# CHARACTERIZATION AND EVALUATION OF *Escherichia coli* BIOTYPE I STRAINS FOR USE AS SURROGATES FOR ENTERIC PATHOGENS IN VALIDATION OF BEEF CARCASS INTERVENTIONS

A Dissertation

by

ELISA CABRERA DIAZ

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

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Food Science and Technology

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December 2007

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# ABSTRACT

Characterization and Evaluation of *Escherichia coli* Biotype I Strains for Use as Surrogates for Enteric Pathogens in Validation of Beef Carcass Interventions.

(December 2007)

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Antimicrobial interventions implemented in slaughter establishments for the reduction of enteric pathogens on beef carcasses must be validated to demonstrate efficacy under commercial operation conditions. Validation studies can be conducted using surrogates which are nonpathogenic organisms that respond to a particular treatment in a manner equivalent to a target pathogen. The purpose of this study was to identify surrogates for enteric pathogens to validate antimicrobial interventions on beef carcasses. The growth, attachment, resistance properties as well as the response to interventions on beef carcasses of nonpathogenic fluorescent protein-marked *E. coli* strains were evaluated and compared to *E. coli* O157:H7 and *Salmonella* strains.

Growth curves were performed in tryptic soy broth at 37°C and it was demonstrated that in general, growth parameters were not different among surrogates and target pathogens. Thermal resistance was compared in phosphate buffered saline (PBS) at 55, 60 and 65°C; D-values of surrogates were not different or were higher than

those of target pathogens. The acid resistance of surrogates was not different to that of *E. coli* O157:H7 in PBS acidified with lactic acid at pH 2.5, 3.0 and 3.5. Some *Salmonella* serotypes were found to be less acid resistant than the surrogates. Survival of surrogates after storage at low temperatures (4°C and -18°C) was not different or was longer than survival of *E. coli* O157:H7 and *Salmonella*. Additionally, the cell surface hydrophobicity and attachment to beef carcasses surfaces was not different among surrogates and pathogens. Antimicrobial interventions were applied on carcass surfaces under laboratory controlled conditions. After application of hot water washes, D-values were not different among surrogates and pathogens, while no differences were observed in log reductions ($CFU/cm^2$) among surrogates and pathogens when 2% L-lactic acid sprays at 25 and 55°C were applied, regardless of the temperature and volume of the acid solution. The response of surrogates to water washes and lactic acid sprays on beef carcasses was also evaluated in commercial slaughter facilities. Reductions of surrogates were not different to those of aerobic plate count, coliforms and *E. coli*. However, the surrogates showed less variation and provided more consistent results than traditional indicators.

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# INTRODUCTION

Meat and poultry are traditional and important vehicles of pathogens which cause foodborne illness. In order to reduce the occurrence and numbers of pathogenic microorganisms on meat and poultry products as well to reduce the incidence of foodborne illness associated with the consumption of those products, the United States Department of Agriculture established regulations that require all meat and poultry establishments to develop and implement the Hazard Analysis and Critical Control Point (HACCP) system to minimize the presence of pathogens on carcass surfaces because at the present time the production of pathogen-free beef is not feasible.

A variety of antimicrobial interventions have been implemented as Critical Control Points (CCPs) at different points in the beef slaughter process to reduce carcass contamination with enteric pathogens. Physical interventions include trimming, hot water washes, steam-vacuuming and steam-pasteurization. Chemical interventions include the application of compounds such as trisodium phosphate, acidified sodium chlorite, aqueous ozone or organic acids such as lactic, acetic and peroxyacetic, on the surface of beef carcasses. L-lactic acid is one of the most common chemical interventions implemented in beef slaughter operations.

Antimicrobial interventions must be validated to demonstrate efficacy under in-plant operation conditions. Validation procedures are commonly conducted using indicator groups such as aerobic plate count (APC), coliforms and *E. coli*. However,

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This dissertation follows the style and format of the *Journal of Food Protection*.

these indicators may have different resistance properties than pathogens such as *E. coli* O157:H7, and their use in validation procedures may have limitations.

Surrogates are nonpathogenic microorganisms with similar growth, survival and resistance properties to target pathogens. The use of surrogates in validation studies offers the opportunity to quantify the effect of antimicrobial interventions on nonpathogenic organisms with similar behavior to target pathogens in commercial food processing environments where pathogens cannot be used due to safety concerns.

In this study, *E. coli* biotype I strains were evaluated for use as surrogates for *E. coli* O157:H7 and *Salmonella* in the validation of beef carcass interventions. The growth, attachment and resistance properties of nonpathogenic *E. coli* strains expressing fluorescent proteins were compared to those of *E. coli* O157:H7 and *Salmonella*. The response of the fluorescent protein-marked *E. coli* strains to the application of hot water washes and 2% L-lactic acid sprays on beef carcass tissues was compared to the response showed by the target pathogens under laboratory controlled conditions. Additionally, the fluorescent protein-marked *E. coli* strains were used in commercial slaughter establishments to evaluate their response to water washes and lactic acid interventions on beef carcasses under operation conditions and reductions observed were compared to those of traditional indicators including APC, coliforms and *E. coli*.

# REVIEW OF LITERATURE

**Foodborne illnesses related to meat and meat products.** It has been estimated that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations and 5,000 deaths each year in the United States (U.S.) *(168)*. Factors that contribute to the emergence of foodborne diseases include: changes in human demographics and behavior, changes in technology and industry (increase of intensive food production systems), increase of international travel and commerce, microbial adaptation, economic development and land use, and the breakdown of public health measures *(2, 136)*. Foodborne diseases have high economical costs for industry, health services and society. The Economic Research Service (ERS) of the U.S. Department of Agriculture (USDA) has estimated that five foodborne bacterial pathogens including *Campylobacter jejuni*, *Escherichia coli* O157, Shiga toxin-producing *E. coli* non-O157, *Listeria monocytogenes* and *Salmonella*, cost $6.9 billion annually in the U.S. *(236)*

During 1993-1997 a total of 2,751 foodborne illness outbreaks were reported to the Centers for Disease Control and Prevention (CDC) resulting in 86,058 cases and 29 deaths *(55)*. During 1998-2002, the total number of reported foodborne illness outbreaks increased to 6,647 producing 128,370 illness cases and 88 deaths *(52)*. According to the CDC, this increase in the number of outbreaks may be attributed to the implementation of measures to enhance the surveillance systems and to an increased availability of improved diagnostic methods.

