ESCHERCHIA COLI BIOTYPE I SURROGATES AS PREDICTORS OF NON-O157 SHIGA TOXIN-PRODUCING *E. COLI* FOR GROWTH, ACID RESISTANCE, FREEZING, REFRIGERATED STORAGE

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Abstract – The percentage of non-O157 serotype infections is increasing in many countries. This study was conducted to determine if previously identified *Escherichia coli* biotype I surrogates can be used for he growth parameters, acid-resistance, and storage conditions of the non-O157 STECs (O26, O111, O121, O145, O103, and O45). Non-O157 Shiga toxin-producing *E. coli* (STECs) and, *E. coli* biotype I surrogates were cultured in tryptic soy broth (TSB) at 37°C for 18 h. Growth curves were obtained, and growth parameters of *E. coli* biotype I surrogates were similar to those of all non-O157 STECs throughout the evaluation. Stationary and acid-adapted organisms were transferred to 10 ml of pre-warmed phosphate buffer saline (PBS) acidified with L-lactic acid at pH 3.0 and 3.5. For acid resistance, most *E. coli* biotype I microorganisms had similar ($P > 0.05$) reductions to the non-O157 STECs. For freezing (-20 ± 0.5°C) and refrigerated (4 ± 0.5°C) storage, bacterial strains were enumerated. Although some differences were found for individual surrogates, these data suggest that a cocktail of *E. coli* biotype I surrogates may be used to predict growth, acid resistance and storage conditions for the six non-O157 STECs used in this study.

Key Words – beef, nonpathogenic, E coli

I. INTRODUCTION

Meat and poultry are sources of pathogens associated with foodborne illness [1]. Foodborne illness attributed to Shiga toxin-producing *Escherichia coli* (STECs) has increased in the past decade, and acid interventions are often applied to beef as antimicrobial treatments. STECs comprise many pathogenic serotypes, of which O157:H7 is the most studied. The percentage of non-O157 serotype infections is increasing in many countries. In addition to O157:H7, the Centers for Disease Control and Prevention (CDC) has stated that the serotypes most commonly identified in human illness, causing diarrhea and post diarrheal hemolytic uremic syndrome, are O26, O45, O103, O111, O121, and O145. On September 20, 2011, USDA-FSIS declared that raw, non-intact beef products, or raw, intact beef products intended for use in raw non-intact product, contaminated with Shiga toxin-producing *E. coli* ("Big 6") adulterants [2]. *E. coli* biotype I strains have been previously isolated from cattle hides and determined to be appropriate for use as surrogates for *E. coli* O157:H7. These potential surrogates were sent to the *E. coli* Reference Center (Penn State University, University Park, PA) for determining the genes encoding for virulence attributes. All strains tested negative for the virulence attributes and were considered as nonpathogenic; a necessary attribute for surrogate organisms. Six non-O157 STEC serotypes identified as O26, O111, O121, O145, O103, and O45 have been selected as target pathogens for comparison against the known surrogate bacteria. The objective of this study was to compare the resistance and survival properties of non-O157 STECs to those of previously developed *E. coli* biotype I surrogate microorganisms in raw meat.

II. MATERIALS AND METHODS

A. Bacterial cultures

Non-O157 Shiga toxin-producing *E. coli* (STECs) (ATCC # 2192, 2193, 2196, 2215, 2217 and 2219) and, *E. coli* biotype I surrogates (ATCC # BAA-1427, BAA-1428, BAA-1429, BAA-1430, and BAA-1431) were used in the experiments. All experiments were conducted in triplicate.

B. Growth parameters

Each strain was individually cultured in TSB at 37°C for 18 h. Dilutions were made for each culture in 0.1% peptone water and 0.1 ml of the 1:10,000 dilutions were transferred to a 10 ml fresh TSB to achieve ~3 log CFU/ml. All tubes were incubated at 37°C in a water bath, and over a 24-h period, one tube from each strain was removed each hour for enumeration on tryptic soy agar (TSA) using the spiral plate technique. Enumeration was performed at h 0, 1, 2, 3, 6, 7, 8, 10, 12 and 24. Plates were incubated at 37°C for 24 h, and colonies enumerated.

