime, all other monitoring biological measures (indicator counts, etc.) remain within acceptable limits. To investigate the potential mechanisms by which *S. enterica* strains may be surviving in the local environment, floor drain samples from various locations at the plant were collected. Microbial communities in the drain samples and the *S. enterica* strains that had been isolated from the same plant were characterized with regard to their contact surface colonization (biofilm formation) ability and tolerance against a common sanitizer. The species compositions of the mixed biofilm communities were further analyzed and related to the observed phenotypes of *Salmonella* recruitment into the community and their subsequent stress tolerance. This study is intended to help us understand how the intrinsic properties of the *S. enterica* strains and the composition of the environmental microbial community might benefit the pathogens for survival in the plant.

Materials and Methods

Floor drain sample collection and characterization

A beef processing plant (designated Plant C) that had communicated recurring *Salmonella* incidence was recruited for this study. Floor drain samples (n = 12) were collected at Plant C using cellulose sponges (Speci-sponge; Nasco, Atkinson, WI), each wetted with 10 mL of buffered peptone water. All drains were standard industrial drains located at low points of the floor for the collection and removal of run-off. Drains in the hotbox ($n = 4$), cooler ($n = 4$), and cutting room ($n = 4$) areas that were at least 25 m apart and did not share drainage lines were identified. Then, if a drain was accessible and sample collection did not interfere with processing activities, the drain's covering grate was removed and an area of ~500 cm^2 was vigorously swabbed with the sponge, turning it over halfway through the process. The underside of the grate and interior surfaces were sampled to collect attached microorganisms. Sponges were sealed in their whirlpak bag, then transported to the laboratory on wet ice in a cooler. To ensure an adequate sample was

obtained, each drain sample was thoroughly hand massaged, and then portions were removed and serially diluted to measure the levels of total mesophile count (TMC) and psychrophilic bacteria (PB), as well as Enterobacteriaceae (EB), coliforms (CF), and EC using Petrifilm (3M Microbiology, St Paul, MN). Total mesophile count and PB were measured using Petrifilm Aerobic Count plates, EB were measured using Petrifilm EB plates, and CF/EC were measured using Petrifilm EC plates. Incubation times and temperatures for the different bacterial groups were 24 h at 37°C for EB, CF, and EC; 48 h at 30°C for TMC; and 10 d at 7°C for PB (Supplemental Table).

Isolation, identification, and serotyping of *S. enterica* strains from floor drain samples

From each sponge sample, a 300 µL aliquot was removed and added to 2.7 mL of tryptic soy broth for isolation of *Salmonella* as previously described (Bosilevac et al., 2019). Briefly, each sample in tryptic soy broth was incubated in a 48 deep-well block at 42°C for 12 h, and then 1 mL was removed for immunomagnetic concentration of *Salmonella* using anti-*Salmonella* Dynabeads (Thermo Fisher, Carlsbad, CA) that were recovered into 3 mL Rappaport-Vassiliadis soya peptone broth (EMD Millipore Corp., Billerica, MA). The Rappaport-Vassiliadis soya peptone–immunomagnetic separation bead mixture was selectively enriched at 42°C for 48 h and then streaked for isolation on to Xylose Lysine Deoxycholate (XLD; Oxoid Ltd., Hampshire, England) agar plates. Xylose Lysine Deoxycholate plates were incubated overnight at 37°C and then viewed for suspect *Salmonella* colonies (those with black centers). Suspect colonies were confirmed by polymerase chain reaction (Wang et al., 1997) and then serotyped using slide agglutination (O typing) and tube agglutination (flagellar H-typing) methods, with commercial antisera (Difco, BD Diagnostic Systems, Sparks, MD), following manufacturer guidelines (Bosilevac et al., 2019).

Culture conditions for drain samples and the *S. enterica* strains

The *S. enterica* strains isolated from the plant were stored at −70°C in Lennox Broth (LB; Acumedia Manufacturers, Baltimore, MD) without salt (LB-NS) medium containing 15% glycerol. For each experiment, the *S. enterica* strain was streaked from the glycerol stock onto Tryptic Soy Agar (TSA; Difco, Beckton Dickinson, Sparks, MD) plates and grown overnight at 37°C, and then one single colony on the plate was inoculated into LB-NS medium and grown overnight at 37°C with orbital shaking at 200 rpm to reach bacterial stationary phase containing a cell concentration of approximately 5×10^8 cells/mL. The bacterial broth culture was then further diluted in fresh sterile LB-NS medium for each experiment.

Floor drain samples confirmed to be free of *S. enterica* were selected for the biofilm and sanitization study using stainless steel (SS) chips. To best maintain the original microbial composition of the floor drain samples and expand the sample volume for experimental use, each sample was diluted 1:50 in LB-NS medium and incubated at 7°C (to simulate a chilled processing plant environmental temperature and to be consistent with our previous studies) for 5 d with orbital shaking at 200 rpm, then aliquoted and stored at −20°C in LB-NS medium with the addition of sterile glycerol to 15%.

Sanitizer

The quaternary ammonium compound (QAC)-based commercial sanitizer Vanquish™ (Dawn Chemical Corp., Milwaukee, WI) was used in this study. This sanitizer, commonly used in the food and meat processing facilities, contains an alkylbenzyldimethyl–ammonium chloride mixture as its active ingredients. The sanitizer was applied to treat biofilms at 300 ppm for 1 min in the present study.

Biofilm formation and sanitizer treatment

Surface colonization by each individual *S. enterica* strain isolated from Plant C were assessed on SS surface as previously described (Chitlapilly Dass et al., 2020). Briefly, SS (18 × 18 × 2 mm; 2B brushed finish; average roughness of 0.1 to 0.5 µm) chips sterilized by autoclave were prepared as substrates, which were immersed and incubated with 100-fold diluted *S. enterica* overnight cultures (approximately 5×10^6 cells/mL) in LB-NS medium for 5 d at 7°C. Colonized *S. enterica* cell density and cell survival after sanitizer treatment was measured using a colony enumeration method on agar plates as further described later.