*Salmonella* and pathogenic *E. coli* (including enterohemorrhagic strains) are among the most common bacterial pathogens causing foodborne disease outbreaks. For the period 1993-2002, *Salmonella* was related to 942 foodborne outbreaks with 49,431 illness cases, while pathogenic *E. coli* was responsible of 224 outbreaks and 8,114 cases *(52, 55)*. However, the number of reported outbreaks represents only a small proportion of the outbreaks that actually occurred because some of them are never recognized, and those that are recognized frequently go unreported.

Meat and poultry are traditional vehicles of foodborne pathogens. *Salmonella*, *E. coli* O157:H7, *Campylobacter* and *L. monocytogenes*, are significant food safety hazards associated with meat and poultry products. The Food Safety and Inspection Service (FSIS) of the USDA, estimates that contamination of meat and poultry products with these bacteria results in 4,000 deaths and 5 million illnesses each year *(242)*. According to the CDC reports, beef products appeared among the three most common vehicles of foodborne illness and were linked to 274 outbreaks reported for the period 1993-2002. These outbreaks resulted in 7,394 illness cases and 9 deaths (Table 1) *(52, 55)*. Pathogenic *E. coli* was linked to 51 of the 274 outbreaks, while *Salmonella* was linked to 23 outbreaks.

***Escherichia coli* O157:H7.** The recognition of enterohemorrhagic *E. coli* (EHEC) during the 1980s resulted from two epidemiological observations: the recognition of two outbreaks of hemorrhagic colitis linked to under-cooked hamburgers from a fast-food restaurant chain *(203)*, and the association of sporadic cases of

TABLE 1. *Foodborne outbreaks, cases and deaths related to the consumption of beef products in the United States, 1993-2002*[a]

| Year | Outbreaks[b] | Cases[c] | Deaths[d] | No. outbreaks linked to | |
|---|---|---|---|---|---|
| | | | | *E. coli* | *Salmonella* |
| 1993 | 16 | 1,368 | 4 | 5 | 0 |
| 1994 | 22 | 871 | 0 | 7 | 7 |
| 1995 | 14 | 437 | 0 | 8 | 4 |
| 1996 | 7 | 227 | 0 | 0 | 1 |
| 1997 | 7 | 302 | 0 | 1 | 2 |
| 1998 | 26 | 805 | 0 | 1 | 0 |
| 1999 | 62 | 1,332 | 0 | 12 | 3 |
| 2000 | 43 | 696 | 1 | 11 | 1 |
| 2001 | 33 | 525 | 1 | 0 | 1 |
| 2002 | 44 | 831 | 3 | 6 | 4 |
| Total | 274 | 7,394 | 9 | 51 | 23 |

[a] Extracted from: Surveillance for foodborne-disease outbreaks. United States, 1998-1997 and 1998-2002 *(52, 55)*

[b] Total number of reported outbreaks of all etiologies

[c] Total number of reported cases of all etiologies

[d] Total number of reported deaths of all etiologies

hemolytic uremic syndrome (HUS) with cytotoxin-producing *E. coli* strains corresponding to serotype O157:H7 *(138)*.

Since then, *E. coli* O157:H7 has become a major foodborne pathogen in the U.S., with a remarkable impact on public health, food industry and federal regulations regarding food safety *(100)*. The CDC has estimated the total number of illness cases caused by *E. coli* O157:H7 in the U.S. at 73,480 each year, with 2,168 hospitalizations and 61 deaths; for non-O157 Shiga toxin producing *E. coli*, the estimated number of annual cases is 36,740 with 1,084 hospitalizations and 30 deaths *(168)*. Serotypes O26 O103, O111 and O145 are more prevalent and more important as cause of foodborne illness in continental Europe *(19, 42)*.

*Characteristics of the disease*. The illness produced by *E. coli* O157:H7 is characterized by hemorrhagic colitis (bloody diarrhea and abdominal cramps) that can progress to the life-threatening renal disease known as HUS *(176)*. The pathogenicity of EHEC, including *E. coli* O157:H7, is related to the production of several virulence factors: the adherence factor intimin encoded by the *eae* gene located in the locus of enterocyte effacement (LEE) *(72, 73)*, an enterohemolysin encoded in a 60 MDa plasmid *(231)*, and two potent cytotoxins denominated Shiga-like toxins (Stx1 and Stx2) encoded in bacteriophages inserted into the chromosome *(227)*. After the initial adherence of the bacteria to the intestinal epithelial cells, an intimate adherence process is responsible for the attaching and effacing (A/E) lesions observed on the mucosa of the large intestine, disrupting the microvilli, inducing an inflammatory response and producing an initial non-bloody diarrhea *(72)*. The subsequent production of cytotoxins causes the

hemorrhagic colitis by their action on the endothelial cells of blood vessels in the intestinal submucosa *(175).* A single EHEC strain can express Stx1 only, Stx2 only, or both toxins. The cytotoxins produced in the intestine are translocated to the bloodstream and transported to the renal endothelial cells that contain the specific receptor $Gb_3$. The damage produced by the toxin(s) on the glomerular and tubular endothelial cells produces a decrease in the glomerular filtration rate which is responsible of the acute renal failure that is typical of HUS *(176, 229).*

