Reduction of Multidrug-Resistant and Drug-Susceptible
Salmonella in Ground Beef and Freshly Harvested Beef Briskets
after Exposure to Commonly Used Industry
Antimicrobial Interventions

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ABSTRACT

Two separate studies were conducted to examine the differences in survivability of multidrug-resistant (MDR) and drug-susceptible Salmonella in fresh meats in a simulated industry environment. Beef trim from a commercial facility was inoculated with either MDR (AmpC phenotype) or drug-susceptible Salmonella (SUS) cocktails (10⁶ CFU/ml). Antimicrobial interventions included 3% lactic acid (LA), 1,000 ppm of acidified sodium chloride (ASC), ambient water (AW), and an inoculated control with no intervention (CTRL). Each aliquot was ground and formed into patties and packaged using high-O₂ modified atmosphere packaging. Samples for microbiological evaluation were collected on days 0, 7, 10, and 14. In the second study, beef briskets were collected immediately after harvest. Inoculation and antimicrobial application were the same, except treatments were heated and there was an additional hot water treatment. All beef briskets were refrigerated, and samples were collected at 0, 6, and 24 h. For the first study, the overall effectiveness of the treatments (from most effective to least effective) was LA, ASC, CTRL, and AW. Significant differences were observed only between MDR and SUS Salmonella when AW was applied (P = 0.02), and bacterial loads with AW were significantly greater (P < 0.01) for MDR Salmonella. In the second study, the intervention effectiveness ranked LA, ASC, hot water, AW, and CTRL. Significant differences between MDR and SUS Salmonella levels were not detected for any intervention or sampling time point. These data indicate that MDR and SUS variants of Salmonella behave similarly in response to the antagonistic action of antimicrobials commonly used in beef facilities.

Salmonella continues to pose a significant hazard to human safety on a global scale and is a difficult challenge for the food industry. The estimated costs associated with Salmonella infections are billions of dollars annually in the United States (39). Of the four pathogens with current Healthy People 2010 targets (3), Salmonella is furthest from its target goal (13). The situation persists because of the widespread occurrence of the organism in natural reservoirs and intensive husbandry and offal recycling practices implemented in multiple sectors of the food chain (14, 16, 17).

To further complicate the issue, treatment of salmonellosis has become problematic because of the emergence of multidrug-resistant (MDR) Salmonella strains. In the last two decades, the number of antibiotic-resistant Salmonella strains isolated from humans and food animals has significantly increased. MDR organisms can complicate treatment, prolong symptoms, increase an individual’s risk for more severe illnesses and mortality, and impose additional health care costs (40). Another potential burden to the well-being of humans is the possible relationship between increased virulence and drug-resistant strains (43). Patients with MDR Salmonella infections are more likely to experience symptoms for a longer period (29), undergo hospitalization (29, 33), and face a higher fatality risk (25, 26) than those infected with drug-susceptible (SUS) Salmonella strains.

Many scientists have suggested that the misuse and overuse of antibiotics in veterinary medicine is responsible for the global occurrence of antibiotic-resistant microorganisms in the food supply (1, 16, 22, 30, 38, 40, 42, 45). The origins of MDR Salmonella and the reasons driving accumulation of multiple resistance determinants are not certain, but antibiotic use in human and animal medicine may be one factor. Because of the lack of comparable and quantitative data, a link between antibiotic use in food animals and increased risk to human health has not been found (8, 31).

Because most salmonellosis cases in developed countries have been attributed to food products, antibiotic resistance has become an increasingly important albeit
controversial issue in the food industry, especially the meat and poultry sectors. For example, MDR *Salmonella* strains have been identified as the causal agent of multiple outbreaks associated with the consumption of undercooked and raw ground beef in the United States (12, 15, 20). These outbreaks occurred despite the fact that all ground beef suppliers involved in the outbreaks reportedly operated under U.S. Department of Agriculture Food Safety and Inspection Service production guidelines and hazard analysis and critical control point (HACCP) plans.

