SIMULATION OF CONTAMINATION THROUGH THE POST-HARVEST ENVIRONMENT USING SURROGATE ORGANISMS

A Thesis

by

MARIANA VILLARREAL SILVA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Food Science and Technology

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Approved by:

Co-Chairs of Committee,	Margaret D. Hardin
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ABSTRACT

Simulation of Contamination Through the Post-Harvest

Environment Using Surrogate Organisms. (August 2010)

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Co-Chairs of Advisory Committee: Dr. Margaret D. Hardin
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The beef industry has made tremendous strides in reducing pathogen contamination on carcasses. Multiple antimicrobial interventions have been validated for their use during harvesting. Information in regards to cross-contamination with pathogens in the post-harvest environment is limited. Surrogate microorganisms for enteric pathogens are commonly used to validate antimicrobial interventions and might allow for the simulation of cross-contamination through the post-harvest environment.

The purpose of this study was to determine how the post-harvest environment impacts the direct and indirect transmission of pathogens. This was achieved by using fluorescent protein-marked surrogate strains of *Escherichia coli* O157:H7 and *Salmonella* spp. from inoculated carcasses to the adjacent ones and to the equipment and facility in three different abattoirs.

Thirteen hide-on carcasses were inoculated using a gelatin-based slurry containing three nonpathogenic fluorescent protein-marked strains of *E. coli* biotype I. In order to determine direct and indirect cross-contamination, inoculated and adjacent carcasses were sampled (300 cm^2) during the harvesting process at different stages: after

hide opening (AHO), prior to evisceration (PE), after evisceration (AE), after splitting (AS), and after final intervention (AFI). Environmental samples consisting of the floor, walls, and air were tested as well as personal equipment including gloves, boots, and aprons. Equipment including hand knives, air knives, meat hooks, hide puller and split saw were also sampled.

Results showed evidence of cross-contamination between inoculated carcasses and the adjacent non-inoculated ones for all abattoirs. Although this occurred in all abattoirs, surrogate counts on carcasses were below detectable levels (<1.4 log CFU/cm^2) after antimicrobial interventions. Surrogates were found in low levels for all environmental samples. However surrogate counts from equipment such as knives, split saws, meat hooks, and hide puller were more frequently detected (15%) than those found on the floor, air and walls samples (10%). In the case of aprons, boots, and gloves, the prevalence of countable surrogate samples was 7%.

DEDICATION

To my family for their invaluable love

and moral support despite the distance

To Fabian, for his encouragement during

the progression of this thesis

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INTRODUCTION

Escherichia coli O157:H7 and *Salmonella* spp. are among the major foodborne pathogens reported in various countries. *Salmonella* spp. has been found in cattle feed, water, dust, and feces, and some studies have documented the presence of *E. coli* O157:H7 in feces at the feedlot. Several studies have demonstrated that hides are one of the main carriers of pathogens such as *E. coli* O157:H7 and *Salmonella* spp. which can be transferred on and between carcasses during the beef harvesting procedures. This transmission of pathogens is considered a direct way of contamination from the hide onto the carcass. The plant environment may also serve as an indirect source of contamination; from the hide to surfaces, were these pathogens can survive and being further transferred onto the carcass. The beef industry has made tremendous strides in reducing the presence of these pathogens in the post-harvest environment using multiple physical and chemical antimicrobial interventions developed and validated for its use during the beef slaughter process.

Surrogates are nonpathogenic microorganisms with similar growth, survival, and resistance properties to pathogens. Surrogates are used for a variety of purposes in food systems including the validation of the effectiveness of different antimicrobial control measures. As part of a research project between Texas A&M University (College Station, TX) and Iowa State University (Ames, IA), marker organisms were previously isolated from beef hides and identified as nonpathogenic *E. coli* biotype I strains. These

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This thesis follows the style of *Journal of Food Protection.*

surrogates are beneficial for in-plant studies either in validating carcass interventions or studying the potential of pathogens to colonize plant niches. In a previous study, these bacteria were transformed in the Food Microbiology Laboratory at Texas A&M University (College Station, TX) to produce yellow, red or green fluorescent proteins as well as resistance to ampicillin (AMP) (100 μg/liter) which is beneficial for selective purposes during laboratory testing. These marker organisms demonstrated identical thermal and acid resistance to *E. coli* O157:H7 and *Salmonella* spp.

In the present study, three of these novel marker organisms were utilized to trace potential cross-contamination of enteric pathogens from contaminated hides to carcasses and to the plant environment (walls, floors, general equipment, and personal equipment) and from contaminated hide-on carcasses to non-inoculated carcasses in close proximity during the post-harvest processes. The purposes of this study were to determine how the post-harvest environment impacts direct and indirect transmission of pathogens using fluorescent surrogates of *E. coli* O157:H7 and *Salmonella* spp., to determine if cross-contamination occurs from the inoculated hide to its carcass, to the adjacent non-inoculated carcass as well as to the environment. Determination of the differences in the level of contamination related to the abattoir, equipment, and processing step in order to identify areas and conditions that increase the risk of direct and indirect pathogen contamination through the post-harvest environment was also evaluated.

REVIEW OF LITERATURE

Characteristics of *Salmonella* spp.

All serovars of *Salmonella* are regarded as human pathogens, though they differ in the severity of the disease *(2)*. Among the Gram negative rods that cause foodborne illness gastroenteritis, the most important is the genus *Salmonella.* The first reference made to this organism later named *Salmonella* was in reference to the description of typhoid fever, by Bretonneau in 1829 *(12)*. The *Salmonella* genus name was not assigned until 1900 by Lignières who gave this to honor D.E. Salmon, one of the first scientists that described *Salmonella* spp. as the causing agent of hog cholera in pigs *(2)*. They are widely distributed in nature, with humans and animals being their first reservoirs *(38)*.

The genus belongs to the Enterobacteriaceae family. The *Salmonella* cells are small, rod-shaped, non-sporing, facultatively anaerobic, cytochrome oxidase negative, catalase positive, indole negative, Voges-Proskauer negative, methyl red positive, and can utilize citrate as a sole carbon source. Although non-motile strains have been identified, *Salmonella* are generally motile with peritrichous flagella. *(10, 38)*. *Salmonella* are unable to use sucrose and lactose but ferment glucose with production of both acid and gas. *Salmonella* require pH for growth between 6.6 and 8.2 but can survive in pH as low as 4.05 *(38)*. They are mesophilic organisms with optimum temperature to grow of 37^{o}C but growth has been reported above 5^{o}C up to 40^{o}C *(2)*. The minimum water activity (a_w) required to grow is approximately 0.93 but cells survive well in dried

food, the survival rate increasing as the a_w is reduced *(2)*. They can survive to foods with salt levels up to 9% and are easily destroyed by milk pasteurization temperatures. Even so, *Salmonella* can survive to high temperatures for prolonged times if the media provides certain protection, as in high-fat products like chocolate sauce and peanut butter *(2, 62)*.

Classification

The genus *Salmonella* are divided into two different species, *Salmonella enterica* and *Salmonella bongori* based on DNA/DNA hybridization. *S. bongori* has been considered unimportant as a cause of human infection and accounts for less than 1% of *Salmonella* serovars *(2)*. The species *S. enterica* is divided into six subspecies, *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica (57).* Serovars belonging to *S. enterica* susp. *enterica* are designated by a name, related to the region where the serovar was first isolated. The subspecies are divided into various serovars or serotypes using the Kauffman-White serotyping scheme which has proven to be the most useful technique for differentiating within the genus. This scheme describes organisms on the basis of their somatic (O) and flagelar (H) antigens, and by capsular (Vi) antigens. In 1940 the scheme contained 100 serovars and actually this number has risen to more than 2,500 serovars *(2, 38, 57)*. Although, there is a high number of serovars identified; only a few have been related to human and animal disease.

Virulence factors

For epidemiological purposes, *Salmonella* serovars can be classified in three groups. The species that affects humans is *S. enterica* which can cause the illness

salmonellosis and includes organisms such as *S*. typhi, *S*. Paratyphi A, *S*. Paratyphi C as agents of typhoid and paratyphoid fevers which are the most severe of *Salmonella*-caused disease *(38)*. The second group is composed of host-adapted serovars including *S*. Gallinarum (poultry), *S*. Dublin (cattle), *S*. Abortus-equi (horses), *S*. Abortus-ovi (sheep) and *S*. Cholerasuis (swine). The third group is formed by serovars without particular host preference and these are pathogenic for humans and animals. This group includes most foodborne serovars such as Typhimurium, Enteritidis, Newport, Heidelberg, Muenchen, and Montevideo *(18, 38)*.

Salmonella causes disease in both animal and humans. The genes encoding for resistance are mainly located in plasmids. Some serotypes of medical importance such as mentioned before are known to have plasmids that encode for virulence factors that contribute to the pathogenicity of *Salmonella* such as fimbriae, serum resistance, and other factors *(19)*. Isolates from these serovars more commonly express resistance to multiple antibiotics, third generation cephalosporins and aminoglycosides *(18, 19)*. Furthermore, many *Salmonella* contain some genes that encode for pathogenic abilities such as adhesion, invasion and infection *(19, 36)*. Stressful environments located within the host such as low pH in the stomach and low oxygen concentration in the intestinal tract may cause the expression of these particular genes that help *Salmonella* to invade the intestinal epithelium and infect the host *(31)*. The serovars Typhi and Paratyphi A, B and C do not have an animal reservoir; the remaining serovars are considered zoonotic and are commonly known as non-typhoidal *Salmonella (31)*.