*Reservoirs and prevalence in cattle. E. coli* O157:H7 strains are not host specific and they can be isolated from different species including cattle, sheep, goats, deer, chickens, pigs, horses and birds *(24, 57, 82, 83, 131, 213).* The microorganism is more prevalent in ruminants than in other animals and cattle appears as the most relevant reservoir. Cattle are also an important reservoir of non-O157 Shiga toxing producing *E. coli* strains (STEC) and the isolation of as many as 95 "O" serogroups and 250 serotypes of the enterohemorrhagic group has been reported in different countries *(131).*

The prevalence of *E. coli* O157:H7 and other STEC in cattle from different countries has been widely documented. In the U.S., the National Animal Health Monitoring System (NAHMS) conducted a study on feedlots with 1,000 head or more capacity within the 12 top cattle-feeding states in the country *(243).* These operations represented 84.9% of the U.S. feedlots in 1999 and contained 96.1% of the U.S. cattle inventory on feedlots with ≥1,000 head capacity. Overall, 11% (1,148 of 10,415) of fecal samples collected from cattle in the feedlots were positive for Shiga toxin-producing *E. coli* O157. In other studies conducted in the Midwestern U.S., Elder et al. *(93)*

isolated EHEC from 27.8% (91 of 327) of fecal samples from cattle presented for slaughter at four meat processing plants. Barham et al. *(10)* reported a prevalence of *E. coli* O157 of 9.0% in fecal samples collected from cattle at feedlots and a prevalence of 5.5% in cattle arriving to the packing plants. Barkocy-Gallagher et al. *(12)* isolated *E. coli* O157:H7 from 5.9% (70 of 1,189) of fecal samples collected from cattle presented for slaughter at three processing plants. In another survey, Smith et al. *(220)* reported the isolation of *E. coli* O157:H7 from 23% (719 of 3,162) of fecal samples obtained from animals tested in five feedlots. Chapman et al. *(59)* reported that *E. coli* O157 was isolated from 12.9% (620 of 4,800) of fecal samples taken immediately after slaughter during a one-year study in the United Kingdom. Fegan et al. *(98)* isolated *E. coli* O157 from 10% (n=68) of cattle fecal samples collected after evisceration at an Australian abattoir. The prevalence of *E. coli* O157 in cattle was 3.6% (87 of 2,419) in 60 dairy farms investigated by Nielsen et al. *(180)* in Denmark. Callaway et al. *(40)* found *E. coli* O157 in 1.2% (3 of 240) cattle fecal samples collected at two dairy and two beef cattle farms located in Mexico. Rogerie et al. *(208)* reported that STEC was isolated from 7.9% (67 of 851) cattle fecal samples collected immediately after slaughter at seven abattoirs in France, and strains from the serogroup O157 were only recovered from 0.5% of the samples. Pradel et al. *(194)* conducted another study in France and found STEC in 34% (162 of 471) of bovine fecal samples analyzed during a one-year prospective study.

The results from a large number of studies on STEC prevalence in cattle from different countries were summarized by Hussein and Bollinger *(131).* According to this review, in general, prevalence rates for *E. coli* O157 ranged from 0.3 to 19.7% in fecal

samples of cattle in the feedlot and from 0.7 to 27.3% in fecal samples of animals on pasture. The prevalence rates of non-O157 STEC ranged from 4.6 to 55.9% in feedlot cattle, compared to 4.7 to 44.8% in grazing cattle and from 2.1 to 70.1% at slaughter. The differences in reported prevalence rates may be attributed to variation in methods used for isolation, and also to differences in cattle managing practices and/or environmental conditions.

Several factors influence the carriage and fecal shedding of STEC in cattle, including animal age, sex, diet, climate and managing practices. Prevalence rates are higher in younger cattle, particularly weaned calves *(63, 124, 144, 180, 218, 224)*. The increase in prevalence following weaning may be associated to the loss of protective antibodies present in the colostrum, changes in the gut microbiota as a result of changes in diet regimes, and contact of calves with adult animals in the pens *(180)*. It has been reported that diet also affects the prevalence rates of *E. coli* O157:H7 in cattle. Several reports indicate that animals fed on grain diets have higher carriage rates and higher numbers of *E. coli* O157:H7 than animals fed on forage diets, and switching cattle from a high grain diet to a hay-based diet prior to slaughter can reduce *E. coli* O157:H7 populations; however, the economical implications of these practices needs to be evaluated *(41, 77, 80, 123)*. Differences in cattle fecal shedding have also been observed after feeding with barley, corn or barley-cotton diets *(29)* while other reports indicate no effect of diet in steers *(18)*.

Carriage and fecal shedding in cattle is affected by environmental factors and follows a seasonal trend, with the highest prevalence rates observed during the warm

months *(12, 89, 124, 224, 243)*. This peak prevalence during the summer season may be related to day length *(92)*. It has been hypothesized that the seasonal shedding patterns of *E. coli* O157 in cattle are a result of the physiological responses within the host animal to changing day length, and that thyroid hormones may be involved in these physiological changes *(91, 215)*.

Fecal shedding of *E. coli* O157:H7 seems to be correlated with levels of carcass contamination. For this reason, a large variety of pre-harvest interventions to reduce this pathogen in the animals before they are slaughtered have been proposed in recent years *(36-38)*. Preharvest intervention methods can be classified in 3 groups: i) exposure reduction strategies, ii) exclusion strategies and iii) direct inhibition strategies *(148)*. The most common interventions that have been evaluated to control this pathogen in cattle include: vaccination *(193)*, administration of probiotic bacteria *(185, 259)*, dietary modification *(41, 77, 80)*, oral administration of chemical compounds such as sodium chlorate *(39)*, rectal administration of therapeutic agents such as polymyxin B and chlorhexidine *(178)* and recently, the rectal administration of bacteriophages *(217)*.

