Evaluation of non-pathogenic surrogate bacteria as process validation indicators for *Salmonella enteric* for selected antimicrobial treatments, cold storage and fermentation in meat

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ABSTRACT

Pre-rigor lean and adipose beef carcass tissue was artificially inoculated individually with stationary phase cultures of five non-pathogenic *Escherichia coli* cultures which had been previously identified as surrogates for *E. coli* O157:H7 or a mixture of five *Salmonella* strains in a fecal inoculum. Each tissue sample was processed with microbial interventions comparable to those used in the meat industry. The $\log_{10}$ reductions of the *E. coli* isolates were generally not statistically different from the salmonellae inoculum within a specific treatment. Inoculation experiments were also conducted with ground beef stored at either 4C or -20C. When compared to the *Salmonella* inoculum, at least three of the five *E. coli* strains survived in a manner which was not statistically different from the salmonellae. The *E. coli* strains and the *Salmonella* mixed culture were also inoculated into summer sausage batter, and the population enumerated both before and after fermentation. Four of the *E. coli* strains showed a lower population reduction (higher survival) than the *Salmonella* mixed culture. The five non-pathogenic *E. coli* strains may be used as individually or collectively for specific process validation indicators for *Salmonella*.

Introduction

Salmonella enterica is one of the major foodborne bacterial pathogens in the United States food supply (*4*). The significance of this bacterium in meat and poultry products was emphasized by the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS), by incorporating *Salmonella* performance standards in the Pathogen Reduction/HACCP final rule (*6*). In 2006, the USDA-FSIS began a new initiative to reduce the levels of *Salmonella* in meat and poultry products (*7*).

One of the practical limitations of process validation is that the actual pathogens cannot be taken in to a food processing establishment to verify a specific process. While laboratory research can be used as a reference point, it is not a true substitute for actual, in-plant process validation. The availability of non-pathogenic bacteria, which have similar responses to specific food processes as the pathogenic bacteria, offers the food processor the ability to validate a process in-plant, without the use of the actual pathogens. For example, coliforms have been used as process indicators for pasteurization in the dairy industry.

Recently, several non-pathogenic *Escherichia coli* strains were isolated which responded to meat process interventions in a manner similar to *E. coli* O157:H7 (*3*). Since the bacteria were shown to be not statistically different from *E. coli* O157:H7 in their responses to various antimicrobial interventions, it was reasonable to determine if they could also be used as surrogates for salmonellae. The objective of this research was to compare the responses of

these specific non-pathogenic surrogate bacteria to the response of a mixed culture of *Salmonella* to various meat processes. The intent was to determine which, if any, of the non-pathogenic surrogates could be used for process validation for the reduction of *Salmonella* on meat.

Materials and Methods

Culture Preparation: Five generic *Escherichia coli* beef cattle isolates (P1, P3, P8, P14, P68), previously characterized and selected based upon their heat resistance (*3*) and five *Salmonella enterica* strains (Table 1) were used in this study. The *E. coli* isolates were deposited with the American Type Culture Collection (www.ATCC.org), and the accession numbers are given in Table 1. All strains were maintained on Tryptic Soy Agar (TSA, Difco) slants at 4°C until use.

Preparation of Tissue Surface Inoculum: The preparation of the inoculum procedure was adapted from Hardin et al. (*2*). Feces collected from randomly selected beef cattle which had not been treated with or fed antimicrobials from the Iowa State University Beef Research Farm, were brought back to the laboratory and stored at 4°C. The cultures were grown to late logarithmic phase at 37C in 45 ml Tryptic Soy Broth (TSB, Difco), and then centrifuged at 3,000 x g for 15 minutes at 4°C (Sorvall Super T21, Kendro Lab Products, Newtown, CT). The supernatant was discarded and the strains were re-suspended with 9.0 ml of 0.1% sterile peptone water (Difco, Detroit, MI). The prepared inoculum of each isolate (ca. 10^{8} CFU/ml) was added to individual bags containing 10 g of feces

and hand kneaded for 1 minute. All five *S. enterica* strains were grown and harvested as described above, and each strain was re-suspended in 2.0 ml of 0.1% sterile peptone. The individual *S. enterica* strains were combined, and the inoculum (ca. 10^9 CFU/ml) was pooled into one bag containing 10 g of feces and hand kneaded for 1 minute. A control inoculum was prepared by adding 9.0 ml of 0.1% sterile peptone water to 10 g of feces and hand kneaded for 1 minute.