In an attempt to reduce bacterial contamination of meat products, numerous interventions have been developed and implemented. Despite the widespread adoption of common antimicrobial interventions, it is unclear whether the interventions used by beef processing industries are equally effective for controlling MDR *Salmonella* and SUS *Salmonella* variants. The purpose of this study was to compare the survival of naturally occurring MDR and SUS *Salmonella* strains in raw ground beef patties (objective I) and freshly harvested beef briskets (objective II) exposed to common antimicrobial interventions in a simulated industry setting.

### MATERIALS AND METHODS

#### Bacterial strains.

The initial group of *Salmonella* strains consisted of 52 *Salmonella enterica* isolates of various serovars (Reading, Agona, Muenster, Montevideo, Paratyphi B, Anatum, and Cerro) isolated from naturally infected cattle (27). Of this group, 23 strains were susceptible to antibiotics and 29 were coresistant to ampicillin, chloramphenicol, streptomycin, sulfoximides, tetracyclines, a potentiated beta-lactam, and third generation cephalosporins (MDR-AmpC). To choose isolates for the MDR and SUS *Salmonella* cocktails, several screening steps were taken. First, a disk diffusion susceptibility test was conducted on standard methods agar (Remel, Lenexa, KS) and xylene lysine tergitol 4 agar (Difco, Becton Dickinson, Sparks, MD). The lactate resistance of the MDR isolates was compared with that of the susceptible isolates at levels of $1 \times 10^6$ CFU/ml using discs soaked in a 3% lactate acid (LA) solution (PURAC lactic acid 88%, Birko Corp., Henderson, CO). The resulting zones of inhibition were used to determine which five isolates would be included in the *Salmonella* cocktails. These isolates were chosen to mitigate sample bias by hierarchically clustering the isolates and plotting them as dendrograms. These dendrograms provided data on the relationships between the different *Salmonella* isolates, which gave insight into the general behavior of the *Salmonella* strains. Both cocktails were prepared by combining the five chosen strains (in their appropriate volumes to reach the target concentration) using strict aseptic techniques.

One week before the start of each replication of the experiment, both the MDR and SUS *Salmonella* cocktails were tested in duplicate to confirm MDR and SUS status, respectively. Antimicrobial disc diffusion susceptibility testing was conducted following the Kirby-Bauer methodology (7, 9) on Mueller-Hinton agar (Difco, Becton Dickinson), as standardized by the Clinical and Laboratory Standards Institute (formerly the NCCLS) (34, 37). The MDR cocktail remained highly resistant, and the other cocktail displayed susceptibility to all tested antibiotics throughout the duration of the experiment.

#### Product preparation.

All processing for this project was conducted in the Pathogen Processing Laboratory at Texas Tech University (Lubbock). For objective I, 200 lb (90.8 kg) of 81% lean–19% fat beef trim from a commercial beef processing facility was utilized. The background microbial flora was tested in triplicate by in random samples of trim. One hundred pounds (45.4 kg) of beef trim pieces was carefully inoculated with the MDR *Salmonella* cocktail and the other 100 lb received the SUS *Salmonella* cocktail at a level of $1 \times 10^6$ CFU/ml by submersion into a sanitized stainless steel container with the specific pathogen in 900 ml of buffered peptone water (BPW). Each 100 lb of inoculated trim was then removed from the dip, divided into smaller 20-lb (9.1-kg) aliquots, placed on sterile stainless steel mesh racks in large plastic tubs, and held at refrigeration temperature (4 to 8°C) for approximately 1 h to facilitate bacterial attachment to the product. Pathogen distribution in the inoculation solution and on the final product was determined by sampling and testing the liquid inocula and then setting aside an untreated positive control to test for distribution across the surface of the product.