C. Acid resistance

Each strain was individually cultured in TSB at 37°C for 18 h and 0.1-ml aliquots of fresh cultures were individually transferred to 10 ml TSB, and 10 ml TSB+G tubes (supplemented with 1% glucose to induce acid tolerance response [3]). After incubation at 37°C for 18 h, each culture was transferred to conical centrifuge tubes and cells harvested by centrifugation at 1,620 × *g* for 15 min. The supernatant was discarded and the pellets re-suspended in 10 ml PBS (pH 7.4). Each cell suspension was centrifuged again (1,620 × *g* for 15 min) and the procedure repeated two more times. The final pellets were suspended in 10 ml PBS, 0.1-ml aliquots were individually transferred into glass tubes containing 10 ml pre-warmed (37°C) PBS at values 3.0 and 3.5. The pH of the PBS was previously adjusted with 88% L-lactic acid. All tubes were incubated at 37°C in a constant water bath with the water level at least 1 cm above the level of the cell suspension in each tube. One tube of each strain was removed from the water bath immediately after inoculation and every 0.5, 1.0, 1.5 and 2.0 h for the enumeration of survivors. Appropriate decimal dilutions were performed and plated on TSA for enumeration. Colonies were enumerated after incubation of plates at 37°C for 24 h, and leg reduction (CFU/ml) values were calculated by subtracting the log count (CFU/ml) of each microorganism after each exposure time from the initial log count obtained at time zero.

D. Resistance to refrigeration and freezing temperatures

Each strain was individually cultured in TSB at 37°C for 18 h. Aliquots of 0.1 ml of each culture were individually transferred to 10 ml TSB and 10 ml TSB+G and incubated at 37°C for 18 h. Each culture was transferred to conical centrifuge tubes and the cells were harvested by centrifugation at 1,620 × *g* for 15 min. The supernatant was discarded and the pellets re-suspended in 10 ml PBS (pH 7.4). Each cell suspension was centrifuged again (1,620 × *g* for 15 min) and the procedure repeated two more times. The final pellets were re-suspended in 100 ml PBS and 1-ml aliquots of each bacterial suspension were individually transferred to sterile micro centrifuge tubes. Fifty percent of the tubes were stores at 4 ± 0.5°C (refrigeration) and the remaining 50% at -20 ± 0.5°C (freezing). After 0, 7, 14, 21, 28, 60 and 90 d of storage, one tube of each cultures was removed from refrigeration and frozen storage for enumeration of survivors. Frozen cultures were defrosted in ice water for 30 min before enumeration. Decimal dilutions were performed in 0.1% peptone water and were plated on TSA. Plates were incubated at 37°C for 24 h before colonies were enumerated.

E. Statistical analysis

Plate counts were converted to log CFU per milliliter before analysis. The general linear model procedure of the Statistical Analysis System (SAS) was used for data analysis. Least squares means were calculated using the LSMEANS statement of SAS and multiple comparison procedures were conducted to determine whether there were statistical differences ($P < 0.05$) between means.

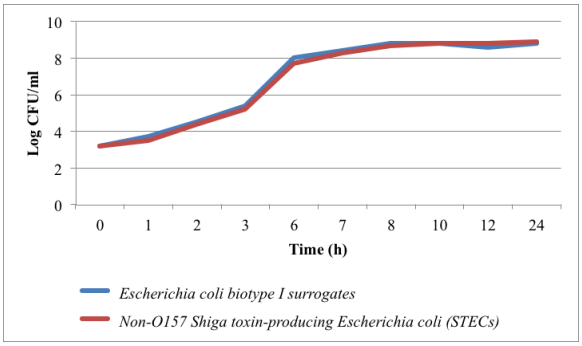
III. RESULTS AND DISCUSSION

A. Growth parameters

For these surrogates to be useful for predicting the behavior of pathogens during processing, the growth of these surrogates must be equivalent or slightly greater, but not less than that of the target pathogens (six non-O157 STECS) [4]. Growth curves of *E. coli* biotype I surrogates were similar

to those of all non-O157 STECs throughout the evaluation, differing by 0.5 log or less at each 1-h evaluation during the 24-h growth period (Figure 1). While some statistical differences ($P < 0.05$) were detected at intervals during growth from an initial population of approximately log 3.0 to log 9.0 over 24 h, trends clearly were within range for use as surrogates.