Carcass tissue inoculation: The cutaneous trunci (lean tissue) and adipose carcass trim was excised from randomly selected carcasses of cows immediately after slaughter (pre-rigor) at a Federally inspected beef slaughter establishment. The tissue samples were placed in individual Whirl-Pak® bags, packed in an insulated cooler and delivered to the Food Safety Research Lab (FSRL; Iowa State University, Ames, IA) within two hours after collection.

The tissue samples were separated into lean and adipose tissue and each tissue type was cut into strips 2 cm wide by 6 cm long by 1 cm thick. A sample was taken of each tissue type prior to inoculation with feces to determine the background aerobic bacterial population of the tissues (typically $\leq 10^2$ CFU/cm^2; data not shown). Tissue samples of lean and adipose were inoculated by applying the fecal suspensions with a foam paint brush, and then allowed to stand 20-30 minutes at 10^oC prior to treatment.

Application of wash treatments: Spray washes were conducted in a BioSafety Level II Pilot Plant of the FSRL maintained at 10°C. The treatments, designed to simulate commercial processes included (a) 90 ^{o}C water wash (b) 90 ^{o}C water wash followed by a 55 ^{o}C, 2% (wt/vol, pH 2.0) lactic acid rinse (c) 90 ^{o}C water wash followed by a 20 ^{o}C, 2% lactic acid rinse (d) 20 ^{o}C water wash (e) 20 ^{o}C water wash followed by a 20 ^{o}C, 2% lactic acid rinse (f) 20 ^{o}C water wash followed by 20 ^{o}C, 20 ppm chlorine rinse (g) 20 ^{o}C water wash followed by a 20 ^{o}C, 10% (wt/vol, pH 11) trisodium phosphate (TSP) wash. Inoculated tissues were placed on sterile metal hooks and were hung in the same orientation as the tissue would hang on a carcass. Each set of hanging inoculated tissues was separated by a square piece of high density polyethylene (HDPE) plastic to prevent aerosol contamination between samples. The wash and rinse treatments were applied using a low pressure, Shur flow diaphragm pump sprayer with a flat fan nozzle (ME Anderson Engineering Inc., Model No. MEA-10-1, Columbia MO) at a pressure of 20 psi for 3 seconds (flow rate 77 ml/s) at a distance of 10 – 15 cm. All of the treatments were conducted within a chamber constructed of PVC pipe and polyethylene plastic sheeting to provide protection from the biological agents.

Microbiological Analysis: A 1 cm x 2 cm sample of each tissue type was excised both prior to inoculation, after inoculation with the fecal suspension, and then after treatment. Each tissue sample was placed in a sterile filter stomacher bag and homogenized for 1 min in 25 ml buffered peptone water (BPW; Difco). Previous research had shown that the BPW neutralized the residual chemicals from the treatments, based on the presence of free available chlorine or pH measurements. Serial dilutions were performed in BPW, and total aerobic populations were determined by spread plating 0.1 ml of the sample from the appropriate dilution onto duplicate plates of TSA. Populations of each indicator organism were determined by spread plating 0.1 ml of the sample from the appropriate dilution onto duplicate plates of Violet Red Bile Glucose (VRBG; Difco) agar. All plates were incubated at 37°C for 24 h. The minimum detection limit for the assay was $\log_{10}$ 1.8 colony forming units/cm^2.

Survival During Refrigerated and Frozen Storage: Frozen ground beef was obtained from local retail establishments. The ground beef was irradiated to a minimum dose of 3 kGy at the Iowa State University Linear Accelerator Facility to eliminate the background gram negative microflora. Twenty five grams of the thawed meat was placed into sterile Whirl-Pak bags (Ft. Atkinson, WI) and inoculated with either the mixed culture of *S. enterica* or individually with the five surrogate *E. coli* bacteria, to an approximate population of ca. 10^7 colony forming units/gram (cfu/g). The samples were stored at either 4°C or -20°C, and sampled at 0, 1,3,5,7,10,14 and 21 days (4°C) or 0,1,5,10,15,30,60 and 90 days (-20°C).

The populations of the bacteria were enumerated on VRBG after incubation at 37°C for 24 h.