To test the biofilm forming ability and sanitizer tolerance of the floor drain samples—and also investigate the potential impact of the interspecies interactions (between the *S. enterica* strains and the environmental microorganisms in the drain samples) on sanitizer tolerance of the *Salmonella* cells in mixed biofilms—the glycerol stocks of the floor drain samples were thawed, diluted 1,000-fold, inoculated into sterile LB-NS medium, then incubated at 7°C for 5 d with orbital

shaking at 200 rpm. The 5-d cultures were aliquoted into 50 mL centrifuge tubes, 15 mL per tube. To develop mixed biofilms with colonized *S. enterica* strains, each of the overnight *S. enterica* cultures prepared as described earlier was added at a 1:100 ratio into each of the aliquoted 5-d drain cultures in the tubes. One sterile SS chip was then placed in each of the tubes to be immersed in the 5-d drain culture containing the added *S. enterica* strain to serve as the platform on which biofilms were developed statically for another 5 d at 7°C.

After the incubation, each chip was rinsed with 10 mL of sterile water, 5 mL each side, and then immersed in sterile water (control) or 300 ppm QAC solution for 1 min. To neutralize the sanitizer activity, each chip was then transferred to a new 50 mL centrifuge tube containing 10 mL Dey/Engley broth (BBL, Difco, Sparks, MD) supplemented with 0.3% soytone and 0.25% sodium chloride, and 1.0 g sterile glass beads (425 to 600 microns; Sigma-Aldrich, St. Louis, MO). Biofilm cells on chip surface were harvested by 1 min sonication in an ultrasonic waterbath (Bransonic Ultrasonics Corp, Danbury, CT) followed by 2 min vortexing at maximal speed, and then the liquid suspension was 10-fold serially diluted in sterile Dey/Engley broth and plated onto TSA and XLD agar plates. The plates were incubated overnight at 37°C for colony enumeration. The total bacteria count in the multispecies biofilms was measured by CFU counts on TSA plates. *Salmonella* cells colonized in the multispecies biofilms were distinguished from background microorganisms by colony morphology on XLD plates (black colonies), and the pathogen's populations in mixed biofilms were determined based on the CFU counts on the selective agar plates.

DNA extraction and 16S ribosomal RNA gene amplicon-based sequencing

To characterize bacterial diversity from the drain samples, biofilm cells from each drain sample before and after QAC treatment were harvested and DNA extraction/purification was performed for amplicon sequencing based on the variable region V4 of the 16S ribosomal RNA (rRNA) gene as previously described (Claesson et al., 2010; Gohl et al., 2016). Briefly, bacteria were harvested as described earlier and centrifuged at 13,000 × *g* at 4°C for 5 min. After the supernatants were removed, the cells were resuspended in phosphate-buffered saline and centrifuged at 13,000 × *g* for another 2 min. DNA was extracted and purified from the cell pellets using the Power soil microbial DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following the manufacturer's protocol, and DNA concentrations were quantified using the Qubit double stranded DNA assay kit with the fluorescence measured on a Qubit fluorometer (Life Technologies Corporation, Carlsbad, CA).

Primers used were 15F (5'-GTGCCAGCMGCC GCGGTAA-3') and 806R (5'-GGACTACHVGGG TWTCTAAT3'), flanking the 515 and 806 regions. Barcodes were attached to the 806R primers. Library preparation and 2 × 250 bp paired-end sequencing was carried out using the Illumina® MiSeq® platform at Novogene (Sacramento, CA). All forward and reverse sequences were subjected to FastQC (version 0.11.9) analyses separately for raw and trimmed sequences. Quality trimmed sequences were analyzed further using dada2 package of R. Analysis of 16S rRNA sequencing results was conducted using Quantitative Insights Into Microbial Ecology (QIIME2.0) (Caporaso, 2010). Paired-end sequences were demultiplexed using MiSeq Control software prior to importing into QIIME. FastQC was used to check per sample sequence quality. Based on the quality checks, forward reads were truncated to 240 bp, and reverse reads were truncated to 200 bp. The sequences were classified using Silva_138 release as the reference database with the pre-trained classifier based on 99% sequence identity. The downstream analyses were performed on the final sequence count and taxonomy table in RStudio using different packages of R. Percentage relative abundance of each genus in each sample was tabulated with Microsoft Excel.

Statistical analysis

Biofilm cell density was measured as $\log_{10}$ CFU/chip based on CFU counts on the agar plates and their corresponding dilution factors. GraphPad Prism software (GraphPad Software, La Jolla, CA) was applied for analysis of variance and comparisons of the logarithmic biofilm cell counts with standard deviations and 95% confidence intervals. Logarithmic biofilm cell counts were analyzed using a one-way analysis of variance with a post-Dunnett's multiple-comparisons test to compare cell densities of total bacteria or *S. enterica* cells among the mixed cultures recovered in the drain samples, as well as log reductions of total bacteria and *S. enterica* cells among the mixed biofilms after QAC treatment. Standard deviation (SD) of log reductions between pre- and post-sanitization samples of viable *S. enterica* cells or total bacteria was calculated using the formula SD = square root of $(SD_{pre}^2 / n_{pre} + SD_{post}^2 / n_{post})$.

Log reductions of *S. enterica* cells in mixed biofilms and in respective single-strain surface attachment were compared using an unpaired *t* test. *P* values less than 0.05 were considered statistically significant.