Cattle hides play an important role as a source of carcass contamination at slaughter, and for this reason, the prevalence of *E. coli* O157:H7 and other STEC serotypes on hides has been widely investigated. Barkocy-Gallagher et al. *(12)* recovered *E. coli* O157:H7 from 60.6% (n=1,288) of hide samples collected from cattle at three different processing plants in the Midwestern U.S. Rivera-Betancourt et al. *(206)* found that prevalence of *E. coli* O157:H7 on the hides of cattle presented for slaughter at two large processing plants in the U.S. was 55.9 and 68.1% (n=1,033), respectively. O'Brien

et al. *(184)* reported a prevalence of *E. coli* O157 in cattle hides of 7.3% (n=1,500) when samples were collected at early stages of the carcass processing over a 17-month period. Elder et al. *(93)* isolated *E. coli* O157 from 11% (n=355) of cattle hide samples collected from animals after stunning at four meat processing plants. Woerner et al. *(254)* collected hide samples from cattle presented for slaughter at three commercial facilities and from the total samples tested, 14.7% (n=150) were positive for *E. coli* O157. Arthur et al. *(7)* found *E. coli* O157 on 75.7% (n=288) of cattle hides from animals coming into two slaughter plants. In a study conducted to address the effect of transportation and lairage environment on the prevalence of *E. coli* O157:H7 on hides, the prevalence of the microorganism increased from 50.3 to 94.4% between the time the cattle were loaded onto tractor-trailers at the feedlot and the time the hides were removed at the processing plant *(6)*. A recent study reports that *E. coli* O157 can be isolated from cattle hides at concentrations ranging from less than 0.1 to 4.2 log CFU/100 $cm^2$ *(184)*.

*Outbreaks related to meat and meat products.* Outbreaks of foodborne illness related to *E. coli* O157:H7 have been traced in most cases to cattle and their edible products. Between 1993 and 2002, a total of 274 foodborne outbreaks were linked to beef products in the U.S. and fifty one were attributed to pathogenic *E. coli* including *E. coli* O157:H7 *(52, 55)*. The first *E. coli* O157:H7 foodborne outbreak reported in 1982 was linked to ground beef, which remains the most common vehicle among foodborne outbreaks. This meat product was related to 75 of 183 (41%) foodborne outbreaks reported to the CDC from 1982 to 2002 and accounted for 33% of 5,269 foodborne-

related cases. Outbreaks involving ground beef showed the highest rate in summer months and 71% occurred from May to August *(199)*.

During the same period, five outbreaks were associated with the consumption of beef products other than ground beef, including roast beef *(207)*, steaks *(53)*, sirloin tips and salami *(56)*. In 2003, an outbreak linked to the consumption of non-intact blade tenderized steaks was identified by the Minnesota Department of Health *(145)*. The tenderizing and injection processes likely transferred the microorganism from the surface to the interior of the steaks. These processing methods create new challenges for prevention of *E. coli* O157:H7 and other enteric pathogens present on the surface of raw meat. Meat products other than beef, including pork and deer have been also involved in *E. coli* O157:H7 outbreaks *(64, 140)*.

***Salmonella.*** Infections caused by *Salmonella* in humans and animals are a major health problem worldwide. *Salmonella enterica* is considered as a "neotype species" according to the nomenclature proposed by Euzéby *(97)* and is classified in more than 2,000 serotypes defined as a result of complex antigen variability. Some serotypes, such as Typhi and Paratyphi are highly adapted to humans and have no other known natural hosts, while the non-typhoid serotypes have a broad host range and can infect a wide variety of animals *(60)*. The CDC has estimated that the total number of illness cases caused by non-typhoid *Salmonella* each year in the U.S. is 1,412,498, with 16,430 hospitalizations and 582 deaths *(168)*. In 2005, from a total of 16,614 laboratory-confirmed human cases of infections identified by the Foodborne Diseases Active

Surveillance Network (FoodNet) *(50)*, 6,471 cases were related to *Salmonella* and of the 5,869 *Salmonella* isolates serotyped, six serotypes accounted for 61% of infections: Typhimurium (19%), Enteritidis (18%), Newport (10%), Heidelberg (6%), Javiana (5%) and serotype I 4,[5],12:i:- (3%).

*Characteristics of the disease*. The infections produced by non-typhoid *Salmonella* are characterized by nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. The symptoms appear after 12-72 hours and the infective dose has been estimated as low as 15-20 cells, depending on the strain and the host condition *(51)*. The acute enteritis is characterized by an inflammatory infiltrate associated with necrosis of the mucosa in large areas of the terminal ileum and colon. Acute symptoms may last for 1 to 2 days and chronic sequel such as arthritic symptoms may follow 3 to 4 weeks after the onset of acute phase. Infection usually remains localized to the intestine and mesenteric lymph nodes, while bacteremia is uncommon. *Salmonella* pathogenesis is a complex and multifactorial phenomenon that has been more extensively investigated in *S.* Typhimurium. Virulence factors are encoded in two chromosomal pathogenicity islands denominated SPI-1 and SPI-2. Effector proteins translocated by the SPI-1 type III secretion system are involved in bacterial penetration into target cells, whereas the function of SPI-2 is to facilitate the replication of intracellular bacteria within vacuoles *(108, 249)*. The acute inflammatory response is associated with mucosal edema and the injury to the intestinal epithelium leads to leakage of extravascular fluids and massive transmigration of neutrophils into the intestinal lumen *(248, 257)*.