Salmonellosis

Salmonellosis is the name given to the infection caused by *Salmonella*. This disease is the second leading cause of foodborne illness in U.S. and one of three main causes of deaths originated from foodborne illness *(49)*. The disease has been reported in humans and almost all animals throughout the world. Over 1.4 million cases of salmonellosis occur per year in the U.S. associated with non-typhoidal *Salmonella*, resulting in 168,000 visits to physicians, 15,000 hospitalizations and 580 deaths annually. Other industrialized countries have similar rates whereas under-developed countries might have an even higher incidence of salmonellosis in humans although very few countries report data of the incidence of this disease *(49)*.

Salmonella is typically acquired through consumption of contaminated food or water. After passage through the stomach, bacteria colonize the intestine, interacting with and translocating across the intestinal epithelium via three routes: active invasion of enterocytes; invasion into specialized epithelial cells called M cells, which sample antigens from intestinal lumen; and through dendritic cells that intercalate epithelial cells by extending protrusions into the gut lumen *(33)*. The infection dose under controlled conditions is approximately 10^5 CFU, but consumption of 50 to 100 cells in food products may cause disease *(38)*. Interaction of *Salmonella* with the epithelium and the underlying resident immune resident cells, and the production of both enterotoxin and cytotoxin, leads to the production of inflammatory cytokines and chemokines, which subsequently recruit and activate other immune cells such as neutrophils, macrophages, dendritic cells and T and B cells *(33)*.

The clinical manifestation of the disease may differ widely. Salmonellosis leads to symptoms as self-limited gastroenteritis, severe gastroenteritis, fever, bacteremia, abortion, meningitis, respiratory disease, cardiac disease, osteomyelitis and severe septicemia *(7, 8, 30, 37, 77)*. The infection causing moderate to severe gastroenteritis is characterized by nausea, vomiting and watery diarrhea. Abdominal cramps, persistent and spiking fever may also appear. The incubation period ranges from 5 h to 7 days, but clinical signs usually begin 12 h to 36 h after ingestion of contaminated products *(31)*. The illness usually lasts 4 to 7 days and is self-limiting in healthy individuals. Elderly, infants and immunecompromised people are more likely to have severe illness *(17)*. Symptoms may disappear but the recovered patient may shed the microorganism in feces for long periods. Non-typhoid salmonellosis can later give rise to chronic disease, including localized infections in specific tissues or organs and reactive arthritis, as well as neurological and neuromuscular illness *(31)*. Although salmonellosis might have only mild and self-limiting presentation, dehydration and death may occur *(31)*. The average mortality rate is 4.1% varying from 5.8% during the 1st year of life, to 2% between the first and the 50th year, and 15% in people over 50 years. Among the different *Salmonella* serovars, *S.* Cholerasuis has been reported to produce the highest mortality rate of 21% due to a higher frequency of septicemia cases *(38)*.

Epidemiology

Salmonella is the most frequently described cause of foodborne illnesses in the world*(1)*. The principal mode of infection is the consumption of contaminated food items. The food vehicles found in different outbreaks include eggs and other poultry

products, milk and dairy products, orange juice, tomatoes, alfalfa sprouts, cantaloupes, chocolate, peanuts and peanut butter, spices, and raw to undercooked ground beef *(38)*. Ground beef patties have been implicated in outbreaks in U.S., Canada and Europe. According to the Centers for Disease Control and Prevention (CDC) in the U. S. for the period of 2001-2005 *Salmonella* was the most reported cause of foodborne illnesses of bacterial origin with 127 outbreaks and more than 3,250 confirmed illness cases. The most common serovars associated with these outbreaks were *S*. Enteritidis, *S*. Typhimurium, *S*. Newport and *S*. Heldelberg *(16)*.

The primary habitat of *Salmonella* is the intestinal tract of animals such as birds, reptiles, wildlife and farm animals, humans and occasionally insects. The organism may also be found in polluted water *(38)*. In cattle, *Salmonella* have been isolated from feces, hides, carcasses, feedlot, and environment related to herds with very variable prevalence reported worldwide. Some studies have reported *Salmonella* prevalence of 2 to 50% and from 0% to 90% in hide cattle in herds *(1, 31, 73, 75)*. Infected cows can remain asymptomatic carriers of these microorganisms for life. Bacteria may be shed from cattle carriers in feces and milk after stress related to poor hygiene, insufficient diet, transportation and lactation. Shedding in dairy herds lasting 50 to 391 days has been documented. Cows shed the bacteria more frequently than calves *(21)*. These healthy cattle can serve as reservoir of *Salmonella* and a source of contamination of the carcass *(1)*. One of the principal ways of contamination of carcasses is via fecal or intestinal contact as in beef carcasses. Another route of contamination is when bacteria are directly

introduced to the food product, for example in eggs. These can become contaminated by transovarial transmission or during the egg washing procedures *(31, 38)*.

Characteristics of *E. coli*

In 1885, *E. coli* was first isolated by the bacteriologist Theodor Escherich from human feces. Although most strains of *E. coli* have been described as harmless commensal organism, they can be an opportunistic pathogens in immunecompromised patients *(2)*. The organism is an inhabitant of the human digestive tract and can also be found in other warm blooded animals. *E. coli* has been used as an indicator of fecal contamination in food and water due to its common occurrence in feces and its survival in water *(2, 38)*. The genus *Escherichia* is part of the family Enterobacteriaceae and includes six species: *E. hermanii, E. fergusonii, E. vulneris, E. blattae, E. albertii,* and *E. coli (2)*.

E. coli is a non-sporing, Gram-negative, facultative anaerobic, mesophilic microorganism. The bacterial cell has a rod shape and flagella, if present, are in a peritrichious arrangement *(2, 38)*. This organism is catalase positive, cytochrome oxidase negative, does not liquefy gelatin, indole positive (with exception of biotype II which reacts negative), Voges-Proskauer negative, and methyl red positive. Strains are unable to utilize citrate as their sole carbon source *(2, 41)*. Most strains ferment glucose with production of acid and gas, and lactose is fermented with production of both acid and gas by most strains. *E. coli* grows at temperatures ranging from 7-10^{o}C and up to 50^{o}C. The optimum temperature for growth is 37^{o}C. These bacteria do not have a marked heat resistance, with a D-value at 60^{o} C of 0.1 min, but can survive refrigeration

and freezing temperatures for prolonged periods *(2)*. The optimal pH for growth is 7.0 but has been shown to grow at pH as low as 4.4 if all other conditions are in optimal ranges. Under optimum conditions the minimum a_w for growth is 0.95. *(2)*.

***Serology of* E. coli**

Kauffman *(41)* established the basis for the serological studies of the lipopolysaccharide somatic-O antigen, capsular-K antigen and flagellar-H antigenic reaction of the *coli* group *(41)*. In his review, the relationship of the K antigen with the strain and, the O antigen classification group and its necrotizing hemolytic and toxicity virulence factors were described. Kauffman continued to describe some strains of the coli group that may play a particular role in diseases as appendicitis, peritonitis and pyelitis *(41)*. The classification of the coli group was difficult to identify due to the H antigen was not always apparent as well as the isolation of highly motile cultures was infrequent. Consequently only 22 H antigens were considered *(41)*.

This classification system is currently in use. First, serogroups are defined by O antigens and then subdivided into serotypes on the basis of H antigens. Strains of each category of pathogenic *E. coli* tend to fall within certain O:H serotypes. This method plays an important role in the detection of pathogens and for epidemiological studies *(2)*. In 1988, 171 O serogroups and 56 H types were recognized *(44)*.

***Pathogenic* E. coli**

Some *E. coli* help to maintain gastrointestinal functions, this are known as generic *E. coli,* or biotype I *E. coli*, while other strains are major causes of different syndromes of diarrheal disease and are called pathogenic *E. coli (44)*. Some of these

pathogenic *E. coli* share some virulence characteristics such as plasmids encoding for critical virulence factors, the particular interaction with the intestinal mucosa, the production of enterotoxins or cytotoxins, and a clear tendency to fall in similar O:H classification *(44)*.

There are six recognized virulence groups for *E. coli*: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), diffuse-adherent (DAEC) and enterohemorrhagic (EAEC) *(44)*. The EPEC group is recognized as the main cause of infantile diarrhea. These strains do not develop the heat-labile and heat-stable enterotoxins found in ETEC. Theydo not exhibit the invasiveness of EIEC. They do produce a toxin almost identical to Toxin 1 from *Shigella dyseteriae* which causes diarrhea by destruction of microvilli without further invasion *(44)*. Common symptoms of EPEC infection include fever, malaise, vomiting and diarrhea with an elevated amount of mucus but without blood *(44)*. In some strains within the group of EPEC, two patterns of adhesion have been identified. The fist pattern corresponds to EAggEC which forms aggregates similar to "stacked bricks" when they adhere to HEp-2 cells. This adherence and ability to form aggregates is related to the production of fimbriae and an outer protein by means of the expression of a 60-MDa plasmid *(23, 50)*. EAggEC do not cause lesions as EPEC and are negative to DNA probes for EPEC, EIEC and ETEC. The second pattern of adherence in HEp-2 cultured cells is known as diffuse-adherent, which leads to the differentiation of these strains from other EPEC. Compared to other diarrhea-causing *E. coli*, DAEC and EAggEC have been only recently described. The epidemiology, risk factors, and pathogenesis are still in early stages of

investigation *(65)*. Strains belonging to the ETEC group are the major cause of infant diarrhea in less developed countries, and are a common cause of traveler's diarrhea *(43)*. The syndrome of ETEC infection includes watery diarrhea, nausea, abdominal cramps and a low-grade fever. The mechanism of infection includes fimbrial colonization and expression of LT toxin, ST toxin or both *(3, 43)*. The EIEC group is formed by strains capable of causing invasive dysenteric diarrhea. This strains are different in serotype to ETEC and EPEC, and are more similar to the *Shigella* virulence plasmid *(43)*. Some authors have suggested that all *Shigella* groups and EIEC strains descend from a common *E. coli* antecessor due to the similarities found for their virulence plasmid and the clinical manifestation of shigellosis and EIEC *(28, 66)* . As with *Shigella*, EIEC invade epithelial cells with a preference for colonic mucosa *(43)*. EIEC infection frequently causes watery to bloody diarrhea and vomiting *(66)*. The EHEC group is very similar to EPEC due to the fact they posses common genes, the type of attachment, and epithelial effacement lesions. Differences between EPEC and EHEC are evident in that EHEC strains only invade the large intestine and produces large amounts of Shiga-like toxins. *E. coli* O157:H7 is the principal member for this group.

