Project Title: Real-time Monitoring of Cross-contamination of *Listeria monocytogenes* between Equipment and Ready-to-Eat Meat Products via a GFP Reporter

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Background

Listeria monocytogenes is a ubiquitous bacterium commonly found on raw meat and poultry products and has been associated with a number of foodborne illness outbreaks and food product recalls (CDC 1999; USDA-FSIS 2003a). This organism possesses a relatively high resistance to heat and can grow at refrigeration temperatures as low as 2°C and under low oxygen tension, such as that found in vacuum-packaged ready-to-eat (RTE) products (Samelis and others 2002). *L. monocytogenes* is given a zero-tolerance status in RTE meat and poultry products because of the high mortality rate associated with listeriosis (Mead and others 1999). Although the cooking processes currently applied by the meat industry generally meet USDA Food Safety and Inspection Service (FSIS) requirements, the processing steps after cooking such as peeling, sorting, loading, slicing, packaging, and so forth, are potential sources of recontamination. An FSIS survey published in 2001 showed that 1% to 10% of retail RTE products were contaminated with *L. monocytogenes* (Levine and others 2001). *L. monocytogenes* is currently the most concerned pathogen of post-cook contaminants among RTE meat and poultry products.

The CDC estimates the annual cost of illness due to *Escherichia coli* O157:H7 infections acquired from food or other sources as $405 million (Frenzen et al., 2005). Because of the high cost of illness due to *E. coli* O157: H7 infections, the federal government has implemented mandatory hazard analysis and critical control point (HACCP) programs and improved pre- and post-harvest processes to lower the contamination levels of meat products with this pathogen in meat and poultry plants and the juice industry (Frenzen, 2005). Most outbreaks of *E. coli* O157:H7 have been the associated with foods of bovine origin. In cases involving non-bovine foods, cross-contamination by beef or contamination with bovine feces during processing has often been suspected. It is believed that *E. coli* O157:H7 contamination of foods occurs from the intestinal tract of healthy cattle during slaughter and processing (Wells et al., 1991; Wang et al., 1996). Cross-contamination in abattoirs (Bouvet et al., 2001; Warriner et al., 2002) and other food processing plants (Beuchat and Ryu, 1997; Warriner et al., 2002) have been reported to be responsible for several outbreaks of *E. coli* O157:H7. *E. coli* O157:H7 is known to produce extracellular polymeric substances (EPS) and form biofilms on food processing surfaces (Junkins and Doyle, 1992; Dewanti and Wong, 1995; Ryu et al., 2004). Microorganisms are known to be more resistant to removal from foods and food contact surfaces in processing plants and to inactivation by sanitizers when contained in a biofilm than when dispersed in a liquid medium (Kumar and Anand, 1998; Carpentier and Cerf, 1992).

The stated objectives for this work were:

1. To establish a GFP-labeled *L. monocytogenes* and *E. coli* O157:H7 system.
2. To determine the degree of *E. coli* O157:H7 cross-contamination of raw beef from a contaminated meat grinder during grinding.

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3. To determine the degree of *L. monocytogenes* cross-contamination of RTE beef from a contaminated meat slicer during slicing.
4. To assess the degree of transfer of *E. coli* O157:H7 and *L. monocytogenes* from contaminated stainless-steel surfaces following cleaning and sanitizing.

Methodology

Bacterial strains and culture conditions

L. monocytogenes V7 was cultured at 37°C in brain heart infusion (BHI) broth and *E. coli* O157:H7 505B was cultured in tryptic soy broth supplemented with 0.5% yeast extract (TSBY). Plating and enumeration of *L. monocytogenes* V7 was done on modified Oxford (MOX) agar, while those for *E. coli* O157:H7-GFP was done on plate count agar supplemented with 50 µg/ml ampicillin and 60 µg/ml L(+)-arabinose (PCAA).