Results

Surface colonization and sanitizer tolerance of S. enterica strains

A total of 3 *S. enterica* strains were isolated from Plant C floor drains. One Cerro strain (strain C-Cerro) and one Montevideo strain (strain C-Montevideo) were isolated from different hotbox drains, whereas a Typhimurium strain (strain C-Typhimurium) was isolated from a processing floor drain. It should be noted that the hotbox drains found to contain *Salmonella* were removed from further experiments so that the inoculated and colonized *S. enterica cells* in the mixed biofilms can be accurately measured and compared.

Among the 3 strains, strains C-Montevideo and C-Typhimurium exhibited a similar level of SS surface colonization (4.6 and 4.8 $\log_{10}$ CFU/chip, respectively) at 7°C, whereas strain C-Cerro showed significantly stronger surface attachment (5.4 $\log_{10}$ CFU/chip) than the other 2 strains under the same experimental condition ($P < 0.05$, Table 1).

Sanitizer tolerance of the colonized *S. enterica* cells also varied significantly among the tested strains. C-Typhimurium appeared to be the most tolerant strain (0.3 log reduction) after 300 ppm QAC treatment. Statistical analysis showed no significant difference in log reductions between strains C-Cerro (1.6 log) and C-Montevideo (1.1 log, $P \geq 0.05$, Table 1).

Biofilm formation and sanitizer tolerance of floor drain microorganisms

The environmental microorganisms in the 6 floor drain samples without *S. enterica* presence all developed significant biofilm matrix on SS surface at 7°C (Table 2). One cooler sample (13C) and all 3 hotbox samples (15C, 17C, and 18C) developed biofilms containing bacterial densities above 6.0 $\log_{10}$ CFU/chip; cooler sample 14C developed the lowest biofilm matrix (5.7 $\log_{10}$ CFU/chip) (Table 2). However, statistical analysis showed no significant difference in biofilm formation among the 6 samples.

Sanitizer tolerance of the drain multispecies biofilms was sample dependent. Log reductions of total viable bacteria in the multispecies biofilms by each drain sample ranged from 0.8 to 2.0 $\log_{10}$ CFU/chip after QAC treatment. Higher log reductions were observed in biofilms formed by hotbox sample 17C (2.0 $\log_{10}$ CFU/chip) and cooler sample 13C (1.9 $\log_{10}$ CFU/chip). Conversely, the more tolerant mixed biofilms were formed by cooler sample 12C (0.8 log reduction) and hotbox sample 15C (0.9 log reduction) because statistical analysis showed that these two samples were significantly more tolerant than samples 13C and 17C (Table 2).

Colonization of S. enterica strains in drain multispecies biofilms

All 3 *S. enterica* strains isolated from Plant C were able to establish themselves efficiently in the multispecies biofilms formed by the Plant C environmental microorganisms. The amount of *S. enterica* cells of strain C-Montevideo and C-Typhimurium in the 6 multispecies biofilm communities ranged from 3.8 to 4.7 $\log_{10}$ CFU/chip, while the strongest single-strain colonizer C-Cerro overall established itself in mixed biofilms more efficiently (4.0 to 5.8 $\log_{10}$ CFU/chip). Notably, significantly higher amounts of *S. enterica*

Table 1. *S. enterica* strains isolated from Plant C

Strains	Drain location	*Salmonella* cells on SS	*Salmonella* log reduction
C-Cerro	Hotbox	5.4 (0.1)[a]	1.6 (0.3)[a]
C-Montevideo	Hotbox	4.6 (0.1)[b]	1.1 (0.1)[a]
C-Typhimurium	Processing floor	4.8 (0.1)[b]	0.3 (0.3)[b]

Data are shown as mean $\log_{10}$ CFU/chip (standard deviation) (biofilm on SS) or mean $\log_{10}$ CFU reduction (standard deviation) ($n = 3$). Means within the same column labeled with different superscript letters are statistically different ($P < 0.05$).

SS = stainless steel.

Table 2. Floor drain samples collected from Plant C, biofilm cell density on SS, and log reduction of total bacteria after 300 ppm QAC treatment

Drain samples	Drain location	Biofilm on SS	Log reduction
12C	Cooler-1	5.9 (0.1)	0.8 (0.3)[a]
13C	Cooler-2	6.3 (0.9)	1.9 (0.6)[b]
14C	Cooler-3	5.7 (0.4)	1.6 (0.2)[ab]
15C	Hotbox-1	6.2 (0.5)	0.9 (0.4)[a]
17C	Hotbox-2	6.4 (0.1)	2.0 (0.3)[bc]
18C	Hotbox-3	6.8 (0.2)	1.5 (0.2)[ab]

Data are shown as mean $\log_{10}$ CFU/chip (standard deviation) (Biofilm on SS) or mean $\log_{10}$ CFU reduction (standard deviation) ($n = 3$). Means within the same column labeled with different superscript letters are statistically different ($P < 0.05$). Means within the same column without superscript letter labels are not statistically different ($P \geq 0.05$).

QAC = quaternary ammonium compound; SS = stainless steel.

cells (5.8, 5.2, and 5.6 $\log_{10}$ CFU/chip) of strain C-Cerro were colonized in mixed biofilms with cooler samples 12C, 13C, and 14C compared with the other drain samples ($P < 0.05$), and the colonized C-Cerro *S. enterica* cells became a major component in these mixed biofilm communities. Meanwhile, among the 6 floor drain samples, relatively low amounts of *S. enterica* cells were colonized within mixed biofilms formed by hotbox sample 15C when each of the 3 *S. enterica* strains was individually inoculated into the drain samples (Table 3).