Characteristics of* E. coli *O157:H7

E. coli O157:H7 first gained public recognition as an important human foodborne pathogen in the U.S. in 1982 following two outbreaks of hemorrhagic colitis in Oregon and Michigan associated to the consumption of undercooked beef hamburger patties from a fast food restaurant chain *(60)*.

Virulence factors

Studies within the time of the first outbreak described *E. coli* O157:H7 as being different from EIEC strains and clinical manifestations were different from EPEC. Fever was not present as in EIEC infection and profuse bloody diarrhea resembling gastrointestinal bleeding has not being found in EIEC or EPEC infection symptoms *(60)*. Further studies described the expression of toxins similar to *Shigella dysenteriae* (Shiga-toxin). These toxins were different from any previously described *E. coli* toxins and are named Stx1 and Stx2 *(38)*. After this, the EHEC group was recognized, placing *E. coli* O157:H7 as the most important strain of the group *(44)*. *E. coli* O157:H7 toxins are toxic to Vero (African green monkey kidney) tissue cultured cells and lethal to mice. Other verotoxin-producing *E. coli* (VTEC) strains have been related with hemorrhagic colitis and hemolytic uremic syndrome (HUS); however, *E. coli* O157:H7 is currently recognized as the most common cause of VTEC-associated human illness *(25)*. Virulence genes of the pathogenic island on the chromosome of EHEC include the *eae* gene that encodes the intimin protein that is essential for attachment/effacement (A/E). The pathogenicity of EHEC is related to the Stx toxins, endotoxins, and host-derivated cytokines such as the tumor necrosis factor alpha (TNF-a) and interleukin-1 β. Toxins Stx1 and Stx2 inhibit protein synthesis in endothelial cells. The host receptor for these toxins is globotriasylceramide (Gb3). Human renal tissue contains large amounts of Gb3 and thus it is highly sensitive to the Stx toxins producing HUS symptoms such as hemolytic anemia, thrombocytopenia and acute renal failure *(38, 56)*.

Epidemiology

The occurrence of *E. coli* O157 within the food supply generates particular concern for both the public and the beef industry. The annual cost of illness in the U.S. due to O157 Shiga toxin producing *E. coli* (STEC) in 2003 was estimated at $405,000,000 including $370,000,000 for premature deaths, $30,000,000 for medical care and $5,000,000 in loss of productivity *(32)*.

E. coli O157:H7 infection can have different clinical manifestations. These differences are related to the patient, the dose of the pathogen and the infecting strain. The highest risk groups for infection are children under 5 years, elderly, and immunocompromised individuals *(34)*. *E. coli* O157:H7 infections are transmitted via three primary routes: directly from animals (farm animals, domestic pets, deer, sheep, dogs, wild birds), by person-to-person route such as day care centers and nursing homes, and from contaminated foods and water *(38)*. Because more foodborne outbreaks of EHEC syndromes have been linked to beef more than any other single food source, cattle have typically been considered the primary reservoir for *E. coli* O157:H7 in U.S. and it has been found in other ruminants in several countries *(25)*. Ground meat has been the principal vehicle of several outbreaks in humans *(56)*. Raw milk also has been related to outbreaks and HUS syndrome in humans and *E. coli* O157:H7 has been isolated from healthy dairy cattle *(74)*. Overall, weaned calves have a higher prevalence of EHEC strains in their feces than either calves or adult cattle, and this is due the immature rumen biota of weaned calves *(38)*.

According to Elder et al. *(26) E. coli* O157:H7 is transferred to beef via fecal contamination of carcasses during slaughter processing. *E. coli* O157:H7 is found in cattle gastrointestinal tract and excreted in feces and been isolated from 0.2 to 3% of fecal samples collected from healthy calves or cattle in the U.S., Canada, United Kingdom, Germany, and Spain *(38)*. Shedding of the bacterium varies by season, type of management production system, geography, diet, sex, age. Prevalence may also be related to the screening and isolation method used and its sensitivity *(5)*.

Cattle hides play an important role as source of *E. coli* O157:H7 and other STEC's. The relationship between the incidence of this pathogen on hides and the contamination of derived carcasses has been demonstrated *(26, 54)*. In Turkey, Akkaya et al. *(4)* found *E. coli* O157 in the environment, equipment and abattoir workers with frequencies of 0.31, 1.0, 1.42% respectively, in five commercial abattoirs. Bosilevac et al. *(11)* sampled 1995 hides and 1995 carcasses in 7 U. S. abattoirs finding prevalences of enumerable levels of *E. coli* O157:H7 and *Salmonella* of 12 and 36% in hides of stunned animals, and of 2 and 8% for carcasses on pre-evisceration process. Elder et al. *(26)* investigated cattle lots in Midwestern U.S. processing plants and found 72% of lots with at least one fecal sample positive for *E. coli* O157 and 38% of the same lots with at least one hide sample positive for this pathogen.

The pre-harvest environment

The manipulation of the cattle, during its life and until the harvesting process may define the final characteristics of the food products. Controlling zoonotic organisms in animal reservoirs have a substantial effect in the food safety system. The control and

care of healthy livestock will be reflected in the quality of product obtained after the slaughter *(20)*. The problems related to the infection with pathogens of cattle arise when environmental conditions expose them to the source of contamination. Circumstances such as the continuous contact with soil and contaminated water in addition to deficient husbandry practices increases the possibility of acquiring pathogens which survives in soil such as *Salmonella* and *E. coli* 157:H7 *(6, 20)*. The exposure of humans to cattle, livestock facilities or through the consumption of beef products have been identified as risk factors for this disease. Although carcass interventions in the slaughter plant have been demonstrated to be highly effective, the presence of *E. coli* O157:H7 and *Salmonella* related to meat products is still linked to foodborne illness and recalls each year *(40)*. For these reasons, the pre-harvest management practices have to focus on pathogen contamination with the purpose of reducing the occurrence of pathogens and to diminish the possibilities of in-plant contamination since a greater carriage of pathogens in live animals increases the risk of contamination during slaughter *(45)*.

Some management practices on farms have been studied, with varied results. Many researchers have reported that the initial sources of exposure to *E. coli* O157:H7 in cattle might possibly occur in the feedlot environment *(22)* . A reduction in *E. coli* O157:H7 has been recognized with a rapid dietary change from a grain a forage diet given to the cattle. However, this diet change must be added just prior to slaughter which leads to stress and therefore weight losses with the consequent negative economic effects for the producer *(15)*.

In some instances, animals can be tested to detect shedding of the pathogen shedding in order to prevent carrier cattle being introduced to the slaughter plant. However, this sampling practice is not viable since it would need to sample all cattle to detect only a few of those that are shedding the pathogen. This becomes the sample collection large, expensive and highly time-consuming *(29, 45)*. The reduction of the fecal contamination occurrence has demonstrated to reduce possible pathogen contamination but the chance of controlling cattle hides to become contaminated with feces is unfeasible *(63)*. Small and Buncic *(63)* have related the fecal contamination degree of holding pens and longer transportation periods to a higher fecal contamination of hides and with the increased presence of *E. coli* O157:H7. Muddy pen and crowded cattle lots may increase the possibility of contaminated cattle and carcasses related to these, but this is still being studied *(45, 64)*. Despite this, cattle standing in cleaner pens could not be consistently related with lower risk rate of pathogen contamination in carcasses *(29)*. Although cross-contamination of cattle may occur due to contact between cattle before slaughter, this contact between animals cannot be effectively controlled because common practices of transportation of cattle and loading of cattle allows animal-to-animal contact in normal conditions *(29)*.

Another practice applied to reduce *E. coli* O157:H7 is the use of probiotics such as *Lactobacillus* as a diet supplement. Younts-Dahl et al. *(76)* demonstrated the affectiveness of this practice. In this study *Lactobacillus acidophilus* reduced 57% less likely the occurrence of *E. coli* 157:H7 in cattle shedding than control.

Another pre-harvest practice based on the biological theory of competitive exclusion has been applied in order to reduce pathogens in cattle. This proposal describes that similar bacteria compete for the same niche inside the host. This competition of similar strains has been studied using artificially infected calves with pathogenic *E. coli*. Tkalcic et al. *(67)* demonstrated that generic *E. coli* can be used as a niche competitor for *E. coli* O157:H7 in cattle gastrointestinal tract with the reduction in *E. coli* O157:H7 shedding in treated cattle *(67)*. Vaccination is another tool that is still in investigation. Additional research is still needed however, the use of a vaccine has demonstrated the reduction of *E. coli* O157:H7 in cattle in experimentally infected animals *(48)*. The use of sodium chlorate to reduce *E. coli* O157:H7 is another way to reduce pathogens. When administered in feed and drinking water, it reduced populations of *E. coli* O157:H7 in the feces and in the intestinal content of cattle. This may help in reducing *E. coli* O157:H7 as well as other anaerobic facultative pathogens as *Salmonella (42, 45)*.