*Reservoirs and prevalence in farm animals.* The primary habitat of *Salmonella* is the intestinal tract of humans, farm animals, birds, reptiles and occasionally insects. Although their primary habitat is the intestinal tract, they may be found in other parts of the body; for example, the organism has been detected in the spleen, liver, bile, tonsils and lymph nodes of slaughtered pigs *(158, 182, 247).*

The prevalence of *Salmonella* in farm animals has been widely documented. In broiler chickens, prevalence rates are up to 30%. A multistate epidemiological investigation of *Salmonella* in poultry operations in the U.S. showed a prevalence of 6.6% (168 of 2,546) in fecal samples *(9).* A study conducted by Siemon et al. *(219)* to compare *Salmonella* prevalence between pasture and conventional poultry farms in the U.S. showed that 16% of (83/512) fecal specimens collected from pasture and 30% (125/419) of those collected from conventional poultry farms were *Salmonella*-positive. Chambers et al. *(58)* found an overall *Salmonella* prevalence of 4.3% (27 of 635) in broiler chickens from 21 processing plants located in Canada *(58).* In another study conducted in the same country, the prevalence reported by Arsenault et al. *(4)* for *Salmonella*-positive flocks was 50% for chickens and 54% for turkeys in 81 broiler chicken and 59 turkey flocks sampled. Limawongpranee et al. *(152)* sampled the cecal contents of broiler chickens from 28 flocks in 12 farms in Japan to determine the prevalence of *Salmonella*; the organism was isolated from 14.3% (336 of 2,345) of the analyzed samples. Common serotypes isolated from broiler chickens include Enteritidis, Hadar, Heidelberg, Kentucky, Senftenberg, Thompson and Typhimurium *(9, 54, 58).*

In pigs, the *Salmonella* prevalence rates have been found to be up to 53%. According to Davies et al. *(71)*, between 1994 and 1995, *Salmonella* was isolated from 24.6% (565 of 2,288) of fecal samples collected from finishing swine raised in 28 farms in the U.S. In a different survey, Davies et al. *(70)* reported that *Salmonella* was isolated from 12% (95 of 792) of fecal samples collected at seven swine housed farms with a prevalence ranging from 3.4 to 22%. After conducting an intensive longitudinal investigation on breeding and growing pigs in two multiple-site swine production systems, Funk et al. *(107)* found prevalence rates of *Salmonella* from 0 to 48%. In this study, a total of 5,496 rectal fecal samples were analyzed and *Salmonella* was isolated from 8% total samples over the time. According to the authors, point estimates of prevalence cannot be considered as reliable indicators of the *Salmonella* status on farms due to the high variable patterns of prevalence and serotype profiles observed over time and among production systems. Hurd et al. *(130)* compared the prevalence of *Salmonella* in finishing swine at the farm and at the abattoir; the microorganism was isolated from 5.3 and 39.9% fecal samples, respectively. Vieira-Pinto et al. *(247)* isolated *Salmonella* from 26.7% (27 of 101) of pigs presented for slaughter at one abattoir, while Oliveira et al. *(186)* estimated the prevalence of *S.* Typhimurium in about 53.5% of pigs presented for slaughter after an enterocolitis outbreak occurred in the farm during the growing phase. Common serotypes isolated from pigs include Agona, Anatum, Cholerasuis, Derby, Infantis and Typhimurium *(54, 70, 107)*.

In beef cattle, *Salmonella* prevalence has been reported to be up to 46%. *Salmonella* was recovered from 4.4% of fecal samples from cattle monitored at three

processing plants in the Midwestern U.S., according to Barkocy-Gallagher et al. *(12)*. In a study conducted by Fegan et al. *(99)* , *Salmonella* was isolated from 6.8% (21 of 310) of cattle fecal samples with counts that varied from <3 to $2.8 \times 10^3$ MPN/g. Fecal samples from cattle at 73 feedlots located in 12 different states in the U.S. were analyzed for *Salmonella* and it was reported that a total of 6.3% (654 of 10,417) were positive for the microorganism *(69)*. In North Ireland, Madden et al. *(160)* isolated *Salmonella* from 3% (6 of 200) of fecal samples collected postmortem from cattle at seven different abattoirs. The effect of transportation on *Salmonella* shedding in cattle was investigated by Barham et al. *(10)*; the organism was recovered from 18% (n=200) of fecal samples prior to transportation of animals to the slaughter plant and from 46% of the fecal samples taken upon arrival of animals to the plant. Other studies showed that *Salmonella* serovars isolated from on-farm samples and those serovars isolated after slaughter varies and denotes the risk of exposure to *Salmonella* during transport and lairage of animals *(110)*. Common serotypes isolated from beef cattle include Anatum, Kentucky, Montevideo, Muenchen, Senftenberg and Typhimurium *(54, 99)*.

The prevalence of *Salmonella* on cattle hides has been widely documented. The pathogen was isolated from 50.3% (n=523) *(206)*, 71.0% (n=1,066) *(12)* and 91.8% (n=510) *(206)* of cattle hides when the animals were presented for harvest at different commercial beef processing plants in the U.S. On the hides of steers and heifers, the prevalence reported by Barham et al. *(10)* was 6% (n=200) two weeks before shipping to a commercial packing facility, and increased to 89% upon arrival of animals to the packing plant.

*Outbreaks related to meat and meat products.* According to the CDC, between 1993 and 1997, *Salmonella* was the leading cause of foodborne outbreaks in the U.S., accounting for 357 outbreaks (13% of the total reported), 32,610 cases (37.9%) and 13 deaths (44.8%) *(55)*. During the period 1998-2002, *Salmonella* was again the primary cause of foodborne illnesses and was involved in 585 outbreaks (8.8% of the total reported), 16,821 cases (13.1%) and 20 deaths (22.7%) *(52)*. As mentioned before, the increment in the number of reported outbreaks may be attributed to the implementation of measures to improve surveillance systems. The most common vehicles linked to *Salmonella* foodborne outbreaks included eggs, poultry, beef, pork and dairy products, as well as fruits and vegetables. Between 1993 and 2002, from a total of 274 outbreaks linked to beef products, 23 (8.4%) were attributed to *Salmonella (52, 55).*

**Microbial contamination of meat during beef slaughter operations**.

Pathogenic bacteria can be transferred to meat during the carcass dressing process at the slaughter plant. The main source of pathogenic bacteria is the gastrointestinal tract of the animal. Contamination of the carcass may occur directly from intestinal contents or indirectly from feces present on the animal hide. Carcasses can also be cross-contaminated by contact with adjacent carcasses, equipment or workers. Other sources of contamination may include ingesta, saliva and mucus from the nose or mouth, and bile *(112)*. Numerous studies have been conducted to address the prevalence of *E. coli* O157:H7 and *Salmonella* on beef carcasses. These studies show considerable variation,

which may be attributed to a number of factors, including differences in sampling procedures and analytical methods.