Figure 1. Average graphical representation of *Escherichia coli* biotype I surrogates versus non-O157 Shiga toxin-producing *E. coli* (STECs) log CFU/ml plotted over 24 h time period



B. Acid resistance

The acid resistance of *E. coli* biotype I surrogate microorganisms, and the non-O157 STECs were compared in PBS acidified with L-lactic acid at pH values 3.0 and 3.5. This pH range was selected based on the expected carcass surface after application of 2% L-lactic acid [5]. To be used in the validation of lactic acid interventions, surrogate organisms must have resistance to lactic acid similar to that of the target pathogens (six non-O157 STECs). Mean reductions (log CFU/ml) for acid-adapted *E. coli* biotype I surrogates, and non-O157 STECs are shown in Table 1. For acid resistance, most *E. coli* biotype I microorganisms had similar ($P > 0.05$) reductions to the non-O157 STECs; however, there were some instances of greater ($P < 0.05$) reductions.

Table 1. Mean reductions (log CFU/ml) for acid-adapted *Escherichia coli* biotype I surrogates, and non-O157 STECs after exposure to phosphate buffered saline acidified with lactic acid at pH 3.0 and 3.5

	Time (min)				
	0	0.5	1.0	1.5	2.0
Organism[1]					
pH 3.0					
BAA1427	1.8 $_{CD}$[2]	4.0 $_{DE}$	4.6 $_{DE}$	5.4 $_{BC}$	5.1 $_{C}$
BAA1428	2.1 $_{B}$	4.4 $_{BCD}$	5.3 $_{BCD}$	6.4 $_{AB}$	6.4 $_{ABC}$
BAA1429	2.0 $_{BCD}$	4.7 $_{BC}$	5.3 $_{BCD}$	6.7 $_{A}$	7.0 $_{AB}$
BAA1430	1.9 $_{BCD}$	4.6 $_{CD}$	5.6 $_{ABC}$	6.6 $_{A}$	7.4 $_{A}$
BAA1431	1.8 $_{D}$	3.7 $_{E}$	4.2 $_{E}$	5.3 $_{C}$	7.0 $_{AB}$
STEC2219	1.9 $_{BCD}$	4.3 $_{CD}$	4.7 $_{CDE}$	5.1 $_{C}$	5.2 $_{C}$
STEC2217	1.9 $_{BCD}$	4.9 $_{AB}$	6.0 $_{AB}$	6.1 $_{ABC}$	6.1 $_{BC}$
STEC2215	2.1 $_{BC}$	4.5 $_{BCD}$	5.3 $_{BCD}$	6.4 $_{A}$	5.9 $_{BC}$
STEC2196	2.5 $_{A}$	5.3 $_{A}$	6.1 $_{AB}$	6.9 $_{A}$	6.9 $_{AB}$
STEC2193	1.9 $_{BCD}$	4.5 $_{BCD}$	5.2 $_{BCDE}$	6.6 $_{A}$	7.0 $_{AB}$
STEC2192	1.9 $_{BCD}$	4.5 $_{BCD}$	6.4 $_{A}$	6.8 $_{A}$	6.0 $_{BC}$
pH 3.5					
BAA1427	1.9 $_{ABC}$[2]	1.9 $_{B}$	2.6 $_{A}$	2.6 $_{AB}$	3.5 $_{A}$
BAA1428	2.1 $_{A}$	2.6 $_{A}$	2.2 $_{A}$	2.5 $_{AB}$	2.9 $_{A}$
BAA1429	1.8 $_{BC}$	1.9 $_{B}$	2.1 $_{A}$	2.2 $_{B}$	3.5 $_{A}$
BAA1430	1.9 $_{ABC}$	2.4 $_{AB}$	2.3 $_{A}$	2.8 $_{AB}$	3.3 $_{A}$
BAA1431	2.0 $_{ABC}$	2.0 $_{B}$	2.2 $_{A}$	2.4 $_{AB}$	3.6 $_{A}$
STEC2219	2.1 $_{A}$	2.1 $_{AB}$	2.4 $_{A}$	2.5 $_{AB}$	3.4 $_{A}$
STEC2217	1.8 $_{C}$	2.0 $_{B}$	2.4 $_{A}$	3.1 $_{A}$	3.6 $_{A}$
STEC2215	2.1 $_{A}$	2.2 $_{AB}$	2.5 $_{A}$	2.7 $_{AB}$	3.6 $_{A}$
STEC2196	2.1 $_{AB}$	2.3 $_{AB}$	2.5 $_{A}$	2.7 $_{AB}$	3.3 $_{A}$
STEC2193	2.0 $_{ABC}$	2.1 $_{AB}$	2.4 $_{A}$	2.9 $_{AB}$	3.3 $_{A}$
STEC2192	1.9 $_{ABC}$	2.0 $_{B}$	2.3 $_{A}$	2.9 $_{AB}$	3.7 $_{A}$