Regardless of problems associated with approval and implementation of pre-harvest interventions, some practices such as the supplementation with probiotics in feeds are currently in use *(45)*. However, the simple application of one or more of these practices cannot completely eradicate the problem. The widespread distribution of pathogens such as *E. coli* O157:H7 and *Salmonella* in the environment, the apparent re-infection of cattle, and wildlife animals serving as a reservoir makes this elimination of these pathogens unrealistic *(42)*.

The post-harvest environment

The prevalence of pathogens such as *E. coli* O157:H7 and *Salmonella* found on beef carcasses and the unpredictable results from pre-harvest practices to eliminate these pathogens highlight the importance to develop strategies for hazards management. The implementation of sanitary processing procedures helps to prevent cross-contamination within the abattoir environment *(24)*. The maintenance of Good Manufacturing Practices (GMP's) during all receiving, processing, packaging, and transporting is one of the most important factors associated with consumer protection *(20)*. One strategy implemented in cattle abattoirs is the reduction of visible contamination on the hides of cattle entering the slaughter facility with water washes. However, a visible clean hide might not necessary be pathogen free and could still result in a potential hazard for the cross-contamination on carcasses *(59)*.

Sources of contamination in the post-harvest environment

The risk analysis and the application of systems that controls hazards as the Hazard Analysis and Critical Control Point (HACCP) system have demonstrated their effectiveness as well-conducted controls inside the abattoirs to reduce the risk of contamination with pathogens *(20)*. The application of practices as GMP's and HACCP, an assessment of the possible sources of contamination is necessary to evaluate the correct application of safety measures and to conduct procedures that improve the effectiveness of these practices. As mentioned before, extensive studies have demonstrated that one of the main sources of contamination is the hide. Pathogenic bacteria can be transferred onto the meat during carcass dressing and processing at the

slaughter plant. Feces contaminating the hide are the principal transport vehicle. Elder et al. *(26)* reported a positive correlation between fecal and hide prevalence of *E. coli* O157:H7 and the subsequent contamination of carcasses with the bacteria following slaughter and processing. It is also likely that contamination spreads from animal to animal during transport and lairage, either directly through animal contact before slaughter or indirectly through contact with contaminated floors and other surfaces. During the slaughter process, carcass can become contaminated via carcass-to-carcass contact especially in the flank and rump areas, which are the most likely to be contaminated areas *(26, 63)*.

Contamination of carcasses during hide removal has been found to be the most important source of contamination for workers and equipment leading to cross-contamination *(4, 26, 47)*. Carcass contamination is likely to occur during the dehiding process for several reasons such as cuts made through the animal skin from outside dirty areas to inside cleaner areas, alternate use of hands for handling the hide and the carcass surface, and ineffective roll back procedure of the hide during hide removal. The slaughter and dressing processes can lead to contamination even under the best sanitary conditions *(47, 54)*.

Interventions to reduce occurrence of pathogens during the post-harvest environment are the most useful tools developed to control pathogen hazards and to reduce microbial loads in the carcasses at the slaughter plant. These interventions include the use of combined treatments to reduce the risk of pathogen presence in the meat. It may include but is not limited to the use of trimming, water washes, steam

pasteurization and steam vacuum, use of antimicrobial substances such as chlorinated compound and organic acids *(24, 27, 35, 68, 71)*.

Best practices in the post-harvest environment

The use of best practices and current technology lead to improve beef safety. The U.S. Department of Agriculture (USDA) has implemented several guidelines related to the reduction of visible contamination of carcasses which includes sanitation standard operating procedures (SSOP) and the implementation of a HACCP system, and must meet microbiological performance criteria for *E. coli* and *Salmonella* as a mean to verify the effectiveness of these sanitary procedures *(9)*. Microbial decontamination technologies include animal cleaning, chemical dehairing at slaughter, spot-cleaning of carcasses by knife trimming, the use of steam or hot water vacuuming, carcass spraying, washing, or rinsing before evisceration and before chilling with water, chemicals, and steam *(9)*. These technologies are most effective when combined, and this practice is known as hurdle technology *(9, 38)*. In order to increase the effectiveness of these decontamination technologies, the plant environments including the facility, employees and equipment need to be taken into consideration. Facility design, processing flow, and overall operation must contribute to the production of a safe product. Equipment should be properly sanitized and well-maintained and employees must be continuously trained, especially those employees that are in close contact to the carcasses, and those who has to operate equipment for the decontamination interventions *(52)*.

Surrogate microorganisms

Surrogates are organisms, mainly bacteria often used specifically to evaluate the effects and responses to selected processing treatments *(13)*. Surrogates play an important role as biological indicators that can mimic the behavior of one or more pathogens and can help to identify process deviations which occur inside the plant since pathogens cannot be intentionally introduced to any food processing facility *(72)*. For example, surrogates can be utilized in challenge or in process-validation studies in situations where the use of the target pathogens can compromise the safety of workers and the environment inside the processing plant or laboratory *(61, 70)*. Surrogates are more often used to evaluate food safety and sanitation processes than food quality *(38)*.

The ideal surrogate

According to the U.S. Food and Drug Administration (FDA), the ideal surrogate must be the pathogen itself with its pathogenic abilities removed by genetic engineering modifications. This is not feasible due to the biological reversion to virulence conditions of the pathogen and because it presence can lead to false positives during routine testing *(70)*. A better approach to the ideal surrogate includes the following characteristics: nonpathogenic, with thermal or chemical inactivation equal numerically and kinetically to target pathogen, durability in foods equal to target organism such as pH stability, refrigeration stability and atmosphere tolerance. The surrogate must be easily identifiable, isolated and enumerable under rapid and inexpensive detection systems, easy differentiable from natural occurring bacteria and very stable so results can be repeatable *(38, 70)*.

The practical use of surrogates

Surrogates have been used in low-acid canned foods to validate the destruction of *Clostridium botulinum* spores. The use *C. sporogenes* and *Bacillus stearothermophilus* apores as surrogates of *C. botulinum* helped in the evaluation of the effectiveness of treatments to obtain a safe, commercially sterile product in the canning industry *(70)*. In the pasteurization of food, surrogates are also used to obtain quantitative information of proper treatment, which may be a good substitute for the enzymatic inactivation measures or chemical reactions research that are in use today. Surrogate organisms utilized in pasteurization validation and some other antimicrobial processes such as irradiation include *Streptococcus thermophilus, Lactobacilli bulgaricus, L. lacti,* and *Pediococcus* spp. In the case of *Listeria monocytogenes*, the surrogate used is *Listeria inoccua (13, 61, 70).*

The more practical indicator of fecal contamination found is *E. coli* biotype I, also known as generic *E. coli* because is commonly found in the gastrointestinal tract of food animals. They may serve as surrogates due to their nonpathogenic characteristics in order to evaluate certain decontamination processes *(13)*.

The identification of appropriate surrogates for beef carcasses starts with the isolation and identification of possible surrogates from similar environments. Marshall et al. *(46)* isolated 113 Gram-negative organisms from cattle hides. Based on thermal resistance and growth curves, five possible indicators were compared with five isolates of *E. coli* O157:H7. The isolates were challenged to seven different antimicrobial treatments and the results showed that these indicators in a combined cocktail served to

evaluate and validate antimicrobial intervention for beef carcasses. These isolates have been used in further studies. Niebuhr et al. *(51)* used these *E. coli* biotype I isolates to compare the responses of these surrogates to a mixed culture of *Salmonella* in order to determine if these can be used to validate interventions to reduce *Salmonella* in meat. Four of the five surrogates used had a lower population reduction (higher survival) than the *Salmonella* mixed culture when exposed at the same antimicrobial interventions *(51)*.

As mentioned before, a very important characteristic for possible surrogates of pathogens is the capability of being easily recognized and enumerated. Rang et al. *(58)* isolated gastrointestinal bacteria and genetically constructed them to express the gene that encodes for the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* but results of this study showed low expression of this protein and lower or impaired growth *(58)*. In other study, GFP-positive strains of *E. coli* O157:H7 shown similar growth rates to *E. coli* O157:H7. GFP-positive *Salmonella* strains also showed similar growth kinetics to the GFP-negative *Salmonella* control *(53)*. Cabrera-Diaz et al. *(14)* evaluated the possibility of genetically modifying surrogates of *E. coli* O157:H7 and *Salmonella* to improve their phenotypic characteristics of being easily distinguishable and easily enumerable surrogates. The growth curves and the bacterial reductions resulting from antimicrobial treatments of these modified surrogates were compared with *E. coli* O157:H7 and *Salmonella* strains with successful results *(14)*. This study concluded that these surrogates are useful tools to validate hot water and lactic acid interventions. The possibility of their use as indicators of cross-contamination in the post harvest environment is evaluated in the present study.