Sanitizer tolerance of Salmonella cells in drain multispecies biofilms

Salmonella log reductions in mixed biofilms after QAC treatment varied substantially, depending upon the *S. enterica* strains as well as the drain samples that harbored the *Salmonella* cells. Log reductions of the C-Montevideo strain in 3 mixed drain biofilms (14C, 15C, and 18C, Table 4) were lower than that in its single-strain attachment (Table 1), exhibiting the enhanced *Salmonella* tolerance in these multispecies communities. In particular, statistical analysis using unpaired *t* test showed that the tolerance of C-Montevideo in mixed biofilm with sample 14C (0.7 log reduction, Table 4) was significantly greater ($P < 0.05$) than its single-strain colonization (1.1 log reduction, Table 1). However, such enhanced tolerance of *S. enterica* cells in mixed biofilms compared with their single-strain attachment was not observed when the other 2 strains were inoculated into the 6 drain samples.

16S rRNA analysis of the mixed biofilm communities

For each floor drain sample, one pre-QAC and one post-QAC biofilm sample were sequenced. Therefore, a total of 12 drain-associated biofilm samples were analyzed to assess the bacterial communities before and after the QAC treatment (Figure 1).

The range of the input reads was 58,626 to 147,502, and the retained reads range was 39,660 to 111,732 (46.1% to 78.1%) for the 6 pre-QAC-treated biofilms by the floor drain samples. Sequences were de-replicated into unique amplicon sequence variants (traditionally referred to as operational taxonomic units), and a list of representative sequences were created with 52 features. The 2 most dominant phyla observed were Proteobacteria and Firmicutes, representing more than 96% of relative abundance in any pretreatment samples.

The most abundant genus was *Pseudomonas*, representing 57.7% of relative abundance considering all 6 pretreatment samples together, followed by *Brochothrix* (28.6%), *Serratia* (7.2%), *Lactococcus*

Table 3. Total bacteria and *S. enterica* cell density in multispecies biofilms after each *Salmonella* strain was inoculated into the floor drain samples to form mixed biofilms

Samples	C-Cerro added		C-Montevideo added		C-Typhimurium added	
	Total	***Salmonella***	**Total**	***Salmonella***	**Total**	***Salmonella***
12C	6.2 (0.2)	5.8 (0.3)^{a}	5.4 (0.8)	4.5 (0.1)	6.0 (0.3)^{ab}	4.1 (0.2)
13C	5.8 (0.2)	5.2 (0.4)^{ab}	6.0 (0.8)	4.4 (0.3)	6.8 (0.5)^{a}	4.1 (0.5)
14C	6.1 (0.2)	5.6 (0.3)^{a}	5.8 (0.8)	4.4 (0.1)	5.6 (0.3)^{b}	4.7 (0.2)
15C	5.8 (0.8)	4.0 (0.7)^{c}	6.1 (0.2)	3.8 (0.6)	6.1 (0.3)^{ab}	3.9 (0.7)
17C	6.0 (0.3)	4.4 (0.2)^{bc}	6.2 (0.1)	3.9 (0.5)	6.3 (0.3)^{ab}	4.0 (0.1)
18C	6.4 (0.2)	4.5 (0.3)^{bc}	6.8 (0.3)	4.2 (0.4)	6.8 (0.1)^{a}	3.9 (0.4)

Data are shown as mean $\log_{10}$ CFU/chip (standard deviation) ($n = 3$). Means within each column labeled with different superscript letters are statistically different ($P < 0.05$). Means within the same column without superscript letter labels are not statistically different ($P \geq 0.05$).

Table 4. Log reductions of *S. enterica* cells in multispecies biofilms after treatment with 300 ppm QAC

Strains	*Salmonella* log reduction in multispecies biofilms					
	12C	13C	14C	15C	17C	18C
C-Cerro	1.7 (0.3)^{a}	1.4 (0.3)^{a}	1.4 (0.2)^{a}	1.6 (0.6)^{a}	3.0 (0.7)^{b}	1.7 (0.4)^{a}
C-Montevideo	1.2 (0.1)	1.5 (0.3)	0.7 (0.1)	0.9 (0.6)	1.3 (0.3)	0.9 (0.3)
C-Typhimurium	0.7 (0.3)	1.2 (0.4)	0.7 (0.3)	1.0 (0.5)	1.0 (0.1)	0.7 (0.4)

Data are shown as mean $\log_{10}$ CFU reduction (standard deviation) ($n = 3$). Means within the same row labeled with different superscript letters are statistically different ($P < 0.05$). Means within the same row without superscript letter labels are not statistically different ($P \geq 0.05$).

QAC = quaternary ammonium compound.