Construction of GFP-labeled strains

Numerous attempts were made to construct L. monocytogenes strains that express the GFP gene using plasmids pAMGFP3 and pAMGFP8 that we obtained from Dr. Nancy Freitag (University of Illinois at Chicago). However, the cells did not excrete the protein extracellularly, thus fluorescence was not observed by plating as we had originally proposed. Because of this, a non-GFP labeled *L. monocytogenes* strain V7 was used for the study. On the other hand, we successfully constructed a GFP-labeled *E. coli* O157:H7 strain using a commercial kit (Bio-Rad) and this strain was also added in this study. GFP-labeled *E. coli* O157:H7 cells were propagated in TSBY broth containing 50 µg/ml ampicillin (TSBYA). Because of the GFP phenotype, samples were plated on PCAA instead of a more selective medium, such as MacConkey–sorbitol agar, allowing for more of the target pathogenic cells to be recovered. Typical colonies brightly fluoresced green when observed under long wave UV light (Figure 1), making colony enumeration easy and accurate on PCAA.

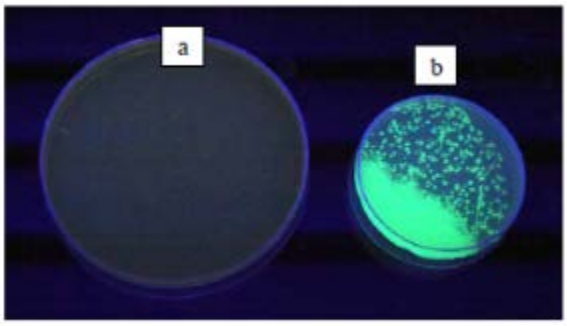

Figure 1. ***E. coli*** **O157:H7 wild type strain (a) compared with GFP-modified variant strain (b) under UV illumination.**

A. Transfer of E. coli O157:H 7-GFP between meat grinder and raw beef

Preparation of inoculum

E. coli O157:H7-GFP was inoculated into 10 ml TSBYA and grown overnight at 37°C under static conditions. Freshly grown cells were inoculated at a concentration of 2% into 100 ml TSBYA broth and grown for 12 h at 37°C with shaking. Cells were pelleted by centrifugation and washed twice in normal saline. Cell pellets were resuspended in 50 ml normal saline to a final concentration of 109 CFU/ml. The cell suspension was used as the inoculum for the grinder.

E. coli *O157:H7-GFP inoculation and meat grinding*

Different components (sausage stuffing funnel, mincing chamber, blade and meat plate) of the meat grinder (Model JR-12) were equally sprayed with 10 ml of 109 CFU/ml *E. coli* O157:H7-GFP using standard plastic bottle sprayers as depicted in Figure 2 below. The sprayed surfaces were allowed to dry for 1 h at room temperature. Raw boneless chuck

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roast beef, purchased from a local grocery store, was used for grinding. Each package of beef (1.5 kg on average) was cut into 4 pieces (approximately 3/8 kg each) and ground. An interval of 30 min was allowed between the grinding of each set of four pieces of beef. The first and last piece of beef from each package that were ground, were selected as samples for plate counting.

Figure 2. Meat grinder parts inoculated with *E. coli* O157:H7-GFP.

Cell enumeration
Twenty-five grams of ground beef sample were transferred into a sterile Whirl-Pak bag, combined with 225 ml peptone water and homogenized for 2 min in a stomacher. Appropriate dilutions of each sample were spread-plated on PCAA.

B. Transfer of L. monocytogenes *between meat slicer and RTE beef*

Preparation of inoculum
Freshly grown *L. monocytogenes* V7 (2 ml) was transferred into 100 ml BHI broth and grown for 12 h at 37 °C with shaking. Cells were pelleted by centrifugation at 3700 rpm for 25 min and washed twice in normal saline. The cell pellets were resuspended in 50 ml saline to a final concentration of 108 CFU/ml and used for inoculating the meat slicer.

Culture inoculation and meat slicing
Different components (blade and meat plate) of the meat slicer (General Slicing, Murfreesboro, TN) were equally sprayed with 10 ml of L. monocytogenes V7 using standard plastic bottle sprayers. The sprayed culture was allowed to dry at room temperature for 30 min. Cooked roast beef (Charlie's Pride Meat, CA) from a local grocery store was used for slicing. Each sliced piece was around 200 cm2 in area, 4 mm in thick and 30 kg in weight. The first two pieces in every eight pieces sliced were selected for plating and enumeration of cells on MOX agar.