For objective II, the beef briskets were obtained from the Gordon W. Davis Meat Science Laboratory (Texas Tech University). Two cattle were harvested, and the brisket primal (deep pectoral muscle) were collected from each side, for a total of four briskets. No standard washes or antimicrobial interventions were applied to the briskets before further processing. The product was transferred to the Pathogen Processing Laboratory in sealed foam containers to minimize loss of moisture. The large briskets were fabricated for uniformity into subprimals with minimal trimming of the outside fat from the surface of the product. Inoculation methodologies and levels were the same as those used for objective I.

#### Antimicrobial interventions.

Each group of samples was randomly assigned an antimicrobial intervention, which was applied by means of a stainless steel spray cabinet (series 800, Intralox, Inc., Harahan, LA). This system provides a spray rate of 0.66 gal (2.5 liters) per min at 20 psi, and the product travels through spray stations (six total spray nozzles) on a wire belt conveyor. Product is loaded onto the conveyor, travels through the first spray station, drops and becomes submerged in the intervention solution for 2 to 3 s, travels up and out of the solution through the second spray station, and then exits the cabinet. The four interventions utilized for objective I were (i) 3% LA, (ii) 1,000 ppm of acidified sodium chlorite (ASC; Dan Mar Co., Arlington, TX), (iii) sterile ambient water (AW), and (iv) no intervention (CTRL). For objective II, the five interventions used were heated versions of the same treatments: (i) 3% LA at 51 to 53°C (125 to 127°F), (ii) 1,000 ppm of ASC at 28 to 29°C (84 to 85°F), (iii) sterile AW, (iv) sterile hot water (HW) at 71 to 74°C (160 to 165°F), and (v) CTRL. The temperature was continuously monitored during the heating process and never surpassed the maximum threshold temperature for each antimicrobial intervention.

#### Further processing of product and packaging.

For objective I, each 20-lb sample of trim was subjected to mixing and coarse grinding (Koch Equipment LLC, Kansas City, MO). In the mixing stage, rosemary extract (NatureGuard Rosemary Extract B, Newly Weds Foods, Edmonton, Alberta, Canada) was slowly added to a level of 0.5% of the total weight. The presence of rosemary extract, a preservative with antimicrobial properties (19), delays the onset of oxidative rancidity in beef patties in a high-oxygen package compared with patties without the rosemary (11). Patties were then formulated from the ground product, and each patty was placed in a plastic tray and sealed using a modified atmosphere packaging machine (G. Mondini S.p.A., Cologne,
Salmonella loads when AW was applied to the beef (0.01), but not SUS (0.02) but not MDR (0.07); |P, which could interfere with the growth or lead to overestimation of the pathogen when counting colonies (37). The thin agar layer method also was utilized, which allows higher recovery of bacterial cells that have been injured or stressed by refrigerated storage temperatures and the interventions (43, 44). Total Salmonella present was determined using the spiral plating method (Autoplate 4000, Spiral Biotech, Norwood, MA). After incubation (35 ± 2°C for 18 to 20 h), plate colonies were counted using Q Count (Spiral Biotech).

Experimental design and analysis. Three replicate experiments were conducted for each objective. The experimental design was a completely randomized block using a split-plot arrangement with repeated measures. Bacteria type (MDR versus SUS Salmonella) was the main plot factor, and the antimicrobial interventions were the subplot factors. Time was considered a repeated measure in this study. All statistical analyses were conducted with a commercially available software package (SAS Institute, Cary, NC). Colony counts were reported as log CFU per gram for objective I and as log CFU per square centimeter for objective II. The model was then fitted using a backward stepwise methodology by eliminating nonsignificant (P > 0.10) effects, beginning with the highest order three-way interaction (bacteria by intervention by time). Once the best-fit model was selected, pairwise comparisons were conducted on significant effects using differences among least square means.