Summer Sausage Fermentation: The survival of surrogate bacteria during summer sausage were compared to the mixed culture of *S. enterica* using the "beaker sausage" method (*1*) Briefly, 100 g of prepared summer sausage batter (50:50 beef : pork; 72:28 lean:fat; 2% NaCl) obtained from the Iowa State University Meat Laboratory and inoculated with Bactiferm HP starter culture, Chr. Hansen, Milwaukee WI. The summer sausage batter was subsequently inoculated with ca. 10^7 cfu/g of either the mixed *S. enterica* culture or the individual surrogate *E. coli* bacteria, and fermented at 37.8^{o} C until the pH $\leq$ 4.8 (approximately 12 h). Samples were collected at the beginning and end of the fermentation, and the bacterial populations were enumerated on VRBG after incubation at 37°C for 24 h.

Statistical Analysis: All experiments were independently replicated three times. The microbial populations were converted from colony forming units (CFU) per cm^2 or g to log_{10} CFU/cm^2 or g. The mean log_{10} reduction was calculated by subtracting the log_{10} (final) count of each organism after each microbial intervention from the log_{10} (initial) count obtained of each organism before the microbial interventions. The mean log reductions of each *E. coli* isolate and for the *S. enterica* composite culture for each treatment were compared by the general linear model procedure (GLM). Significant differences among means

(P<0.05) were reported using GLM procedures. All statistical analyses were conducted using SAS procedures (*5*).

Results and Discussion

The population reductions in the indicator bacteria were compared to the population reductions in the mixed salmonellae culture. Using this method of calculation, a lesser population reduction of the indicator bacteria, as compared to the salmonellae population, would indicate that the treatment would reduce the population of salmonellae more than that of the indicator. Conversely, a larger population reduction in the indicator bacteria, when compared to the salmonellae population, indicated that the treatment would reduce population of the indicator more than that of the salmonellae. A lesser population reduction in the indicator bacteria suggests that the indicator would under-predict the actual reduction in salmonellae, and this under-prediction could be considered a margin of safety for validating the process.

The appropriateness of the *E. coli* isolates as potential *Salmonella* indicators was found to be dependent upon the microbial intervention utilized. On pre-rigor lean tissue (Table 2), the indicator organisms were not significantly different from the mixed culture of salmonellae for five of the treatments. For the 20 ^{o}C water rinse only, two of the indicators (BAA-1430 and BAA-1431) resulted in a statistically (P<0.05) lower reduction in population than the mixed salmonellae culture. For the 20 ^{o}C water/20 ^{o}C acid and 20 ^{o}C water/20 ^{o}C

chlorine treatments, indicator culture BAA-1430 showed a statistically ($P<0.05$) lower reduction in population than the mixed salmonellae culture.

On adipose tissue (Table 3), the indicator organisms were not significantly different from the mixed culture of salmonellae for four of the treatments. Two of the indicators (BAA-1427 and BAA-1428) resulted in a statistically ($P<0.05$) lower reduction in population than the mixed salmonellae culture for the 90° C water/ 55° C acid. Indicator BAA-1429 showed a statistically ($P<0.05$) greater reduction in population than the mixed salmonellae culture for the 90° C water/20° C acid and 20° C water/20° C acid.

The surrogate E. *coli* bacteria were also evaluated in comparison to the survival of *Salmonella enterica* during storage. During both frozen (Figure 1) and refrigerated (Figure 2) storage, the surrogate bacteria survived in a manner either comparable to the mixed culture of *S enterica*, or in some cases to a greater extent than the mixed culture. That is, the surrogate bacteria would survive not less than the mixed culture of *S enterica*. This would provide a margin of safety, suggesting that the surrogate bacteria are in fact adequate predictors of the survival of *S. enterica* under normal storage conditions.

The surrogate *E. coli* bacteria were also evaluated for their survival during summer sausage fermentation. Figure 3 shows the $\log_{10}$ reductions, calculated as $\log_{10}$ initial population – $\log_{10}$ final population, of both the mixed culture of *S. enterica* and the individual surrogate cultures. As with the storage conditions, the surrogate bacteria had population reductions generally less than those of the mixed salmonellae culture. Cultures BAA-1427, 1428, 1429 and 1430 had

population reductions from fermentation which were significantly less than the population reduction of the mixed *S. enterica* culture. The population reductions for the salmonellae inoculum were approximately 5 log_{10}, while those for the four surrogate bacteria were approximately 3 to 3.5 log_{10}. Only one isolate, BAA-1431, was not statistically different ($P>0.05$) from the reduction in the salmonellae population. Again, this provides a margin of safety, as the reduction in the population of the surrogates is either equal to, or less than those of salmonellae.