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Figure 3. Meat slicer parts inoculated with *L. monocytogenes*.

C. Survival of E. coli *O157:H7 and* L. monocytogenes *on stainless steel surfaces*

A stainless steel table was prepared by dividing its top surface into 25 x 25 cm^2 areas for pathogen survival experiments. Before use, the entire surface was disinfected with 5% Pine-Sol disinfectant and wiped with paper towels. Prior to artificial contamination of cultures, the surfaces were sprayed with 90% (v/v) ethanol and wiped dry with paper towels. Cell suspensions of (109 CFU/ml *E. coli* O157:H7-GFP or 108 CFU/ml *L. monocytogenes*) was prepared as described above. Each 25 x 25 cm^2 surface was sprayed with 2 ml of each cell suspension and allowed to dry at room temperature for 30 min. Each surface was sampled by a sterile sponge mixed in 50 ml peptone water and 10 ml neutralizing buffer at 30 min, 4 h, 8 h and 24 h. Viable counts of *E. coli* O157:H7-GFP and *L. monocytogenes* were determined using PCAA and MOX agar, respectively.

D. Effects of detergent and bleach on survival of E. coli *O157:H7-GFP*

The stainless steel table was prepared and inoculated with *E. coli* O157:H7-GFP as described above. The inoculated stainless steel table was wiped down with 90% Chlorox bleach and 82°C 5% detergent (Zep Manufacturing Co., Atlanta, GA), as would typically be done in a processing or retail establishment. The surfaces were sampled using sterile sponges, mixed in 50 ml peptone water and 10 ml neutralizing buffer, and dilutions were plated on PCAA. The effect of detergent and bleach at removing attached cells was determined by counting fluorescent colonies of *E. coli* O157:H7-GFP under UV light.

E. Effects of cleaners on cross-contamination via stainless steel surface to raw beef

This experiment was carried out to determine the transfer rates of the pathogens from artificially contaminated stainless steel surfaces to raw beef. Stainless steel surfaces (25 x 25 cm^2) were artificially contaminated with *E. coli* O157:H7-GFP suspension as described above. The contaminated surfaces were allowed to dry at room temperature for 30 min. Three different treatments (wiping with 90% bleach, wiping with 82°C 5% detergent and no wiping) were applied. To simulate subsequent contamination of food, pieces of raw beef (1 cm thick, 2 x 2 cm^2) were aseptically cut using sterile metal meat corers and pressed on the contaminated table surfaces with a pressure of about 500 g (weight of the beef) a piece. Numbers of cells transferred to the raw beef were determined by suspending 25 g beef in 225 ml peptone water and subsequently homogenizing in a Stomacher for 2 min. Appropriate dilutions were spread-plated on PCAA. The transfer rates were calculated based on the numbers of target microorganisms present on the surfaces that were recovered on PCAA, using the formula:

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% transfer rate = Nf / Ns x 100%
Where: Nf = CFU recovered from beef; Ns = CFU on surface recovered by PCAA.

Findings

A. Transfer of E. coli *O157:H7 from contaminated meat grinder to raw beef*

Cross-contamination of *E. coli* O157:H7-GFP between an artificially inoculated meat grinder and raw beef during grinding is shown in Figure 4. *E. coli* O157:H7-GFP suspensions with a concentration of 1.0 x 109 CFU/ml were used as inocula. The results indicated that the numbers of *E. coli* O157:H7-GFP transferred from the meat grinder to raw beef decreased gradually during the grinding process. The numbers of *E. coli* O157:H7-GFP from the first ground beef sample declined more than 2 log units compared to the initial number count of 9 log CFU/ml initial inoculum. After about 3 h or 7.5 kg of beef had been ground, the number of *E. coli* O157:H7-GFP from the ground beef reached below the detection level of the plate count which is less than 2,500 CFU/g. Samples that had zero colonies from PCAA were enriched in peptone water for 24 h and these tested positive for *E. coli* O157:H7-GFP by plate streaking on PCAA. These results indicated that the pathogen still remained in the ground beef sample after 3.75 h and grinding of a total of 9 kg of beef even though no colonies were detected on agar plates due to its natural detection limit.