RESULTS

MDR and SUS Salmonella in ground beef patties (objective I). The background microbial flora in the background microbial flora in the beef was a major concern for objective I. A 10-g sample was taken from the center of each ground beef patty and homogenized for 1 min in BPW. Three 1:10 serial dilutions were made, and each dilution was plated in duplicate. For objective II, each swab was diluted with 40 ml of BPW (plus the 10 ml of BPW added before sampling) and homogenized for 1 min. Two 1:10 serial dilutions were made. The microbiological medium utilized was xylose lysine deoxycholate agar (Oxoid Ltd., Basingstoke, Hampshire, UK) with 1% aqueous brilliant green (Sigma-Aldrich, St. Louis, MO) added to the base (1.25 ml/liter) before autoclaving. Brilliant green makes the base more selective for Salmonella and inhibits coliforms and Shigella, which could interfere with the growth. After incubation (35°C), the thin agar layer method also was utilized, which allows higher recovery of bacterial cells that have been injured or stressed by refrigerated storage temperatures and the interventions (43, 44). Total Salmonella present was determined using the spiral plating method (Autoplate 4000, Spiral Biotech, Norwood, MA). After incubation (35 ± 2°C for 18 to 20 h), plate colonies were counted using Q Count (Spiral Biotech).

Storage and sampling. Sample were taken from the ground beef patties for microbiological analysis on days 0, 7, 10, and 14 for objective I. From day 0 to day 7, all packaged patties were stored in shipping boxes in a dark walk-in cooler (maintained at 4.6°C to simulate the shipping environment of product in the industry. After storage in the dark cooler, all trays were moved to a three-level retail display case (Hussmann, Ingersoll-R and Co., Piscataway, NJ) maintained at 2 to 5°C.

For objective II, all brisket samples were held under refrigeration conditions on sterile stainless steel mesh racks in large plastic tubs to simulate a beef carcass hanging in a cooler at a processing facility before further fabrication and after treatment with appropriate interventions. Samples of brisket were randomly collected for microbiological analysis at 0, 6, and 24 h. Samples were taken with sterile hydrated swabs (Spongesicle, Biotrace International Plc, 3M, St. Paul, MN) that were used to swab a 50-cm² area of the surface of the meat as delineated with a disposable plastic grid (Biotrace International).

Microbiological enumeration. For objective I, a 10-g sample was taken from the center of each ground beef patty and homogenized for 1 min in BPW. Three 1:10 serial dilutions were made, and each dilution was plated in duplicate. For objective II, each swab was diluted with 40 ml of BPW (plus the 10 ml of BPW added before sampling) and homogenized for 1 min. Two 1:10 serial dilutions were made. The microbiological medium utilized was xylose lysine deoxycholate agar (Oxoid Ltd., Basingstoke, Hampshire, UK) with 1% aqueous brilliant green (Sigma-Aldrich, St. Louis, MO) added to the base (1.25 ml/liter) before autoclaving. Brilliant green makes the base more selective for Salmonella and inhibits coliforms and Shigella, which could interfere with the growth or lead to overestimation of the pathogen when counting colonies (37). The thin agar layer method also was utilized, which allows higher recovery of bacterial cells that have been injured or stressed by refrigerated storage temperatures and the interventions (43, 44). Total Salmonella present was determined using the spiral plating method (Autoplate 4000, Spiral Biotech, Norwood, MA). After incubation (35 ± 2°C for 18 to 20 h), plate colonies were counted using Q Count (Spiral Biotech).

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RESULTS

MDR and SUS Salmonella in ground beef patties (objective I). The background microbial flora in the

FIGURE 1. Behavior of MDR Salmonella (A) and SUS Salmonella (B) in ground beef patties over time in response to ambient water (AW), 1,000 ppm of acidified sodium chlorite (ASC), and 3% lactic acid (LA) treatments (standard error = 0.22).

uninoculated beef trim was 1 × 10² to 1 × 10³ CFU/g, with naturally occurring Salmonella accounting for <1 × 10³ CFU/g. An effect of treatment was detected (P < 0.01), but no effects of bacteria type or time were detected (P = 0.37 and 0.16, respectively). Evidence of two-way interaction of intervention and bacteria was found (P = 0.07); however, this interaction did not depend on time (P = 0.30). The growth of the two Salmonella types in the ground beef patties during the sampling period in response to the antimicrobial treatments was plotted to demonstrate the growth-decline curves of the bacteria (Fig. 1).