MATERIALS AND METHODS

Bacterial cultures

Three nonpathogenic protein-marked *E. coli* biotype I strains (RFP-1, GFP-3 and YFP-66) previously transformed to express yellow, green or red fluorescent proteins and AMP were selected based on previous collaborative studies *(14, 46)*. The strains were obtained from the Food Microbiology Culture Collection (Texas A&M University, College Station, TX) and maintained at -80^{o}C in cryocare vials (Key Scientific Products, Round Rock, TX). One bead of each strain was transferred to tryptic soy broth (TSB, Difco, Sparks, MD) and incubated for 24 h at 37^{o}C. One loopful of each 24 h culture was transferred to tryptic soy agar (TSA, Difco) supplemented with AMP (100 μg/liter) (Sigma-Aldrich Inc., St Louis, MO) and isopropyl β-D-1 thyogalactoside (IPTG, Novagen EBM Biosciences, Inc., Madison WI). After incubation for 18-24 h at 37^{o}C, the strong and stable fluorescent colony was picked and transferred again to TSA+AMP+IPGT. After incubating 18-24 h at 37^{o}C, again the strong and stable fluorescent colony was transferred to TSA slants as working stock cultures for propagation. Slants were incubated at 37^{o}C for 18-24 hr and kept at 25^{o}C to be used within 30 days.

Preparation of the gelatin matrix

A gelatin slurry made was used for inoculation of carcasses inside commercial abattoirs without introducing actual feces. This slurry was prepared by mixing equal volumes of each 18 h culture in a gelatin-based matrix previously prepared. This gelatin

matrix was prepared by dissolving 112 g of food-grade unflavored gelatin powder (Kraft Food North America, Tarrytown, NY) in 1 liter of 0.1% sterile peptone water (PW, Difco) at room temperature (25-27^{o}C). The pre-hydrated gelatin was added to 6.8 liters of boiling sterile PW and stirred for 5 min.

The hot gelatin slurry was poured into a polyethylene tank (2-gallon Ortho Heavy Duty Sprayer, The Fountain Group, Inc., New York Mills, NY) and left overnight at room temperature. A bacterial cocktail was prepared by inoculating one loopful of each fluorescent protein-marked *E. coli* strain in TSB and incubating for 18 h at 37^{o}C. Immediately before the inoculation, the surrogate cocktail was mixed and aseptically added to the sprayer containing the gelatin slurry. The tank was capped and agitated by hand to distribute and homogenized the inoculum over the gelatin slurry. The average concentration of surrogates was approximately 7.0 log CFU/ml of gelatin slurry.

After the first trial, the yellow-fluorescent protein strain (YFP-66) was found to show low to null fluorescence under UV light, and therefore the remaining trials were conducted with the green and red fluorescent strains only (Fig. 1).

Abattoirs and experimental design

The project was conducted in 3 USDA inspected beef commercial abattoirs. The average number of head processed were 25, 98, and 140 for abattoir 1, 2, and 3 respectively. At each abattoir, 13 hide-on carcasses were inoculated leaving one or more non inoculated carcasses between each inoculated one.

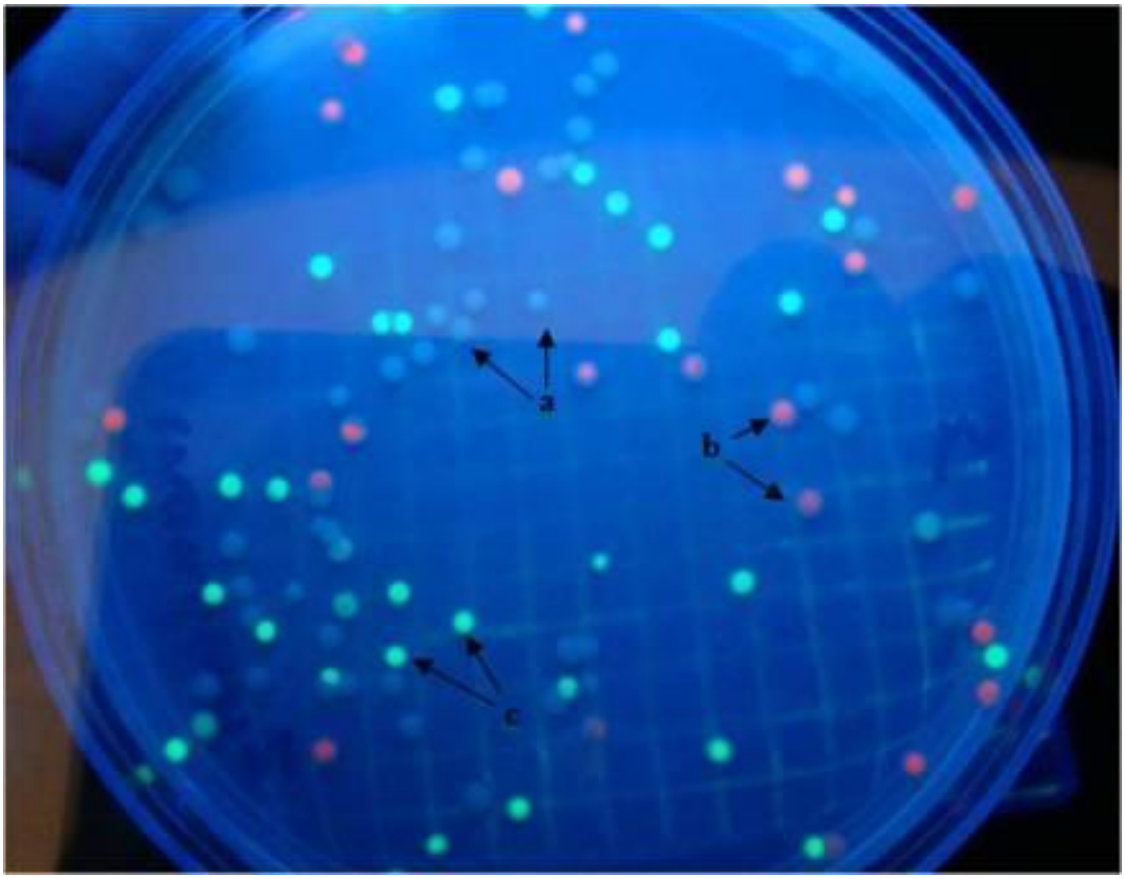

FIGURE 1. *Typical colonies of fluorescent protein-marked* E. coli *biotype I green (GFP-3), red (RFP-1), and yellow (YFP-66 strains grown on tryptic soy agar supplemented with ampicillin (100 µg/liter) and observed under UV light (365nm), showing low or null fluorescence in YFP-66 colonies (a) and the evident fluorescence in RFP-1 and GFP-3 colonies (b and c).*

The slurry containing the fluorescent protein-marked *E. coli* cocktail was sprayed to cover as much as possible at the median line (brisket and plates) after stunning prior to sticking. The hide of each animal was sprayed for 16-18 s at an approximate rate of 1 liter per 16-18 s which resulted in approximately a 9.0 log CFU per carcass. The flow of the slurry exiting the sprayer was calibrated before the first carcass inoculation for each abattoir. Two samples of 1ml of gelatin slurry were taken to verify level and homogeneous distribution of the surrogates.

Each inoculated carcass was followed through the normal harvest process and sampled in different stations including after hide opening (AHO), prior evisceration (PE), after evisceration (AE), after splitting (AS), and after final intervention (AFI). The carcass immediately following each of the inoculated carcasses was also tested at the same points to determine cross-contamination between inoculated and non-inoculated carcasses.

Each carcass sample consisted of a 300 cm^2 surface sample collected following the USDA-FSIS procedure *(69)*. Briefly, one sterile premoistened sponge (3M™, Saint Paul, MN) was used to swab the carcass at each one of the steps in the process. To avoid swabbing the same area more than one time, the following areas were assigned to be sampled for all abattoir: right brisket at AHO, right plate at PE, right flank at AE, left brisket at AS, and left plate at AFI station. The swabbing procedure included removing the premoistened sponge from the bag while squeezing out excess of diluents using a sterile glove. The area to sample was swabbed 10 times in vertical direction and 10 times in horizontal direction using each side of the sponge for each direction (vertical or

horizontal) with strength as to remove dried blood. The sponge was returned to the sterile bag and closed. In total, 26 carcasses were sampled at five processing stations at each abattoir.

Additionally, environmental samples were collected after the last carcass sample was collected. Sanitary procedures were allowed to employees as regularly, then, hooks, knives and saws were cleaned as done during the normal process time. At each establishment, 3 samples each were collected from floors, walls, boots, aprons and gloves, 2 each of knives, meat hooks, air knives, split saw and hide puller, and 5 of plant air. All floor, walls, equipment and utensils were collected by swabbing an area of 300-cm^2 with a pre-moistened sterile sponge using the same procedure of 10 vertical and 10 horizontal passes. Glove samples consisted of a sponge sample of one entire glove passing the sponge through the entire surface. Hand knives and meat hooks were sampled by swabbing their entire surface of the blade and hook only with 10 or more passes.

The air samples were collected at different stations (hide removal, evisceration, carcass splitting, carcass trimming and carcass final intervention) using a Mattson-Garvin air sampler (M-G Model 220, Barramundi Corporation, Homosassa Springs, FL) with 150 mm x 15 mm disposable culture plate containing TSA. The plate with TSA was aseptically placed in position with the lid removed, the sampler was turned on for 5 min. and the plate was removed and the lid was returned.

All sponges and TSA-plate samples were packed in an insulated cooler (Igloo Products Corp, Katy, TX) with refrigerant packs (UTEK 30^oF, Polyfoam Packers Co.

Wheeling, IL) and transported to the Food Microbiology Laboratory at Texas A&M University, College Station, TX. After arrival, samples were kept at 4^{o}C and processed within 24 h.