B. Transfer of L. monocytogenes *from contaminated meat slicer to RTE beef*

L. monocytogenes contamination is generally suspected to occur at the post-processing stage, especially for RTE foods. The cross-contamination of *L. monocytogenes* between an artificially inoculated meat slicer and roast beef during slicing is shown in Fig 5. *L. monocytogenes* V7 suspensions with a concentration of about 1.0 x 108 CFU/ml were used to inoculate the slicer. After drying for 30 min, the number of *L. monocytogenes* declined by about 2.5 log CFU/ml. The number of *L. monocytogenes* from the sliced roast beef samples decreased further during the slicing process. When around 1.2 kg of roast beef had been sliced (approximately 20 min), the bacterial numbers from the sliced beef was below our detection limit of 2.5 x 103 CFU/g. After 2.2 kg of roast beef had been sliced (approximately 40 min), no colonies could be found on MOX agar plates (data not shown). From about 40 min onwards, standard AOAC Listeria enrichment and detection protocols were followed to enrich the samples at 35oC in UVM broth for 26 h, followed by a 24-h enrichment in Fraser's broth. Even though these samples did not produce any colonies on MOX agar plates, they were positive for Listeria after the enrichment steps. This indicated that L. monocytogenes still remained in the sliced roast beef even after 40 min of slicing, even though they were undetectable by plating without enrichment on MOX agar.

C. Survival of E. coli *O157:H 7 and* L. monocytogenes *on stainless steel surfaces*

The survival of *E. coli* O157:H7 and *L. monocytogenes* on stainless steel surfaces is shown in Fig. 6. The contamination level was about 1 x 108 CFU/ml for *L. monocytogenes* and 1 x 109 for *E. coli* O157:H7. The cell number decreased below the detection limit after 24 h. The number of bacteria declined rapidly by about 4 log CFU/ml within 4 h. Compared to *L. monocytogenes*, numbers of *E. coli* O157:H7 decreased more rapidly. Differences in cell surface structures between gram negative and gram-positive bacteria may be responsible for the difference in cell decline rate. Cell surface characteristics, such as flagella, pili and extracellular polysaccharides have been reported to affect the adhesion and survival of bacteria (Peng et al., 2001). Further, clumping of cells might confer some protection to the innermost cells in the clump against drying (Tebbutt, 1991). At any rate, our results highlight the fact that these two pathogens might remain viable on air-dried stainless steel surfaces for considerable periods of time. The presence of such viable pathogens on beef processing equipment obviously poses a recontamination hazard and health risks to consumers.

D. Effects of cleaners on transfer of E. coli *O157:H7 from a contaminated stainless steel surface to raw beef*

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The transfer rates of *E. coli* O157:H7 from stainless steel surfaces to raw beef are shown in Table 1. The transfer rates were based on the ratio between the numbers of the pathogen that were recovered from stainless steel surfaces and those recovered from raw beef samples. Our results indicated a transfer rate about 50% to 80%. The general obstacle for microbial cross-contamination studies is usually the interference of the natural microflora from food samples and food processing equipment, in which case transfer rates of more than 100% are often achieved. By using the GFP-labeled *E. coli* O157:H7, this problem was easily solved and more accurate results were obtained.

Implications

Although we were unsuccessful at constructing a GFP-labeled *L. monocytogenes*, results using a wild type variant of the pathogen still showed the ability of transfer of this organism from contaminated slicer to roast beef during slicing for up to 40 minutes. The rate of decline of *E. coli* O157:H7 transferred from a contaminated meat grinder was slower than that for *L. monocytogenes* on a meat slicer. However, this may be attributed to differences in structural components of the meat grinder and slicer. The results of this study demonstrate the importance of cleaning and sanitization of beef processing equipment and confirms the importance of disassembling, cleaning and sanitization of equipment before, during and after processing. The use of the GFP gene as a marker to monitor the cross-contamination pattern in this study indicates the usefulness of such a system in these types of studies.