In conclusion, at least some of the non-pathogenic surrogate *E. coli* were equivalent to a mixed culture of salmonellae for all of the processes studied in these experiments. This study demonstrates that these bacteria, either individually or collectively, have the potential to be used to validate meat processes for the reduction of salmonellae. There are clearly limitations to these experiments, with the most significant being the use of a larger initial population than would be expected to be encountered in meat, as well as the potential for variation in response among strains of salmonellae not evaluated. These limitations are common with most experiments, in which laboratory data is used to predict performance in a processing establishment. However, indicator cultures evaluated in this research may allow a meat processing establishment to internally validate their own processes for salmonellae reduction.

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Table 1. Sources and identification of *Salmonella enterica* serovars and *Escherichia coli* cultures used as a reference cultures.

Bacterium	Strain Designator[1]	Source
S. enterica var. Typhimurium	G-24	Meat product isolate
S. enterica var. Typhimurium	G-25	Meat product isolate
S. enterica var. Typhimurium	G-26	ATCC 14028
S. enterica var. Typhimurium	G-5	Poultry isolate; USDA-ARS, Russell Research Laboratory, Athens GA
S. enterica var. Heidelberg	G-1	Dairy cattle isolate; Iowa State University Veterinary Diagnostic Laboratory
Bacterium	Strain Designator[1]	ATCC accession number
Escherichia coli	P1	BAA-1427
Escherichia coli	P3	BAA-1428
Escherichia coli	P8	BAA-1429
Escherichia coli	P14	BAA-1430
Escherichia coli	P68	BAA-1431

1 Iowa State University Food Safety Research Laboratories. *Salmonella* strain designators (G series) are from the internal culture collection. *E. coli* strain designators (P series) are as referenced on Marshall et al., 2005.

Table 2. Mean log_{10} reduction in populations of a five strain mixture of salmonellae and five individual non-pathogenic indicators on pre-rigor lean tissue.

Micro organism	90°C water	90 °C water 55 °C acid[1]	90 °C water 20 °C acid	20 °C water	20 °C water 20 °C acid	20 °C water 20 °C chlorine[2]	20 °C water 20 °C TSP[3]
salmonellae	1.40	2.72	1.55	1.61[4b]	2.07[b]	1.24[b]	1.57
BAA-1427	1.77	2.55	1.80	1.02[b]	1.82[b]	0.91[b]	1.21
BAA-1428	1.43	2.45	1.55	0.76[b]	1.4[b]	1.18[b]	1.45
BAA-1429	1.09	2.55	1.29	0.78[b]	1.59[b]	0.87[b]	1.19
BAA-1430	1.19	2.03	1.32	0.41[a]	1.12[a]	0.81[a]	0.99
BAA-1431	1.30	2.04	1.58	0.69[a]	1.24[b]	1.04[b]	0.94

1 The acid rinse was 2% (wt/vol).

2 The chlorine rinse was 20 ppm.

3 The TSP rinse was 10% trisodium phosphate.

4 Means within columns with different superscripts are significantly ($P<0.05$) different. If no superscripts are present, there was no significant difference between the means.

Table 3. Mean log_{10} reduction in populations of a five strain mixture of salmonellae and five individual non-pathogenic indicators on pre-rigor adipose tissue.

Micro organism	90°C water	90 °C water 55 °C acid[1]	90 °C water 20 °C acid	20 °C water	20 °C water 20 °C acid	20 °C water 20 °C chlorine[2]	20 °C water 20 °C TSP[3]
salmonellae	1.10	2.76[4b]	1.93 [b]	1.29	2.31 [b]	1.30	1.56
BAA-1427	0.96	1.20[a]	1.52 [b]	0.61	1.92 [b]	0.95	1.37
BAA-1428	0.92	1.63[a]	2.01 [b]	0.78	2.25 [b]	1.25	2.14
BAA-1429	1.13	2.97 [b]	3.02[a]	0.90	3.33[a]	1.03	1.45
BAA-1430	0.77	2.68 [b]	1.74 [b]	0.99	2.10 [b]	1.05	2.21
BAA-1431	0.96	2.63 [b]	2.06 [b]	0.69	2.67 [b]	1.28	1.59

1 The acid rinse was 2% (wt/vol).

2 The chlorine rinse was 20 ppm.

3 The TSP rinse was 10% trisodium phosphate.

4 Means within columns with different superscripts are significantly ($P<0.05$) different. If no superscripts are present, there was no significant difference between the means.