[1] Organism STEC 2219=O121:H19, STEC 2217=O111, STEC 2215=O103:H11, STEC 2196=O26:H11, STEC 2193=O45:H2, STEC2192=O145.
[2] Means within a column lacking a common letter differ ($P < 0.05$).

C. Resistance to refrigeration and freezing temperatures

Different properties must be evaluated when selecting appropriate surrogate organisms for enteric pathogens. The ability of surrogates and pathogens to survive under both freezing and refrigerated temperatures should be similar, if the surrogates are intended to be utilized in validation studies for meat and meat products, given that storage at low temperatures is necessary for preservation. Mean counts (log CFU/ml) for acid-adapted *E. coli* biotype I surrogates, and non-O157 STECs are shown in Table 2. Both refrigerated and frozen storage resulted in random differences

between counts of *E. coli* biotype I microorganisms and non-O157 STECs; however, there were no notable trend or patterns observed. Findings for both acid resistance and storage conditions support previous research stating that surrogates may be used as a cocktail rather than single strains [6].

Table 2. Mean counts (log CFU/ml) for acid-adapted *Escherchia coli* biotype I surrogates, and non-O157 STECs in phosphate buffered saline at 4 ± 0.5 °C (refrigeration) and -20 ± 0.5 °C (frozen)

	Time (d)						
	0	7	14	21	28	60	90
Organism[1]							
Refrigeration							
BAA1427	7.8 $_{A}$[2]	7.7 $_{A}$	7.6 $_{A}$	7.7 $_{A}$	7.6 $_{AB}$	7.6 $_{A}$	7.5 $_{A}$
BAA1428	7.5 $_{D}$	7.2 $_{CD}$	6.8 $_{EF}$	6.7 $_{E}$	6.6 $_{G}$	6.6 $_{EF}$	6.4 $_{CDE}$
BAA1429	7.9 $_{A}$	7.4 $_{AB}$	7.1 $_{CD}$	7.4 $_{C}$	7.2 $_{CD}$	7.2 $_{BCD}$	7.0 $_{B}$
BAA1430	7.8 $_{A}$	7.4 $_{AB}$	7.0 $_{CDE}$	7.0 $_{D}$	6.9 $_{EF}$	6.9 $_{CDE}$	6.5 $_{CDE}$
BAA1431	7.6 $_{CD}$	7.1 $_{CD}$	6.7 $_{F}$	6.7 $_{E}$	6.7 $_{FG}$	6.2 $_{F}$	6.1 $_{E}$
STEC2219	7.8 $_{AB}$	7.7 $_{A}$	7.4 $_{AB}$	7.5 $_{ABC}$	7.4 $_{C}$	7.1 $_{CD}$	6.3 $_{DE}$
STEC2217	7.6 $_{BCD}$	7.5 $_{AB}$	7.6 $_{A}$	7.5 $_{ABC}$	7.4 $_{BC}$	7.0 $_{CD}$	6.4 $_{CDE}$
STEC2215	7.6 $_{CD}$	7.3 $_{BC}$	7.0 $_{CDE}$	7.0 $_{D}$	7.0 $_{DE}$	7.0 $_{CD}$	6.7 $_{BCD}$