Microbiological testing

In the laboratory, TSA from air samples was aseptically removed and placed in sterile bags, 50 ml of sterile PW were added and bags were pummeled in a stomacher lab blender (A.J. Seward, London, UK) for 1 min. Sponges were hand-massaged for 1 min. Appropriate decimal dilutions were spread plated onto TSA and poured plated in violet red bile agar (VRB, Difco). Both were previously supplemented with AMP (100 µg/liter). All plates were incubated at 37^{o}C for 24 h. Fluorescent strains appearing on TSA+AMP plates were counted under UV light (365nm) (UVP Chromato Vue Cabinet and UV handheld lamp, Upland, FL). Plate counts grown in TSA+AMP were reported as log CFU/per surface sampled of ampicillin resistant fluorescent surrogates. Counts in VRB+AMP were reported as log CFU/surface sampled of ampicillin resistant coliforms.

Statistical analysis

Plates counts were converted to log CFU and units were expressed either log/surface or log/300 cm^2 for sponge samples and log CFU/57 m^3 for air samples. Differences between inoculated carcass and adjacent carcass, between abattoirs and between stages within the same abattoir were calculated using the Least Square Mean function in JMP 8.0 *(39)*. Percent samples with detectable counts for environmental, and personal and general equipment samples were compared using the test of comparison between two binomial proportions *(55)*.

RESULTS AND DISCUSSION

Previous studies have referred to the difficulties of pathogen-specific testing for in-plant verification of pathogens because occurrence of pathogens such as *E. coli* O157:H7 and *Salmonella* is very low and the introduction of these to abattoirs is prohibited due to its public health significance *(46)*. Although laboratory research can be used as reference point for process validation with several reliable results, this is not a substitute for actual in-plant process validation *(51)*. Furthermore, beef slaughter plant conditions cannot be reproduced in the laboratory. In recent studies, fluorescent surrogates of *E. coli* O157:H7 have been validated and can be used to reproduce possible pathogen contamination from hides to carcasses *(14, 46)*. The use of an innocuous vehicle such as the matrix prepared with food-grade gelatin and PW allowed the simulation of fecal matter containing pathogens and the inoculation of hides to study of the potential for direct and indirect contamination at commercial abattoirs.

During the experiment at abattoir 1, the yellow-fluorescent protein strain (YFP-66) was found to be very difficult to distinguish under UV light (365nm) due to its low to null fluorescence (Fig.1). In addition, indigenous bacteria that were resistant to AMP produced colonies that were morphologically indistinguishable from the YFP-66 strain colonies. For these reasons, it was determined to eliminate this strain from the study and therefore all other experiments were conducted using RFP-1 and GFP-3 only. The achievement of the objectives of this study was not hindered in any way by eliminating

the yellow strain. The gelatin slurry preparation was adjusted accordingly to compensate for the use of 2 bacterial suspensions during inoculum preparation.

Carcass samples

Counts of fluorescent organisms from inoculated carcasses and adjacent carcasses (non-inoculated) at the 3 sampled abattoirs are shown in Table 1. It was clear that the inoculated microorganisms were transferred from the hide to the carcass and that these organisms remained on the carcass at least before the final carcass intervention (AFI stage). Surrogates were found in both inoculated and adjacent non-inocualted carcasses. The average counts for the three abattoirs from inoculated hides at the AHO, PE and AE stations were 3.1, 1.9 and 1.8 log CFU/300 cm^2 respectevely. For non-inoculated carcasses, these averages were of 2.2, 1.6 and 1.4 log CFU/300 cm^2 for the same stations. In the AS station, surrogates were only found on the carcasses coming from the inoculated hides, with an average of 1.7 log CFU/300 cm^2. At AFI station neither inoculated nor non-inoculated carcasses had detectable counts (Table 1).

Surrogate counts from abattoir 1, 2 and 3 were different ($P < 0.05$). Abattoir 3 which reached the highest counts compared to 1 and 2 at AHO, AE and AS for both inoculated and non-inoculated carcasses with values of 4.3, 3.3, and 2.6 log CFU/300 cm^2 and of 3.5, 2.1, and 1.7 log CFU/300 cm^2 respectively. Abattoir 2 also had higher counts for both, inoculated and non-inoculated, than abattoir 1. For abattoir 2 the counts of inoculated and non-inoculated carcasses at AHO station were 3.3 and 2.1 log CFU/300 cm^2 and PE station of 1.9 and 1.7 log CFU/300cm^2 (Table 1) while abattoir 1 had 1.6 and <1.4 log CFU/300 cm^2 for AHO, and 1.4 and <1.4 log CFU/300 cm^2 at PE

station. Abattoir 1 was the smallest of the establishments (25 head per h) with a plant layout following a straight line. In contrast, abattoirs 2 and 3 were considerably larger (98 and 140 head per h, respectively). Results of this study suggested that larger abattoirs may have a greater risk of cross-contamination and this may be due to a greater number of employees handling the carcass during the hide removal process, and a more difficult operation since more animals are processed at the same time and more equipment is utilized. Consequently, larger plants may need greater attention for the correct implementation of competitive sanitary procedures.

None of the carcasses yielded detectable counts after the carcass intervention step in any abattoir (Table 1). This highlights the importance of these interventions in reducing pathogens on beef carcasses. The chain speed at abattoir 3 may have facilitated cross-contamination between carcasses due to an increase in line speed which allowed contact between carcasses due to the swinging movements of carcasses hanging in the moving line.

Data in Table 2 show the counts obtained when VRB+AMP was used as counting medium. VRB is one of the medium preferred to enumerate coliforms in food samples. The addition of ampicillin allowed the visualization of ampicillin resistant coliforms. Counts from VRB+AMP seemed to be similar to those obtained with TSA+AMP validating the use of TSA+AMP for enumeration of fluorescent surrogates since VRB does not allows the visualization of the distinctive fluorescence of the surrogates used.

TABLE 1. *Counts (log CFU/300 cm^2) of fluorescent protein-marked* E. coli *biotype I RFP-1 and GFP-3 strains on carcass surfaces in three different abattoirs at different stations[a] grown on tryptic soy agar supplemented with ampicillin (100 μg/liter)*

Treatment *n = 13*	Abattoirs	Stations[a] AHO	PE	AE	AS	AFI
Inoculated	1	$1.6^{c,z}$	$1.4^{cd,y}$	$< 1.4^{cd,y}$	$< 1.4^{d,y}$	$< 1.4^{d,x}$
	2	$3.3^{c,y}$	$1.9^{d,x}$	$< 1.4^{e,y}$	$1.4^{de,y}$	$< 1.4^{e,x}$
	3	$4.3^{c,x}$	$2.3^{e,x}$	$3.3^{d,x}$	$2.6^{e,x}$	$<1.4^{f,x}$
	Average[b]	3.1^{l}	1.9^{l}	1.8^{l}	1.7^{l}	$< 1.4^{l}$
Non-Inoculated	1	$< 1.4^{c,z}$	$< 1.4^{c,y}$	$< 1.4^{c,y}$	$< 1.4^{c,y}$	$< 1.4^{c,x}$
	2	$2.1^{c,y}$	$1.7^{c,x}$	$< 1.4^{d,y}$	$< 1.4^{d,y}$	$< 1.4^{d,x}$
	3	$3.5^{c,x}$	$1.9^{d,x}$	$2.1^{d,x}$	$1.7^{de,x}$	$< 1.4^{e,x}$
	Average[b]	2.2^{m}	1.6^{m}	1.4^{m}	$< 1.4^{m}$	$< 1.4^{l}$

[a]AHO: After hide opening, PE: Prior evisceration, AE: After evisceration, AS: After splitting, AFI: After final intervention.
[b]Averages were calculated using a value of 1.1 log CFU/300 cm^2 when values were below 1.4 log CFU/300 cm^2.
[cdef]Values with different letters within a row differ statistically ($P < 0.05$).
[lm]Values with different letters between averages within a column differ statistically ($P < 0.05$).
[xyz]Values with different letters within a column, within a treatment (inoculated or non-inoculated) differ statistically ($P < 0.05$).
Abattoir 1= 25head/h, Abattoir 2= 98head/h, Abattoir 3= 140 head/h

TABLE 2. *Counts (log CFU/300 cm^2) of ampicillin resistant coliforms on carcass surfaces in three abattoirs at different stations[a] grown on violet red bile agar supplemented with ampicillin (100 µg/liter)*

Treatment *n = 13*	Abattoir	Station AHO	PE	AE	AS	AFI
Inoculated	1	$2.2^{c,z}$	$1.9^{cd,x}$	$1.5^{de,y}$	$< 1.4^{e,y}$	$< 1.4^{e,x}$
	2	$2.6^{c,y}$	$1.6^{d,xy}$	$< 1.4^{e,y}$	$< 1.4^{e,y}$	$< 1.4^{e,x}$
	3	$4.0^{c,x}$	$2.0^{e,x}$	$2.9^{d,x}$	$1.8^{e,x}$	$< 1.4^{f,x}$
	Average[b]	2.7^{l}	1.7^{l}	1.8^{l}	$< 1.4^{l}$	$< 1.4^{l}$
Non-Inoculated	1	$< 1.4^{d,z}$	$< 1.4^{cd,y}$	$< 1.4^{c,y}$	$< 1.4^{cd,x}$	$< 1.4^{d,x}$
	2	$1.7^{c,y}$	$1.6^{c,x}$	$< 1.4^{d,y}$	$< 1.4^{d,x}$	$< 1.4^{d,x}$
	3	$3.5^{c,x}$	$1.5^{e,xy}$	$2.0^{d,x}$	$< 1.4^{ef,x}$	$< 1.4^{f,x}$
	Average[b]	2.1^{m}	1.4^{l}	1.4^{l}	$< 1.4^{m}$	$< 1.4^{l}$

[a]AHO: After hide opening, PE: Prior evisceration, AE: After evisceration, AS: After splitting, AFI: After final intervention.
[b]Averages were calculated using a value of 1.1 log CFU/300 cm^2 when values were below 1.4 log CFU/300 cm^2.
[cdef]Values with different letters within a row differ statistically ($P < 0.05$).
[lm]Values with different letters between averages, within a column, differ statistically ($P < 0.05$).
[xyz]Values with differ letters within a column, within a treatment, differ statistically ($P < 0.05$).