According to the USDA Nationwide Beef Microbiological Baseline Data Collection Program for Steers and Heifers, during the period 1992-1993 the prevalence of *E. coli* O157:H7 on beef carcasses sampled after chilling was 0.2% (n=2,081) *(244).* Subsequent studies showed higher prevalence rates, which may be attributed to the availability of more effective isolation methods. In a survey conducted by Barkocy-Gallagher et al. *(12)* at three processing plants in the Midwestern U.S., *E. coli* O157:H7 was recovered from 26.7% (n=1,281) of beef carcasses sampled immediately after hide removal and from 1.2% (n=1,232) of carcasses sampled immediately prior to or within 2 h of entry into the cooler. In a study Rivera-Betancourt et al. *(206)* reported that prevalence of *E. coli* O157:H7 on beef carcasses at each of two slaughter plants was 3.1% (n=510) and 10.9% (n=523) prior to evisceration, and it was reduced to 0.0% (n=497) and 1.0% (n=520) after application of antimicrobial interventions including steam vacuuming and post-evisceration carcass rinses. Woerner et al. *(254)* found that from a total of 1,328 beef carcass samples collected pre-evisceration, post-evisceration and after the application of microbial interventions consisting of a thermal wash and an organic acid rinse, *E. coli* O157 was isolated from 10.1, 1.4 and 0.3% samples, respectively. In a survey performed by Elder et al. *(93)* to estimate the frequency of *E. coli* O157 within groups of cattle from different sources presented for slaughter at four processing plants, the observed prevalence rates on carcasses were 43% pre-evisceration (immediately after complete hide removal), 18% post-evisceration (after evisceration,

splitting and trimming) and 2% post-interventions (after application of either steam pasteurization, hot water washes or organic acid washes, or a combination of these treatments). In a study conducted by Arthur et al. *(5)* at four large processing plants in the U.S., the prevalence for non-O157 Shiga toxin-producing *E. coli* on beef carcasses was estimated at 53.0% (n=334) pre-evisceration and 8.3% (n=326) after application of antimicrobial interventions which varied between plants and included steam vacuuming, hot water washing, organic acid washing and steam pasteurization. In this study, 41 different O serogroups were recovered but only 11% of the isolates carried a combination of virulence factors associated with strains causing human disease.

Regarding *Salmonella*, the USDA Nationwide Beef Microbiological Baseline Data Collection Program for Steers and Heifers reported a prevalence of 1.0% (n=2,089) on carcasses sampled after chilling, for the period 1992-1993 *(244)*. Results from the USDA Nationwide Sponge Microbiological Baseline Data Collection Program for Cattle reported a national prevalence of 1.2% (n=1,881) on beef carcasses during the period 1997-1998 *(239)*. More recently, the USDA Progress Report on *Salmonella* Testing of Raw Meat and Poultry Products reported a prevalence of 0.4% (n=22,826) on steers and heifers carcasses during the period 1998-2006 *(233)*. In other studies, *Salmonella* was isolated from 12.7% (n=1,060), 23.3% (n=511) and 26.8% (n=522) of samples collected pre-evisceration and from 0.08% (n=1,016), 0.0% (n=499) and 0.8% (n=520) of samples collected immediately prior to entering into the cooler at different processing plants *(12, 206)*.

**The Hazard Analysis and Critical Control Point (HACCP) system in beef slaughter operations**. In order to reduce the occurrence and numbers of pathogenic microorganisms on meat products and to reduce the incidence of foodborne illness associated with the consumption of these products, the USDA-FSIS established new regulations in 1995 *(242)*. These regulations required all meat and poultry establishments to develop and implement the HACCP system, which is focused on prevention of hazards to assure the production of food products that are safe to consume *(225)*. Through application of HACCP, each establishment can implement the necessary controls to prevent hazards from occurring or keeping them within acceptable limits, as well as monitor the performance of controls and maintain records. At the present time, the production of pathogen-free beef is not feasible; therefore, the emphasis of HACCP in slaughter operations is to minimize the presence of pathogens on carcass surfaces *(116)*. The combination of HACCP based process controls with appropriate food safety performance standards is the most effective available approach for controlling and reducing pathogens on raw meat *(242)*.

According to USDA-FSIS regulations, every meat and poultry establishment is required to develop and implement a written HACCP plan covering each product they produce whenever a hazard analysis reveals that one or more food safety hazards are reasonably likely to occur. The HACCP plan must contain: 1) a list of the food safety hazards identified; 2) a list of the Critical Control Points (CCPs) for each of the identified hazards; 3) a list of the critical limits that must be met at each of the CCPs; 4) a list of the procedures that will be used to monitor each of the CCPs to ensure

compliance with the critical limits; 5) all corrective actions to be followed in response to any deviation from a critical limit at a CCP; 6) a list of the verification procedures that the establishment will use to confirm the HACCP plan is working effectively, and 7) a record-keeping system to document all procedures and records implemented as part of the HACCP plan *(242)*.

**Interventions for pathogen reduction on beef carcasses**. A variety of antimicrobial interventions have been implemented as CCPs at different points in the slaughter process to reduce the possibility of carcass contamination with enteric pathogens. The purpose of these interventions is to decontaminate the carcass at different steps in the slaughter operation to remove or reduce any microorganisms carried over from the previous slaughter steps *(126)*. Antimicrobial interventions may include physical or chemical procedures and their efficacy is influenced by the location and attachment of bacteria on the carcass surface *(1)*.