STEC2196	7.2 $_{E}$	7.0 $_{D}$	6.8 $_{DEF}$	6.7 $_{E}$	6.8 $_{FG}$	6.8 $_{DE}$	6.8 $_{BC}$
STEC2193	7.7 $_{ABCD}$	7.5 $_{AB}$	7.5 $_{AB}$	7.7 $_{AB}$	7.7 $_{A}$	7.6 $_{AB}$	7.5 $_{A}$
STEC2192	7.7 $_{ABC}$	7.5 $_{AB}$	7.3 $_{BC}$	7.5 $_{BC}$	7.4 $_{C}$	7.3 $_{ABC}$	7.0 $_{B}$
Frozen							
BAA1427	7.8 $_{AB}$[2]	5.9 $_{ABC}$	5.4 $_{BC}$	5.0 $_{AB}$	4.7 $_{ABC}$	4.3 $_{BC}$	3.9 $_{ABCD}$
BAA1428	8.0 $_{A}$	5.5 $_{C}$	5.0 $_{C}$	5.0 $_{B}$	4.2 $_{BC}$	4.4 $_{BC}$	3.5 $_{CD}$
BAA1429	7.9 $_{AB}$	6.3 $_{A}$	6.2 $_{A}$	5.3 $_{AB}$	4.8 $_{AB}$	4.7 $_{AB}$	4.8 $_{A}$
BAA1430	7.9 $_{A}$	5.7 $_{BC}$	5.1 $_{C}$	5.0 $_{AB}$	4.1 $_{C}$	4.6 $_{ABC}$	3.3 $_{D}$
BAA1431	7.5 $_{BC}$	5.8 $_{BC}$	5.2 $_{BC}$	5.0 $_{B}$	4.4 $_{ABC}$	4.3 $_{BC}$	3.8 $_{BCD}$
STEC2219	7.8 $_{AB}$	6.2 $_{AB}$	5.5 $_{ABC}$	5.0 $_{B}$	4.6 $_{ABC}$	5.1 $_{A}$	4.4 $_{ABC}$
STEC2217	7.8 $_{AB}$	6.1 $_{AB}$	5.7 $_{ABC}$	5.5 $_{A}$	4.2 $_{BC}$	4.0 $_{C}$	3.5 $_{CD}$
STEC2215	7.6 $_{ABC}$	6.0 $_{ABC}$	5.2 $_{BC}$	5.1 $_{AB}$	4.8 $_{ABC}$	4.9 $_{AB}$	4.4 $_{ABC}$
STEC2196	7.3 $_{C}$	6.1 $_{AB}$	5.2 $_{BC}$	5.4 $_{AB}$	4.9 $_{A}$	4.8 $_{AB}$	4.6 $_{AB}$
STEC2193	7.8 $_{AB}$	6.1 $_{AB}$	5.9 $_{AB}$	5.0 $_{B}$	4.6 $_{ABC}$	4.9 $_{AB}$	3.7 $_{BCD}$
STEC2192	7.8 $_{AB}$	5.8 $_{ABC}$	5.5 $_{BC}$	5.1 $_{AB}$	4.6 $_{ABC}$	4.0 $_{C}$	3.6 $_{CD}$

[1] Organism STEC 2219=O121:H19, STEC 2217=O111, STEC 2215=O103:H11, STEC 2196=O26:H11, STEC 2193=O45:H2, STEC2192=O145.
[2] Means within a column lacking a common letter differ ($P < 0.05$).

IV. CONCLUSION

These data suggest that using a cocktail of *E. coli* biotype I surrogates may be used to predict growth of non-O157 Shiga toxin-producing *E. coli* (STECs). Furthermore, a cocktail of *E. coli* biotype I microorganisms may also serve as surrogates for acid resistance and storage conditions for these six non-O157 STECs. Additional data collection will provide information critical for use of the surrogates in validation studies.

ACKNOWLEDGEMENTS

This project was funded, in part, by The Beef Checkoff.

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