Since the characteristics of the colonies on VRB did not permit the visualization of fluorescence, some colonies with typical coliform characteristics may not have been the surrogate strains (Fig. 2). However, regression analysis showed a significant correlation between counts on both media ($P < 0.05$) (data not shown). The use of TSA+AMP is recommended in further studies which allowed visualization of the ampicillin resistant surrogates.

When the surrogates counts obtained from carcasses were hides where inoculated and non-inoculated hides were compared abattoir 3 showed significantly ($P < 0.05$) higher counts than abattoir 1 for both inoculated and non inoculated carcasses at all stages except AFI ($P > 0.05$). In AHO, PE, AE, and AS station the counts for inoculated carcasses for abattoir 1 were 2.2, 1.9, 1.5, and <1.4 log CFU/300 cm^2 while abattoir 3 counts were 4.0, 2.0, 2.9, and 1.8 log CFU/300 cm^2 for the same stations (Table 1). When counts of ampicillin resistant coliforms for all 3 abattoirs were combined, carcasses coming from animals with inoculated hide were significantly higher ($P < 0.05$) than those coming from non-inoculated hides at AHO, PE and AE. At the AFI station, counts were consistently at levels below the detection limit (<1.4 log CFU/300 cm^2) (Table 2).

Beef products may become contaminated by microorganisms during harvesting depending upon the hygiene conditions of the slaughter process and its environment. Among the factors that can explain the differences between abattoirs are the facility's structures, techniques of hygiene application, sanitation practices and sizes of harvested animals.

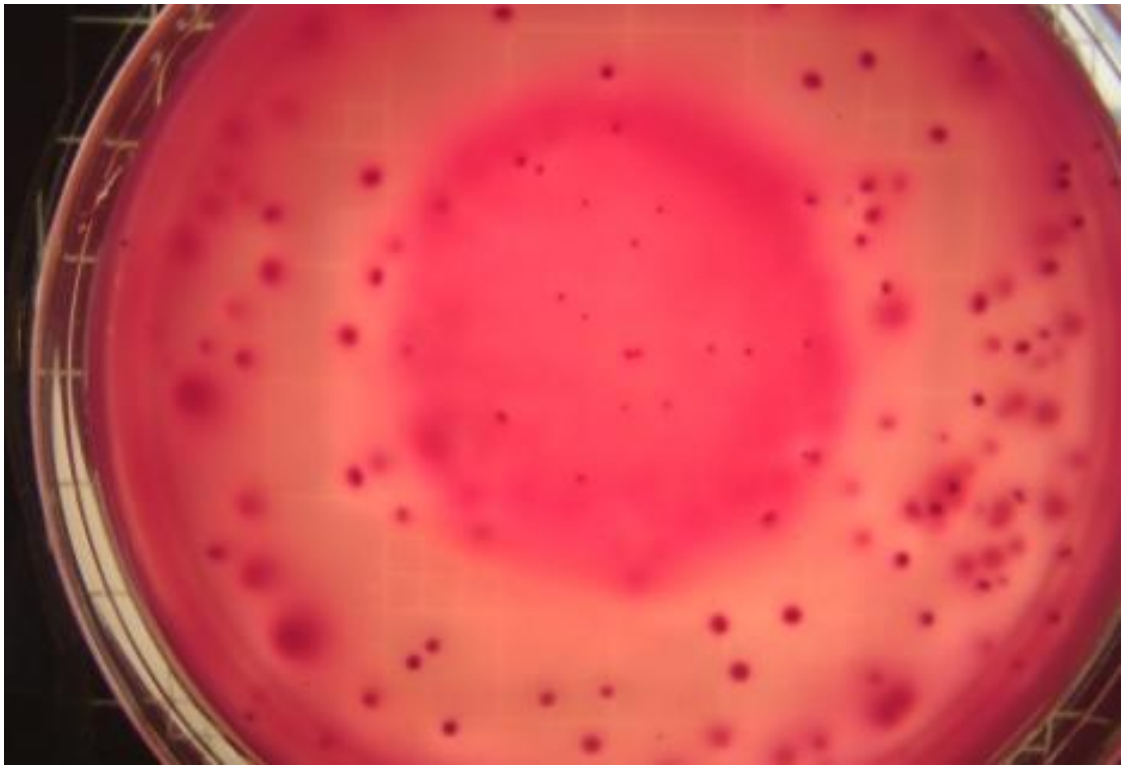

FIGURE 2. *Typical colonies of ampicillin resistant coliforms grown on violet red bile agar supplemented with ampicillin (100 μg/liter).*

Hides are considered the major source of fecal pathogens in carcasses. Elder et al. *(26)* have reported low correlation between the prevalence of pathogens on hides and on carcasses. Nonetheless, other studies have demonstrated that *E. coli* O157:H7 can be transferred to the carcass during hide removal operations and carcass contamination with *E. coli* O157:H7 may result from a source other that the rumen and the rectum *(47)*. This study demonstrated that fluorescent biotype I *E. coli* surrogates are transferred from contaminated hides to the carcass. Microbial transfer to non-inoculated carcasses may have occurred by transfer from contaminated hides to non-contaminated hides, or by cross-contamination between carcasses after hide removal, from the hide, employees or equipment. This reflects the importance of sanitary dressing techniques in order to avoid cross-contamination between carcasses. As shown in Tables 1 and 2, when more fluorescent microorganisms were found during hide removal (AHO stage, abattoir 3) these had a greater count along the process. On the other hand, when there were low counts of surrogate organisms as observed in abattoir 1, the counts remained low or even below detectable levels along the process. Therefore, it is essential to consider hide removal as one of the points with the highest chances for cross-contamination from hides to carcasses and from carcass to carcass.

Environmental samples

The results of environmental samples were very low and many were below detectable counts or showed counts very close to the detection limit of the counting method. For this reason, the prevalence of fluorescent surrogates was reported as percentage of detectable counts (≥ 1.4 log CFU/surface sampled) (Tables 3 and 4). The

presence of fluorescent strains was detected in several equipment and environment samples. Overall, surrogate presence was detected in 10% of aprons and in 11% of boots sampled. For environments samples, 13% of wall and 25% of floor samples had detectable surrogate counts. The samples with the highest proportion of detected surrogates were the hide puller samples (80%). In the case of VRB counts, the proportion of samples with detected ampicillin resistant coliforms were 50% for aprons, 66% for boots, 50% of split saw and 100% for hide puller samples. The prevalence of samples with detectable counts of ampicillin resistant coliforms for floor, walls, and air samples were 62, 13, and 7% respectively (Table 4).

When a high count was observed, this was usually from samples collected from the hide puller, which is the piece of equipment that is in direct contact with the hide and also, it is the only piece of equipment or utensils that is not constantly sanitized between uses. Although the presence of fluorescent surrogates on environmental samples was rather sporadic, it still indicates that pathogens can be potentially transferred from a contaminated animal to the processing environment and may account for an indirect mechanism for carcass contamination.

When least square means were compared for counts of fluorescent surrogates on TSA+AMP or VRB+AMP, no statistical differences were seen between abattoirs, treatments and counts (Table 5). However, this lack of differences may be due to the low frequency of samples with countable numbers. In other words, the number of samples with counts above the detection limit was not enough to determine differences between abattoirs.

TABLE 3. *Proportion of environmental samples with detectable counts (>1.4 log CFU/surface sampled) of fluorescent protein-marked* E. coli *biotype I RFP-1 and GFP-3strains grown on tryptic soy agar supplemented with ampicillin (100µm/liter)*

Sample[a]	Abattoir 1			Abattoir 2			Abattoir 3			Overall		
	No. samples			No. samples			No. samples			No. samples		
	n	Detectable[b]	%[c]	*n*	Detectable[b]	%[c]	*n*	Detectable[b]	%[c]	*n*	Detectable[b]	%[c]
Personal equipment												
Aprons	3	0	0	4	1	25	3	0	0	10	1	10
Boots	3	0	0	3	0	0	3	1	33	9	1	11
Gloves	3	0	0	3	0	0	3	0	0	9	0	0
Total	9	0	0	10	1	10	9	1	11	28	2	7
Equipment												
Knife	2	0	0	2	0	0	2	0	0	6	0	0
Air Knife	2	0	0	2	0	0	2	0	0	6	0	0
Hide Puller	2	1	50	1	1	100	2	2	100	5	4	80
Meat Hook	2	0	0	1	0	0	2	0	0	5	0	0
Split Saw	1	0	0	1	0	0	2	0	0	4	0	0
Total	9	1	11	7	1	14	10	2	20	26	4	15
Environment												
Floor	3	0	0	2	0	0	3	2	67	8	2	25
Wall	3	0	0	2	0	0	3	1	33	8	1	13
Air	5	0	0	5	0	0	5	0	0	15	0	0
Total	11	0	0	9	0	0	11	3	27	31	3	10
Grand Total	29	1	3	26	2	8	30	6	20			

[a]Detection limit was 1.4 log CFU/300 cm^2 for aprons, boots, equipment (except knives), floor and walls, 1.4 log CFU per one glove (gloves), per knife blade (knives) and per one hook (meat hooks), and 1.4 log CFU/57 m^3 of air.
[b]Growth of at least 1 fluorescent colony.
[c]Percentage of samples with detectable fluorescent surrogates counts (>1.4 log CFU/surface sampled).