*Physical interventions.* Physical interventions for pathogen reduction are those methods which result in the inhibition, irreversible inactivation, or mechanical removal of microorganisms without requiring the use of antimicrobial additives *(8)*. These methods include trimming, water washing, steam-vacuuming and steam-pasteurization procedures. Trimming consists of the mechanical removal of any visible fecal or ingesta contamination using a sanitized knife. If performed properly, is effective in reducing microbial populations on beef carcasses, but effectiveness depends on the skill of the workers. If trimming is performed incorrectly, it results in cross-contamination *(241)*.

Aerobic plate counts (APC) and *E. coli* were reduced from beef carcasses in approximately 1.3 log CFU/cm$^2$ by trimming, in a study conducted at six large slaughter operations *(200)*, while *E. coli* O157:H7 and *S.* Typhimurium were reduced by 2.6-3.6 log CFU/cm$^2$ in a study conducted under laboratory controlled conditions *(44)*.

Water washes can remove bacterial populations from beef carcasses; however, efficacy is affected by several factors, including pressure and temperature of the water. According to Castillo et al. *(44) E. coli* O157:H7 and *Salmonella* were reduced by 1.7-3.0 log CFU/cm$^2$ when water was applied at room temperature and at a pressure of 250-400 psi. Cabedo et al. *(31)* found that *E. coli* counts were reduced 1.2-3.5 log CFU/cm$^2$ by application of water at 35°C and 300 psi, while Gorman et al. *(120)* reported that APC were reduced in 2.1 log CFU/cm$^2$ by washing beef carcasses with water at 16°C and 300 psi.

Application of hot water washes pre- or post-evisceration has been proposed as an effective intervention. USDA-FSIS regulations indicate that water applied to the surface of beef carcasses at a temperature greater than 165°F (>74˚C) for more than 10 s can be an effective antimicrobial intervention *(241)*. However, efficacy reported by several studies differs and the variation observed may be a consequence of differences in treatment parameters such as water temperature, pressure, contact time, distance between nozzles and carcass surface, and others. Barkate et al. *(11)* reported reductions of 1.3 log CFU/cm$^2$ on APC after application of hot water at 95ºC, reaching a carcass surface temperature of 82ºC for 10 s. Dorsa et al. *(86)* reported that application of water at 82.2ºC for 10 s (20 lb/in$^2$) elevated the carcass surface temperature to 72ºC and produced

reductions of 2.0 and 2.7 log CFU/cm$^2$ on APC and coliforms, respectively. Castillo et al. *(44)* observed reductions of 1.6, 2.2, 1.6 and 1.9 log CFU/cm$^2$ on APC, coliforms, *E. coli* O157:H7 and *Salmonella,* respectively, after application of water at 95ºC for 5 s (24 lb/in$^2$) obtaining a carcass surface temperature of 82ºC. Bosilevac et al. *(22)* observed reductions of 2.7 log CFU/100 cm$^2$ on APC and coliforms when water was applied at 74ºC for 5.5 s (700 lb/in$^2$) and the carcass surface temperature was raised to 70ºC.

Steam pasteurization consists of exposing all carcass surfaces to "saturated" steam followed by rapid cooling in order to minimize the impact of heat on meat color. In a study conducted by Nutsch et al. *(183)*, the application of steam raised the temperature of the carcass surface to 82.2°C for 6.5 s and produced reductions on APC and coliforms of 0.4-1.3 and 0.6-1.6 log CFU/100 cm$^2$, respectively. Minihan et al. *(172)* found that when beef carcasses were exposed to steam pasteurization at 90°C for 10 s, reductions of *E. coli* and *Enterobacteriaceae* ranged from 0.5 to 2 log CFU/1,000 cm$^2$. The authors concluded that steam pasteurization significantly reduced the level of these microbial populations at more contaminated sites, but did not result in complete decontamination of the carcass surface. Retzlaff et al. *(201)* reported that application of steam at 85 and 87.8°C reduced APC by 1.4 and 1.5 log CFU/cm$^2$, respectively, while *Enterobacteriaceae*, total coliforms, and *E. coli* were reduced to undetectable levels (<0.4 CFU/cm$^2$) on all carcasses sampled. However, when the steam treatments were applied at temperatures of 71.1, 73.9, 76.7, and 79.4°C, they were ineffective at reducing microbial populations. Steam pasteurization is commonly used in large beef slaughter

facilities, however, it does not constitute a feasible intervention for small establishments due to the high economical cost *(162)*.

*Chemical interventions.* Chemical interventions are those methods that apply chemical compounds with antimicrobial activity on beef carcass tissues. These compounds include trisodium phosphate, acidified sodium chlorite, aqueous ozone, lactoferrin, cetylpyridinium chloride, sodium hydroxide, ammonium hydroxide, sodium hypochlorite, hydrogen peroxide and organic acids. The compounds that have been approved by the USDA for use as antimicrobials on beef carcasses are presented in Table 2, and the results from different evaluation studies are described in this section.

The application of trisodium phosphate on inoculated lean beef tissue reduced *S.* Typhimurium and *E. coli* O157:H7 populations by 1-1.5 log CFU/cm$^2$ *(78)*. Treatment of carcass surfaces with ozonated water produced reductions of 1.1, 1.3 and 2.5 log CFU/cm$^2$ on APC, *E. coli* biotype I and streptomycin resistant *E. coli* counts, respectively *(120, 200)*. Other studies have indicated, however, that reductions of microbial populations by application of ozonated water are not significantly different from those achieved with a water wash alone *(49)*. Acidified sodium chlorite (ASC) showed to be effective in reducing *E. coli* O157:H7 and *S.* Typhimurium populations on beef carcasses by 1.5-2.3 log CFU/cm$^2$ in experiments conducted under laboratory controlled conditions *(46, 151, 226)*. However, the application of 0.16% ASC on chilled beef carcasses under processing conditions at two commercial plants showed reductions on APC and coliforms of only <0.5-1.0 log CFU/cm$^2$ *(113)*.