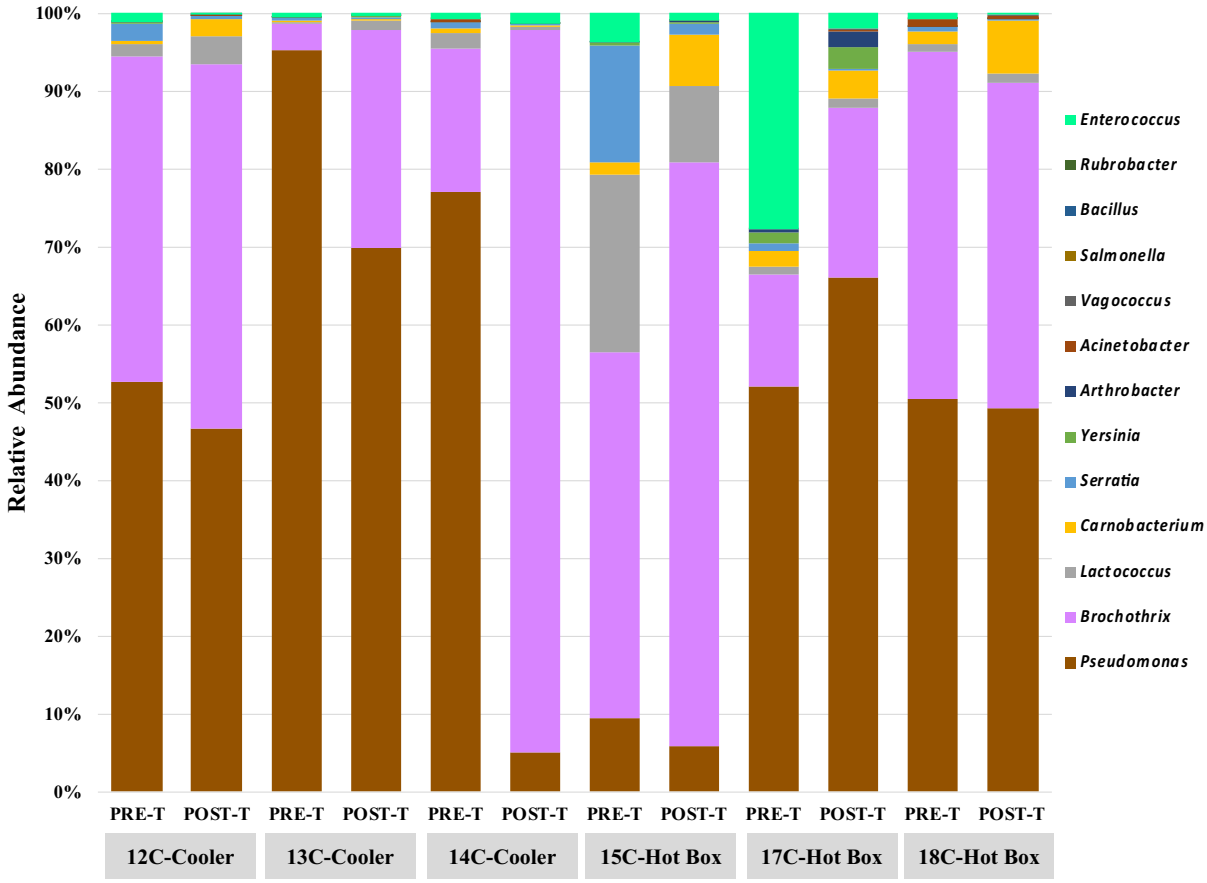

Figure 1. Impact of QAC treatment on species relative abundance changes of the mixed biofilms. For each drain biofilm sample, duplicates of pretreatment samples (PRE-T) or posttreatment samples (POST-T) were analyzed and averaged with 16S rRNA gene amplicon sequencing to demonstrate the changes in the relative abundance of microorganisms in mixed biofilms before and after QAC treatment.

(4.2%), and *Carnobacterium* (1.1%). The remaining 13 genera represented less than 1% of the total relative abundance. Overall, the pretreatment biofilms in the hotbox samples (15C and 17C) displayed higher relative abundance than the cooler samples.

Even though the 6 environmental biofilm communities before QAC treatment all contained the same 5 major species that represented over 98% of total relative abundance, the percentage of each species varied significantly in the different biofilm samples. Notably, samples 13C (95%) and 15C (9.5%) contained the highest and the lowest percentage of *Pseudomonas*, respectively. Samples 14C (0.8%) and 15C (15%) had the least and most percentage of *Serratia*, respectively, and sample 15C had the highest percentage (23%) of *Lactococcus* among all 6 samples.

Effect of quaternary ammonium compound treatment on species diversity of the mixed biofilm community

While the QAC treatment overall reduced approximately 1 to 2 log of total viable bacteria in the drain mixed biofilms (Table 2), the relative proportion of the various species in these multispecies communities were altered considerably after the sanitization (Figure 1). The relative abundance of the 5 major species was not significantly impacted by the QAC treatment in sample 18C, except for a minor increase of *Carnobacterium* and a minor decrease of *Brochothrix*. However, *Brochothrix* was increased in the other 5 post-treatment samples, and 14C was the sample that had the most increased proportion of *Brochothrix* (from 18% to 93%), followed by samples 13C (from 3% to 28%) and 15C (from 47% to 75%).

Conversely, the proportion of the most abundant *Pseudomonas* species in pretreatment samples was decreased after QAC treatment in all mixed biofilms except for sample 17C, which exhibited a slight increase of *Pseudomonas*. The largest decrease of relative abundance of *Pseudomonas* was observed in sample 14C (from 77% to 5%), followed by sample 13C, which had the highest pretreatment proportion of *Pseudomonas* (from 95% to 70%). Meanwhile, the *Lactococcus* genus was also decreased after the

treatment, most substantially in sample 15C, which had the highest pretreatment proportion of this genus (from 23% to 10%), whereas the low proportion of the *Lactococcus* genus in other pretreatment samples (< 2%) was slightly increased or unchanged after QAC treatment. The *Enterococcus* genus represented overall low relative abundance in the pretreatment samples except in sample 17C, within which *Enterococcus* constituted 28% of the relative abundance that was substantially reduced to 2% after QAC treatment.

Discussion

Currently, the contamination source of *S. enterica* and its transmission mechanisms in food processing facilities are not well understood. While the primary source of *Salmonella* contamination of beef products is traditionally attributed to animal hides or lymph nodes, available results have shown that pathogens under certain conditions may survive and present in facilities via biofilm formation (Bosilevac et al., 2007, 2009; Wang et al., 2017), which have long been associated with food safety and quality (due to microbiological spoilage) issues in food processing environments. However, it remains unclear how enteric pathogens efficiently establish biofilms in beef processing plants and gain survival advantages. Therefore, the present study selected one experimental model that simulated the situation most likely encountered in the industry settings where *S. enterica* may interact and compete with the preexisting environmental microorganisms. As a result, the pathogens may colonize and form mixed biofilms within the multispecies community for survival advantages.