TABLE 4. *Proportion of environmental samples with detectable counts (> 1.4 log CFU/surface sampled) of total coliforms grown on violet red bile agar supplemented with ampicillin (100 μg/liter)*

Sample[a]	Abattoir 1			Abattoir 2			Abattoir 3			Overall		
	No. samples			No. samples			No. samples			No. samples		
	n	Detectable[b]	%[c]	*N*	Detectable[b]	%[c]	*n*	Detectable[b]	%[c]	*n*	Detectable[b]	%[c]
Personal equipment												
Aprons	3	0	0	4	1	25	3	1	33	10	2	50
Boots	3	1	33	3	2	67	3	3	100	9	6	66
Gloves	3	0	0	3	0	0	3	0	0	9	0	0
Total	9	1	11	10	3	30	9	4	44	28	2	7
Equipment												
Knife	2	0	0	2	0	0	2	0	0	6	0	0
Air Knife	2	0	0	2	0	0	2	0	0	6	0	0
Hide Puller	2	2	100	1	1	100	2	2	100	5	5	100
Meat Hook	2	0	0	1	0	0	2	0	0	5	0	0
Split Saw	1	0	0	1	1	100	2	1	50	4	2	50
Total	9	2	22	7	2	28	10	3	30	26	7	27
Environment												
Floor	3	2	67	2	1	50	3	2	67	8	5	65
Wall	3	0	0	2	0	0	3	1	33	8	1	15
Air	5	1	20	5	0	0	5	0	0	15	1	67
Total	11	3	27	9	1	11	11	3	27	31	7	26
Grand total	29	6	21	26	6	23	30	10	33			

[a]Detection limit was 1.4 log CFU/300 cm^2 for aprons, boots, equipment (except knives), floor and walls, 1.4 log CFU per one glove (gloves), per knife blade (knives) and per one hook (meat hooks), and 1.4 log CFU/57 m^3 for air.
[b]Growth of at least 1 typical coliform colony.
[c]Percentage of samples with detectable coliforms counts (>1.4 log CFU/surface sampled).

To enable comparison of samples with low counts, the percentages of samples that produced at least 1 colony on the countable plate were calculated and compared between abattoirs. For the environment samples collected from abattoir 3, 20% yielded detectable counts. This value was significantly higher ($P < 0.05$) than the 3 and 8% observed for abattoirs 1 and 2, respectively (Fig. 3).

Environmental samples are related to contact with and between surfaces, areas from the same carcasses, from carcass to carcass, from personal equipment to air, walls and floor and other possible mechanisms for microbial transmission. Akkaya et al. *(4)* reported the presence of *E. coli* O157, *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* on floors, walls, meat hooks, knives, hands, transport wagons and other environments at 5 different abattoirs in Turkey *(4)*. Although environmental samples in the current study showed the presence of the fluorescent surrogates, this was not found in gloves maybe due to the use of disposable gloves and its continuous changes from employees in all abattoirs. Due to the low counts observed for environmental, personal equipment and general equipment samples, the role of these sites as possible sources of carcass contamination could not be determined in this study.

Further research about mechanisms for microbial transfer within the harvesting processing plant is needed. However, the data collected during this study show that personal equipment and various surfaces in the facility can play a role as a mechanism for contamination of carcasses with bacterial pathogens such as *E.coli* O157:H7. High counts at the AHO stage, as found in abattoir 3, may represent an indicative of poor hygiene during hide opening, which leads to carcass contamination.

TABLE 5. *Counts (log CFU/surface sampled) of fluorescent protein-marked* E. coli *RFP-1 and GFP-3 in tryptic soy agar supplemented with ampicillin (100 µg/liter) (TSA+AMP) and total coliforms on violet red bile agar supplemented with ampicillin (100 µg/liter) (VRB+AMP) of environmental samples in three different abattoirs*

	Abattoir 1		Abattoir 2		Abattoir 3		Averages[g]	
Category	TSA	VRB	TSA	VRB	TSA	VRB	TSA	VRB
Personal Equipment	< 1.4[a]	< 1.4[a]	< 1.4[a]	1.9[a]	1.5[a]	< 1.4[a]	< 1.4[e]	< 1.4[x]
General Equipment	< 1.4[a]	1.7[a]	1.5[a]	2.0[a]	< 1.4[a]	1.4[a]	< 1.4[e]	1.7[x]
Environment	< 1.4[a]	1.5[a]	< 1.4[a]	1.4[a]	< 1.4[a]	1.5[a]	< 1.4[e]	1.5[x]
Averages[g]	< 1.4[p]	1.4[j]	< 1.4[p]	1.8[j]	< 1.4[p]	< 1.4[j]		

[a]Values with same letter within a column at each abattoir do not differ statistically ($P > 0.05$)

[e]Averages with the same letter within a row within in TSA medium do not differ statistically ($P > 0.05$)

[g]Averages were calculated using a value of 1.1 log CFU when values were <1.4 log CFU/surface sampled.

[j]Averages with the same letter within a column within the same medium (VRB) do not differ statistically ($P > 0.05$).

[p]Averages with the same letter within a column within the same medium (TSA) do not differ statistically ($P > 0.05$)

[x]Averages with the same letter within a row within in VRB medium do not differ statistically ($P > 0.05$).

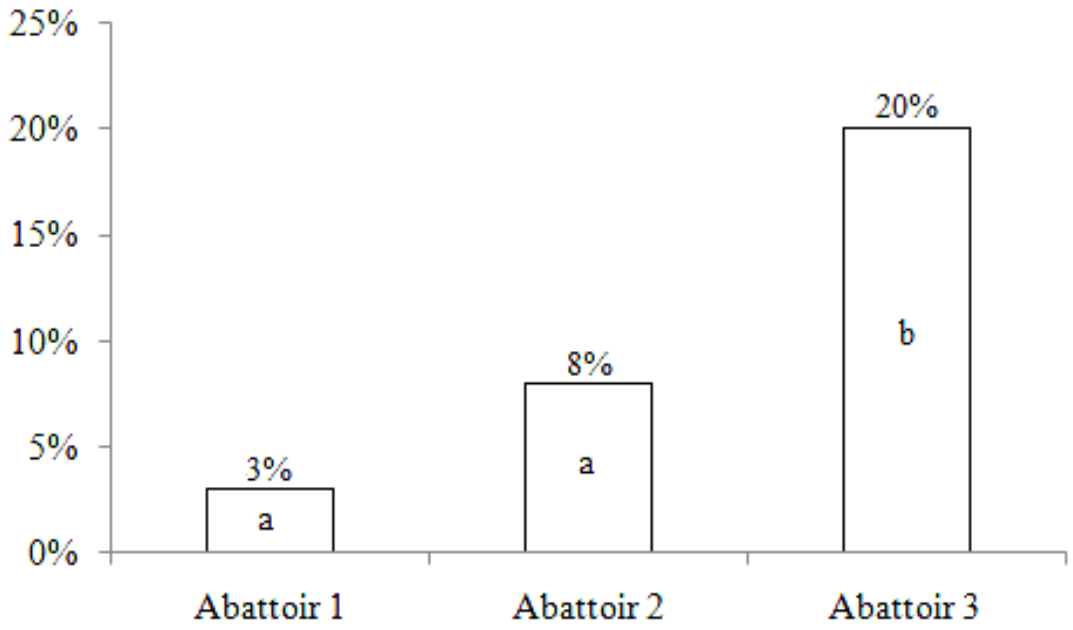

FIGURE 3. *Proportion of environmental samples with detectable counts (>1.4 log CFU/surface sampled) of fluorescent protein-marked* E. coli *biotype I RFP-1 and GPF-3 strains grown in tryptic soy agar supplemented with ampicillin (100 µg/liter) in three different abattoirs.*

[a,b] Columns with different letter differ statistically ($P < 0.05$)

In contrast, when the counts are low, as found in abattoir 1, the fluorescent surrogates were not found on adjacent carcasses or at further steps sampled during the harvest process. The relationship between the number of workers, and the size and distribution of the abattoir facility with the risk of cross-contamination may require further investigation. Larger processors are supposed to have more rigorous systems and superior financial resources to control pathogens. Some researchers favor small processors because small plants are supposed to have more time for careful hide removal as well as more time for inspection scrutiny due to the slower line speed *(11)*. This may explain the results of this study where abattoir 1 had the slower head per h (25 head) rate and the lowest environmental detectable counts for fluorescent surrogates (35) and total coliforms (21%). Besides, these results highlight the importance of following proper procedures for sanitary dressing of beef carcasses in preventing carcass contamination.

CONCLUSIONS

Results from this study showed that the simulation of carcass contamination in the postharvest environment is possible due to the utilization of nonpathogenic fluorescent surrogates of *E. coli* O157:H7 and *Salmonella* spp. Additionally, the use innocuous vehicle such as a gelatin slurry may be used to simulate fecal matter throughout the inoculation of this slurry with surrogate organisms will allow processors to trace cross-contamination thought beef abattoirs. Further studies may explain the differences found in surrogate counts by abattoirs and its relationship to the number of carcasses processed per hours and the number of in-line employees per plant. Antimicrobial interventions for reducing pathogens on carcasses seemed to be very effective since surrogates were not detected after the antimicrobial treatment. Each establishment must evaluate their sanitary dressing procedures to assure the efficacy of these interventions by improving every procedure that can control cross-contamination.

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