Figure 1. Survival of *Salmonella enterica* and surrogate *E. coli* bacteria in ground beef at -20 °C.

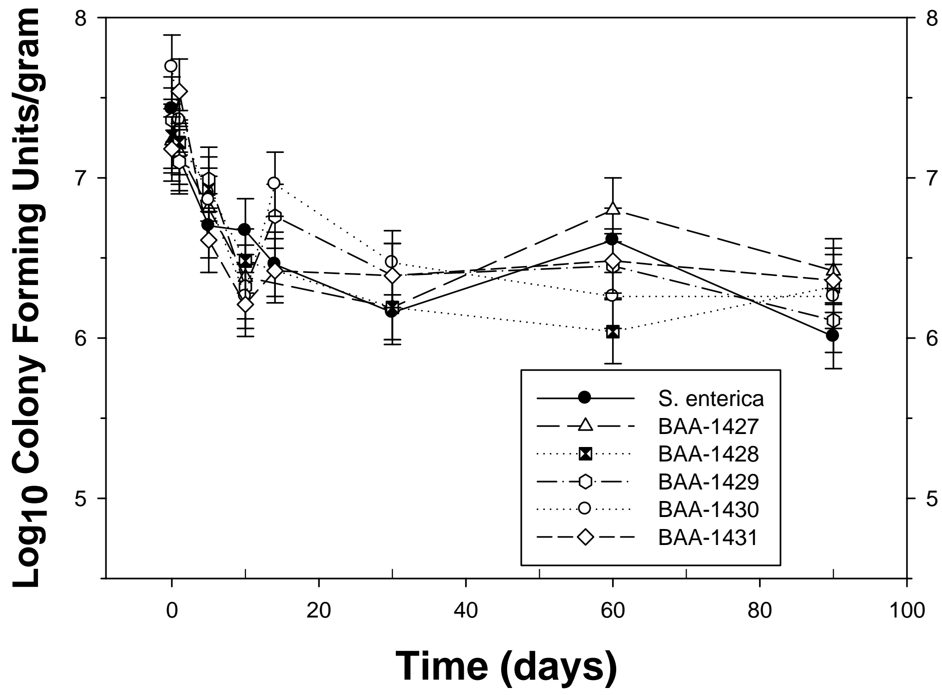

Figure 2. Survival of *Salmonella enterica* and surrogate *E. coli* bacteria in ground beef at 4°C.

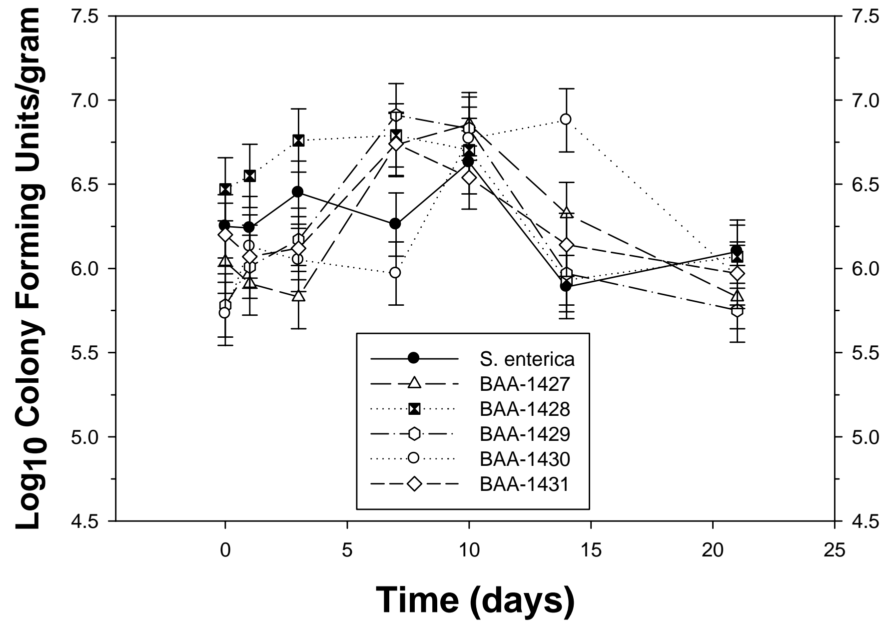

Figure 3. Reduction of *Salmonella enterica* and surrogate *E. coli* bacteria during summer sausage fermentation.