The impact of biofilm formation by unique environmental microorganisms and their interspecies interactions on stress tolerance and prevalence of EC O157:H7 at beef processing plants was previously demonstrated (Chitlapilly Dass et al., 2020). To understand the potential relatedness between environmental microbial community and recurrent *Salmonella* prevalence at a commercial beef plant, floor drain samples were collected at a plant with self-reported *Salmonella* recurrence in beef products. From the environment (floor drains), a total of 3 *S. enterica* strains of serovars (Cerro, Montevideo and Typhimurium) were isolated, supporting the hypothesis that pathogen presence in the environment could be a contamination source at commercial establishments, as also suggested by the findings of previous studies (Marouani-Gadri et al., 2009; Yang et al., 2015; Wang et al., 2017). Interestingly, the USDA Food Safety Inspection Service baseline study (USDA, 2011) showed that Cerro and Montevideo were the two *S. enterica* serovars most often isolated from boneless beef trim samples. Indeed, serovars Montevideo and Typhimurium are among the top 20 most frequently isolated *S. enterica* serovars causing human infections as reported by the Centers for Disease Control and Prevention (CDC, 2016).

All 3 *Salmonella* strains were able to colonize contact surface (SS) under the common processing conditions. They were also able to establish themselves efficiently in the multispecies mixed biofilms. However, the addition of the *S. enterica* strains into the multispecies communities did not significantly alter the total biomatrix of the mixed biofilms. Notably, in most cases, the amount of *S. enterica* cells of each strain in the mixed biofilms was lower than that recovered from their individual single-strain attachment. This observation was consistent with previous findings for EC O157:H7 in mixed biofilms (Visvalingam et al., 2016; Chitlapilly Dass et al., 2020) and is likely due to the competition for nutrient resources and colonization space within the multispecies microbial communities.

The strongest single-strain colonizer among the 3 tested strains, C-Cerro was present in overall higher numbers than the other 2 strains in mixed biofilms. In particular, cell densities of C-Cerro in mixed biofilms with cooler samples 12C, 13C, and 14C were higher than or similar to its single-strain attachment. Notably, even though the drain samples exhibited no significant difference in biofilm-forming ability and the same cell density (5×10^6 cells/mL) of each *Salmonella* strain was inoculated into the mixed suspensions, after biofilm development for 5 d, C-Cerro became the more dominant species in these mixed biofilm communities compared with the other two *S. enterica* strains. These results indicate that the unique properties of the individual strain and its strong colonizing ability could enhance its capability to compete with environmental microorganisms in the mixture. Therefore, this strain was more capable of outcompeting the companion species during the colonization stage so it could maintain a high cell density in the mixed biofilms as observed previously in other *S. enterica* strains (Wang et al., 2013). Such outcompeting capability and the subsequent high retention of the *S. enterica* cells in the multispecies biofilms might potentially favor the survival of certain pathogen strains in the environment.

Sanitizer (QAC) tolerance measurement of surface colonized *Salmonella* cells of each individual strain showed that C-Cerro was not the most tolerant strain

even though it was the strongest colonizer among the three. This is consistent with previous findings that a greater surface attachment did not necessarily parallel with a higher sanitizer tolerance as observed in Shiga toxin-producing EC strains (Wang et al., 2012). Meanwhile, C-Typhimurium appeared to be the most tolerant (0.3 log reduction) among the 3 strains. *S. enterica* strains of the same serovars from a diverse strain collection examined in the previous study (Wang et al., 2017) mostly exhibited log reductions between 1.1 and 1.5 log after the 300 ppm QAC treatment. These strains were isolated from beef trim samples at commercial plants located in geographically distant regions and at various time points, thus representing the breadth of *S. enterica* strains/serovars that have been causing product contamination under the current commercial settings. Thus, the observation that C-Typhimurium was more tolerant than most of these strains suggests that the strain-specific higher stress tolerance might play a role in its survival in the environment of Plant C.

In the present study, the drain samples were enriched to expand volumes for experimental repetitions. Analysis by 16S rRNA gene amplicon-based sequencing showed that species diversity in post-enrichment samples were slightly decreased compared with the original samples, likely due to the growth competition and selection during the enrichment step, but the change was not significant (data not shown). Nevertheless, the species diversity and variation of the drain samples within the same processing plant may be a reflection of the various microhabitats found at the specific drain locations and the different selective pressure resulting from the organic load, sanitizing routines, and antimicrobial agents applied to beef carcass and equipment. For instance, the higher diversity of the hotbox drain samples may be due to the process in which the carcasses enter the hotbox to chill, and the spray chill water may carry away carcass surface bacteria and concentrate them in the floor drains. Furthermore, the presence and dynamic of personal (shoes, uniforms, etc.) and any improper sanitization may all act as additional factors to shape the community. Notably, the least amount of colonized *S. enterica* cells among the mixed biofilms was observed in hotbox sample 15C while it formed mixed biofilms with each of the 3 *S. enterica* strains. The cell density of strain C-Cerro in the mixed biofilm with 15C (4.0 log) was significantly lower than its single-strain attachment (5.4 log) even though this strain was the major component of the mixed biofilms with cooler drain samples 12C, 13C, and 14C. Species composition analysis indicated that among the 6 drain samples, hotbox sample 15C contained the highest percentage (20.48%) of *Lactococcus*, a genus belonging to the lactic acid bacteria (LAB) group. LAB have been reported to have strong antagonistic activity against many microorganisms, including *S. enterica*, *Listeria monocytogenes*, and EC O157:H7 due to their ability to co-aggregate with the pathogens and also produce antimicrobial compounds (Gómez et al., 2016; Camargo et al., 2018). A previous study (Chitlapilly Dass et al., 2020) suggested there was an association between the presence of the LAB members and low EC O157:H7 survival in a beef plant. The high LAB presence in sample 15C may have led to lower counts of the 3 *S. enterica* strains as a result of competitive exclusion, thus representing a potential natural alternative strategy for pathogenic biofilm prevention.