The LA treatment was most effective for both MDR and SUS salmonellae; results for this treatment were significantly different (P < 0.05) from those obtained for the ASC, AW, and CTRL groups. The AW intervention was the least effective for both MDR and SUS Salmonella. Significant differences were observed between MDR and SUS Salmonella loads when AW was applied to the beef trim further processed into ground beef (P = 0.02) but not for the LA (P = 0.69) or ASC (P = 0.56) treatments when compared with the CTRL group. The bacterial levels after AW application were significantly higher (P < 0.01) for...
MDR Salmonella but not for the SUS variants ($P = 0.45$) when compared with the CTRL group (Fig. 2).

MDR and SUS Salmonella in freshly harvested beef briskets (objective II). Background microflora recovered from the briskets was $1 \times 10^3$ CFU/g, and no Salmonella was recovered ($<1 \times 10^1$ CFU/g) before inoculation. A treatment effect was detected ($P < 0.01$), but bacteria type and time did not explain significant model variation ($P = 0.84$ and 0.19, respectively). There was some evidence that the effect of time was dependent on bacteria type ($P = 0.08$), but no three-way interaction was found with bacteria type, intervention used, and time ($P = 0.96$). A plot of bacterial growth versus Salmonella type in the briskets during the sampling time period in response to the antimicrobial treatments is shown in Figure 3.

No interaction between intervention and bacteria was detected ($P = 0.36$). LA treatment provided the greatest effect on both types of salmonellae, with significant differences between the results for the LA group ($P < 0.05$) and those for the ASC, AW, HW, and CTRL groups. The AW intervention was the least effective for both MDR and SUS Salmonella, but populations were still significantly lower than those in the CTRL group ($P = 0.01$). The Salmonella populations in the HW and ASC groups also were significantly lower that those in the CTRL group ($P < 0.01$) (Fig. 4). Significant differences were not found between MDR and SUS Salmonella loads for any intervention when applied to the beef briskets. The two-way interaction between sampling hour and bacteria strain was significant for the 24-h sampling time point when compared with the 0-h and 6-h times. The load of SUS Salmonella on the briskets was significantly higher at 24 h than at 0 h ($P = 0.04$) and 6 h ($P = 0.03$) and significantly higher than the MDR Salmonella load at 6 h ($P = 0.04$). However, when taking the standard error into account, there were no significant differences between MDR and SUS

**FIGURE 2.** Composite least-squares means for MDR and SUS Salmonella populations in ground beef patties in response to ambient water (AW), 1,000 ppm of acidified sodium chlorite (ASC), and 3% lactic acid (LA). For each antimicrobial intervention, means with the same letter are not significantly different ($P > 0.05$) (standard error = 0.11).

**FIGURE 3.** Behavior of MDR Salmonella (A) and SUS Salmonella (B) in freshly harvested beef briskets over time in response to ambient water (AW), hot water (HW), 1,000 ppm of acidified sodium chlorite (ASC), and 3% lactic acid (LA) treatments (standard error = 0.24).

**FIGURE 4.** Composite least-squares means for MDR and SUS Salmonella populations in response to ambient water (AW), hot water (HW), 1,000 ppm of acidified sodium chlorite (ASC), and 3% lactic acid (LA). Means with the same letter are not significantly different ($P > 0.05$) (standard error = 0.13).
Salmonella in this two-way interaction at 0 h (P ~0.12), 6 h (P ~ 0.88), and 24 h (P = 0.18) (Fig. 5).