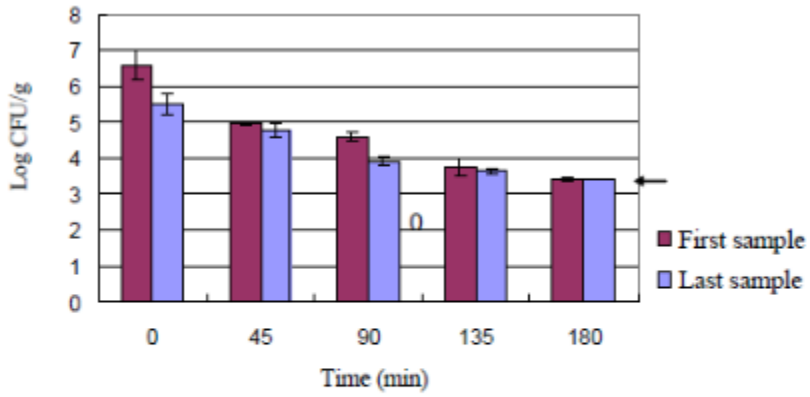

Figure 4. **Cross contamination of raw beef by meat grinder inoculated with *E. coli* O157:H7. Bars indicate standard deviations from 2 replications. The arrow indicates the detection limit of sampling.**

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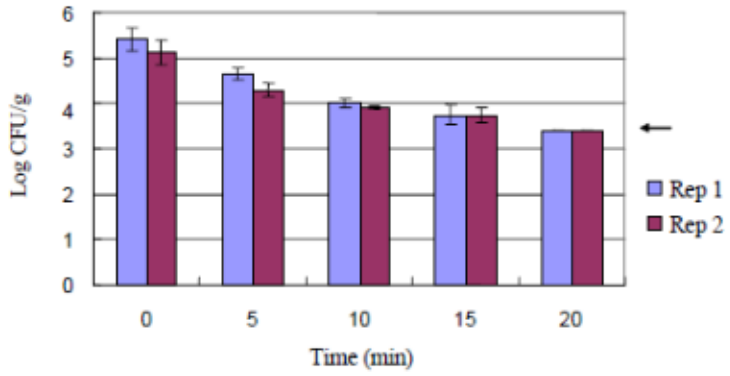

Figure 5. **Cross contamination of RTE sliced roast beef by meat slicer inoculated with *L. monocytogenes*. Bars indicate standard deviations from 2 replications. The arrow indicates the detection limit of sampling using MOX agar plates.**

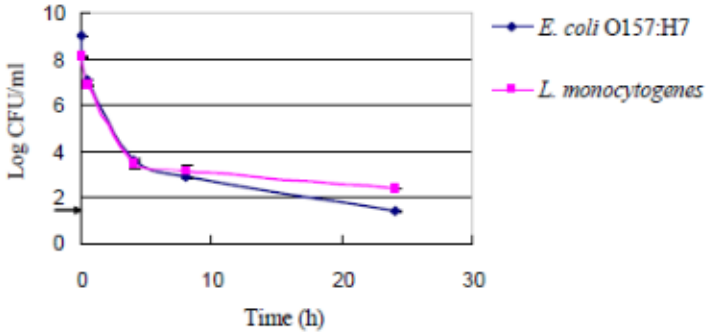

Figure 6. **Survival of *E. coli* O157:H7 and *L. monocytogenes* on stainless steel surfaces. Bars indicate standard deviations of results from 2 replications. The arrow indicates the detection limit of sampling for both pathogens.**

Table 1. Transfer rate of *E. coli* O157:H7 from stainless steel surface to raw beef under different cleaning and sanitizing treatments.

	Cell number (CFU/ml)		Transfer rate (%)
Treatment	Surface	Raw beef	Surface to raw beef
No treatment	(2.5±0) x 10^7	(2.1 ± 0.15) x 10^7	85.6±6.0
90% bleach	(2.4 ±0.99) x 10^4	(1.4± 0.42) x 10^4	59.5±11.4
5% hot soap	(2.7 ± 0.21) x 10^4	(2.0±0.18) x 10^4	74.3±9.5

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