Since the six drain samples in the present study were collected from the same processing plant, it is not surprising that these pre-treatment samples had similar species compositions and all contained the 5 major species. In some cases, similar percentages of the major species within the cooler and hotbox samples were observed (e.g., 12C and 18C). However, it has been known that the various environmental bacterial species and their ability to compete and form mixed biofilms can affect the stress tolerance of the pathogens colonized within the community (Wang et al., 2013; Chitlapilly Dass et al., 2020). For instance, the unique compositions of the environmental microorganisms at different beef plants were previously found to either protect or inhibit EC O157:H7 (Chitlapilly Dass et al., 2020) and thus might further affect pathogen colonization and prevalence. Previous studies (Pang et al., 2017, 2020) further indicated that the presence of *Pseudomonas* in dual-species mixed biofilms could increase QAC tolerance of the companion *Salmonella* cells, thus contributing to the enhanced *Salmonella* survival in food processing plants. Interestingly, *Pseudomonas* was the most abundant genus, accounting for over 57% of relative abundance considering all 6 pretreatment samples collected from Plant C with *Salmonella* reoccurrence history. Therefore, the potential correlation between *Salmonella* prevalence and the high presence of the *Pseudomonas* family in the environment at Plant C requires further investigation.

Despite the same 5 major species and the similar family composition of the drain samples collected from Plant C, species percentage variations and different patterns of relative abundance change after sanitization were also observed, as well as enhanced tolerance of the colonized *S. enterica* cells even though not

statistically significant in some cases. In particular, strain C-Montevideo exhibited enhanced tolerance, compared with its single-strain attachment, in mixed biofilms with samples 14C, 15C, and 18C. Notably, log reduction of C-Montevideo in mixed biofilm with sample 14C was significantly lower than that in its single-strain attachment ($P < 0.05$). Cooler sample 14C contained a relatively high percentage of *Pseudomonas* and the lowest percentage of *Serratia*. Thus, the presence and the different combinations of these species as well as their interaction with the unique *S. enterica* strains/serovars that might lead to the altered stress tolerance warrant further investigation. In particular, pre- and post-treatment biofilms with and without the addition of the different *S. enterica* strains should be analyzed with 16S rRNA gene amplicon-based sequencing to more thoroughly understand the impact of interspecies interactions and relative abundance alteration due to sanitization on *S. enterica* survival.

Conclusions

Three *S. enterica* strains belonging to serovars frequently found in beef product contamination were isolated from the environment at a processing plant with a history of *Salmonella* recurrence. The present study revealed various factors underlying the survival advantages of these pathogenic strains, including strong contact surface colonization, outcompeting capability against background microorganisms, strain-specific sanitizer tolerance, and the interspecies interactions with environmental microbes resulting in enhanced stress tolerance via mixed biofilm formation. Since *S. enterica* is a diverse group of pathogens with over 2,600 serovars, it is not surprising that the various *Salmonella* strains and serovars would apply and adapt multiple strategies, either as individual strains with unique intrinsic properties or interacting with the environmental microorganisms, for higher survival capability. Such enhanced capability to tolerate environmental stress and outcompete and coexist with environmental companion bacteria for extra protection might consequently result in product contamination and pathogen recurrence.

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Literature Cited

Bosilevac, J. M., M. N. Guerini, D. M. Brichta-Harhay, T. M. Arthur, and M. Koohmaraie. 2007. Microbiological characterization of imported and domestic boneless beef trim used for ground beef. J Food Protect. 70:440–449. https://doi.org/10.4315/0362-028x-70.2.440.

Bosilevac, J. M., M. N. Guerini, N. Kalchayanand, and M. Koohmaraie. 2009. Prevalence and characterization of *Salmonellae* in commercial ground beef in the United States. Appl. Environ. Microb. 75:1892–1900. https://doi.org/10.1128/aem.02530-08.

Bosilevac, J. M., S. Zhilyaev, R. Wang, B. E. Luedtke, T. L. Wheeler, and M. Koohmaraie. 2019. Prevalence and characterization of *Salmonella* present during veal harvest. J Food Protect. 82:775–784. https://doi.org/10.4315/0362-028x.Jfp-18-478.

Camargo, A. C., S. D. Todorov, N. E. Chihib, D. Drider, and L. A. Nero. 2018. Lactic acid bacteria (LAB) and their bacteriocins as alternative biotechnological tools to control *Listeria monocytogenes* biofilms in food processing facilities. Mol. Biotechnol. 60:712–726. https://doi.org/10.1007/s12033-018-0108-1.

Caporaso, J. G. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.

Centers for Disease Control and Prevention (CDC). 2016. National Salmonella surveillance annual report, 2013. Atlanta, Georgia: US Department of Health and Human Services, CDC; 2016.

Centers of Disease Control and Prevention (CDC). 2019. Outbreak of *E. coli* infections linked to ground beef. https://www.cdc.gov/ecoli/2019/o103-04-19/index.html. (Accessed 19 June 2019).

Chitlapilly Dass, S., J. M. Bosilevac, M. Weinroth, C. G. Elowsky, Y. Zhou, A. Anandappa, and R. Wang. 2020. Impact of mixed biofilm formation with environmental microorganisms on *E. coli* O157:H7 survival against sanitization. Npj Science of Food 4(1), 16. https://doi.org/10.1038/s41538-020-00076-x.

Claesson, M. J., Q. Wang, O. O'Sullivan, R. Greene-Diniz, J. R. Cole, R. P. Ross, P. W. O'Toole. 2010. Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res. 38:e200. https://doi.org/10.1093/nar/gkq873.

Fox, E. M., K. Solomon, J. E. Moore, P. G. Wall, and S. Fanning. 2014. Phylogenetic profiles of in-house microflora in drains at a food production facility: comparison and biocontrol implications of *Listeria*-positive and -negative bacterial

populations. Appl. Environ. Microb. 80:3369–3374. https://doi.org/10.1128/AEM.00468-14.