DISCUSSION

Overall, the results from this study indicate that the behavior of naturally occurring MDR Salmonella strains is similar to that of naturally occurring SUS Salmonella strains in response to the antagonistic action of antimicrobials. Thus, antimicrobial interventions commonly used in beef facilities would be just as effective for reducing resistant and susceptible variants during normal processing. In a fresh meat system, microbial hazards always will be present and can be only minimized, and the use of various intervention strategies is effective for reducing bacterial contamination (21).

The bacterial loads present on beef trim after the AW rinses were significantly higher for MDR Salmonella than the SUS variants in ground beef. This finding suggests a trend toward more attachment of the MDR Salmonella but is not of serious concern because AW rinse is not considered an antimicrobial intervention.

The potential for antibiotic-resistant foodborne pathogens to have enhanced resistance to antimicrobial agents, sanitizers, and disinfectants has been studied in only a very limited way (2, 10, 41). The results reported here are in agreement with other similar studies. In 2008, Arthur et al. (4) compared the survivability of MDR and SUS Salmonella (serovars Newport and Typhimurium) and Escherichia coli O157:H7 on freshly harvested flank tissue of beef carcasses treated with similar acid and nonacid interventions. The overall conclusions were the same as those of the present study, i.e., regardless of antimicrobial treatment MDR Salmonella, SUS Salmonella, and even E. coli O157:H7 were similarly sensitive. There were no differences in the survival of these pathogens after the acetic acid intervention, but there were differences in the LA treatment groups. LA application had the greatest effect on MDR Salmonella Newport and an intermediate effect on the other salmonellae. Methodology differences could impact comparisons with the results of the present research. In the study conducted by Arthur et al., all treatments were applied in an insertable pod located under a laminar flow hood (laboratory setting), whereas in the present study all treatments were applied in a simulated industry environment.

In another study, Lopes (32) tested an assortment of acid anionic sanitizers formulated for controlling contamination of fruits and vegetables and compared their ability to control nalidixic acid–resistant and antibiotic-susceptible Salmonella Typhimurium. The resistant Salmonella Typhimurium was as susceptible as its susceptible counterpart to the organic acid and anionic surfactant sanitizers. This finding further emphasizes that antimicrobial interventions currently used in the food industry are effective for controlling both drug-resistant and drug-susceptible pathogens and sometimes are more effective against the antibiotic-resistant organisms.

Although the inability of AW treatment to control MDR and SUS Salmonella in the ground beef patties was expected, the effects of ASC and LA were not expected. Under the experimental conditions, minimal reductions of both MDR and SUS Salmonella were achieved with ASC, and highly significant reductions for both microorganisms were achieved with LA. Gill and Badoni (24) found that ASC had very little effect on the natural microflora of chilled beef briskets, and 4% LA reduced E. coli, coliforms, and aerobic bacteria by ≥1.5 log units. We did not achieve as great a reduction on chilled surfaces with the ASC in the present study, but we achieved a significant reduction with the LA treatment.

The persistence of resistant microflora in the food supply and the link between these resistant forms and food-related sanitizer and/or antimicrobial use have been the subjects of much debate (2). Research on this topic is limited, but according to Bacon et al. (5, 6) and Samelis et al. (36) the ability of Salmonella to survive heat stress or low pH exposure is not correlated with antibiotic resistance. It is of key importance to the beef industry to determine whether MDR pathogens should be addressed in the same category as SUS variants with respect to HACCP plans. The research presented here is an important component of this validation process because it addresses some of these critical knowledge gaps. Because MDR bacteria and drug-susceptible variants appear to respond similarly to industry controls, scientists in the area of food microbiology should continue to focus on improving current industry antimicrobial interventions to improve the overall safety of food. Although interventions currently implemented in the meat industry can significantly reduce pathogen populations, additional controls should be evaluated with the goal of improving the biological significance of the reductions achieved with these interventions.

REFERENCES