Gohl, D. M., P. Vangay, J. Garbe, A. MacLean, A. Hauge, A. Becker, T. J. Gould, J. B. Clayton, T. J. Johnson, R. Hunter, D. Knights, and K. B. Beckman. 2016. Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. Nat. Biotechnol. 34:942–949. https://doi.org/10.1038/nbt.3601.

Gómez, N. C., J. M. P. Ramiro, B. X. V. Quecan, and B. D. G. de Melo Franco. 2016. Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes, Salmonella* Typhimurium, and *Escherichia coli* O157:H7 biofilms formation. Front. Microbiol. 7(863). https://doi.org/10.3389/fmicb.2016.00863.

Marouani-Gadri, N., G. Augier, and B. Carpentier. 2009. Characterization of bacterial strains isolated from a beef-processing plant following cleaning and disinfection—Influence of isolated strains on biofilm formation by Sakaï and EDL 933 *E. coli* O157:H7. Int. J. Food Microbiol. 133:62–67. https://doi.org/10.1016/j.ijfoodmicro.2009.04.028.

Pang, X., L. Chen, and H. G. Yuk. 2020. Stress response and survival of *Salmonella* Enteritidis in single and dual species biofilms with *Pseudomonas* fluorescens following repeated exposure to quaternary ammonium compounds. Int. J. Food Microbiol. 325:108643. https://doi.org/10.1016/j.ijfoodmicro.2020.108643.

Pang, X. Y., Y. S. Yang, and H. G. Yuk. 2017. Biofilm formation and disinfectant resistance of *Salmonella* sp. In mono- and dual-species with *Pseudomonas aeruginosa*. J. Appl. Microbiol. 123:651–660. https://doi.org/10.1111/jam.13521.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. A. Widdowson, S. L. Roy, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—Major pathogens. Emerg. Infect. Dis. 17:7–15. https://doi.org/10.3201/eid1701.P11101.

Scallan, E., R. M. Hoekstra, B. E. Mahon, T. F. Jones, and P. M. Griffin. 2015. An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years. Epidemiol. Infect. 143:2795–2804. https://doi.org/10.1017/S0950268814003185.

US Department of Agriculture (USDA). 2011. National prevalence estimate of pathogens in domestic beef manufacturing trimmings (Trim) [December 2005–January 2007]. USDA Food Safety and Inspection Service (FSIS), Office of Public Health Science, Microbiology Division, Washington, D.C.

Visvalingam, J., H. Wang, M. K. Youssef, J. Devos, C. O. Gill, and X. Yang. 2016. Spatial and temporal distribution of *Escherichia coli* on beef trimmings obtained from a beef packing plant. J. Food Protect. 79:1325–1331. https://doi.org/10.4315/0362-028X.JFP-15-598.

Wang, R., J. L. Bono, N. Kalchayanand, S. Shackelford, and D. M. Harhay. 2012. Biofilm formation by Shiga toxin-producing *Escherichia coli* O157:H7 and Non-O157 strains and their tolerance to sanitizers commonly used in the food processing environment. J. Food Protect. 75:1418–1428. https://doi.org/10.4315/0362-028X.JFP-11-427.

Wang, R. F., W. W. Cao, and C. E. Cerniglia. 1997. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J. Appl. Microbiol. 83:727–736. https://doi.org/10.1046/j.1365-2672.1997.00300.x.

Wang, R., N. Kalchayanand, J. W. Schmidt, and D. M. Harhay. 2013. Mixed biofilm formation by Shiga toxin-producing *Escherichia coli* and *Salmonella enterica* serovar Typhimurium enhanced bacterial resistance to sanitization due to extracellular polymeric substances. J. Food Protect. 76:1513–1522. https://doi.org/10.4315/0362-028X.JFP-13-077.

Wang, R., J. W. Schmidt, D. M. Harhay, J. M. Bosilevac, D. A. King, and T. M. Arthur. 2017. Biofilm formation, antimicrobial resistance, and sanitizer tolerance of *Salmonella enterica* strains isolated from beef trim. Foodborne Pathog. Dis. 14:687–695. https://doi.org/10.1089/fpd.2017.2319.

Yang, X., F. Tran, M. K. Youssef, and C. O. Gill. 2015. Determination of sources of *Escherichia coli* on beef by multiple-locus variable-number tandem repeat analysis. J. Food Protect. 78:1296–1302. https://doi.org/10.4315/0362-028X.JFP-15-014.

Supplemental Data

Levels of *Enterobacteriaceae* (EB), coliforms (CF), *E. coli* (EC), total mesophile count (TMC), and psychrophilic bacteria (PB) in recovered from drain samples collected at Plant C.

Sample	EB	CF	EC	TMC	PB
12C	7.0	< 1.4	< 1.4	7.6	8.6
13C	6.6	4.1	< 1.4	≥ 7.6	≥ 8.6
14C	2.7	< 1.4	< 1.4	7.1	6.7
15C	7.2	5.1	< 1.4	≥ 7.6	≥ 8.6
17C	7.1	4.6	< 1.4	≥ 7.6	≥ 8.6
18C	7.6	5.6	< 1.4	≥ 7.6	≥ 8.6

Values represent $\log_{10}$ CFU/cm^2.

Samples were diluted and plated onto appropriate Petrifilm (3M, St Paul, MN), incubated 24 h at 37 °C for EB, CF and EC; 48 h at 30 °C for TMC and 10 days at 7 °C for PB.

The level of detection was 1.4 $\log_{10}$ CFU/cm^2.

The maximum countable levels were 7.6 $\log_{10}$ CFU/cm^2 for EB, CF, EC and TMC; and 8.6 $\log_{10}$ CFU/cm^2 for PB.