TABLE 2. *Compounds approved by the Food Service and Inspection Service of the United Stated Department of Agriculture for use as antimicrobials on beef carcasses*[a]

| Substance | Product | Amount |
|---|---|---|
| Acidified sodium chlorite | • Meat carcasses, trims and organs;<br>• Processed, comminuted or formed meat food products | • 500-1200 ppm in combination with any GRAS acid at a level sufficient to achieve a pH of 2.3 to 2.9 depending on the type of meat product |
| Lactic acid | • Beef carcasses prior to fabrication (pre-chill and post-chill) | • 5% lactic acid solution at 55°C |
| Lactoferrin | • Beef carcasses and parts | • At up to 2% of a water-based antimicrobial spray<br>• As part of an antimicrobial spray that would deliver 1 g of lactoferrin per dressed beef carcass, followed by a wash with tempered water and rinse with lactic acid |
| Organic acids: lactic, acetic and citric acid | • As part of carcass wash applied pre-chill | • At up to 2.5% of a solution |
| Ozone | • All meat products | • In the gaseous or aqueous phase in accordance with current industry standards of good manufacturing practice |
| Peroxyacids: a mixture of peroxyacetic acid, octanoic acid, acetic acid, hydrogen peroxide, peroxyoctanoic acid and 1-hydroxyethylidene-1,1-diphosphonic acid | • Meat carcasses, trim and organs | • Maximum concentration of peroxyacids is 220 ppm as peroxyacetic acid, and the maximum concentration of hydrogen peroxide is 75 ppm |
| Sodium metasilicate | • Raw beef carcasses, subprimals and trimmings | • For use at up to 4% (±2%) of a solution |

[a] Extracted from the FSIS-USDA Directive 7120.1, Amendment 1 *(237).*

The use of cetylpyridinium chloride (CPC) on beef carcass surfaces was evaluated as an antimicrobial intervention in different studies. Stopforth et al. *(226)* reported that application of 0.1% CPC produced reductions of 3.0 log CFU/cm$^2$ on *E. coli* O157:H7. After application of 1.0% CPC, Cutter and Dorsa *(66)* obtained reductions of 5-log cycles on *E. coli* O157:H7 and *S.* Typhimurium, however, the residual concentrations of CPC were excessive for human consumption. CPC has been approved as an antimicrobial agent in poultry processing at a concentration that does not exceed 0.3 g per pound of carcass *(101)*; however, it has not been approved for use on beef carcasses.

Lactoferrin is a protein with antimicrobial activity related to its capacity to bind iron and to produce damage on the outer membrane of gram-negative bacteria *(187, 255)*. By application of 2% activated lactoferrin in combination with 5% lactic acid solution on beef cuts intended for mechanical tenderization, *E. coli* O157:H7 was reduced by 0.9 log CFU/cm$^2$. However, reductions were not different from those achieved by application of other treatments such as trimming, hot water at 82°C and 2.5% lactic acid solution *(127)*.

Sodium metasilicate (SMS) is a strong alkali with antimicrobial activity. Solutions at 0.1, 1.0 and 5% have pH values of approximately 11.3, 12.3 and 12.7, respectively. *In vitro* studies showed an instant reduction of counts of *E. coli* O157:H7 by 3.0 and 5.6 log CFU/ml after exposure to 0.3% and 0.4% SMS, respectively *(251)*. An evaluation of the effectiveness of 4% sodium metasilicate to reduce coliforms and generic *E. coli* from beef adipose tissue, showed reductions of 5.6 and 5.4 log CFU/cm$^2$,

respectively *(43)*. Stopforth et al. *(226)* reported that a spray application of 0.005% sodium hypochlorite and 0.05% ammonium hydroxide showed low ability to reduce indicators and pathogens on beef carcass tissues at chilling temperatures. Reductions of total bacterial populations and *E. coli* O157:H7 counts were <1.1 log CFU/cm$^2$.

Several organic acids have been evaluated as antimicrobial interventions on beef carcasses. Efficacy differs with the type of organic acid and is pH dependent. When the pH of the acid solution is close to the *pKa* of the organic acid, more molecules are undissociated. These undissociated molecules are lipophilic and can diffuse across the bacterial membrane; once inside the cell, the organic acid molecules dissociate because the pH inside the cytosol is close to neutrality (not close to the *pKa* of the organic acid). The dissociation of the organic acid inside the cytosol will produce the reduction of the internal pH of the cell. Organisms that prefer growth at neutral pH, such as *E. coli,* cannot maintain an internal pH of more than two units higher than the external pH. When exposed to an external low pH, *E. coli* will try to maintain the internal pH value with the help of acid-resistance mechanisms. If the internal pH decreases to <4.5, most physiological processes will stop and denaturation of proteins, depurination of DNA (producing breakage of the DNA strand) and damage to cell membrane will occur *(104)*. The use of organic acids as interventions on beef carcasses has several potential advantages: they are not inactivated by reaction with organic matter, do not generate toxic residues on the food product or toxic waste to the environment, and constitute an inexpensive decontamination intervention that is affordable to small and very small plants *(85, 96)*.

The most common organic acids that have been evaluated as potential antimicrobial interventions on beef carcasses include acetic, citric, formic, L-lactic and peroxyacetic. Early studies with short-chain fatty acids under laboratory controlled conditions demonstrated APC reductions of 0.7, 0.9 and 1.6 log CFU/cm$^2$ on beef carcass tissues after application of 1 and 2% formic acid and 3% acetic acid sprays, respectively *(196)*. In other studies, the application of 2% acetic acid at 25°C, 125 psi for 15 s on beef carcasses, reduced acid adapted cells of *E. coli* O157:H7 by 2 log CFU/cm$^2$ *(17)*. When acetic acid was sprayed at concentrations of 1, 3 and 5% on beef carcass tissues, initial populations of 5-log CFU/cm$^2$ of *E. coli* O157:H7 were reduced in 1.6, 1.9 and 2.0 log CFU/cm$^2$, respectively, after storage of tissues for 24 h at 4°C *(68)*. In the same study, the application of 1, 3 and 5% citric acid produced reductions of the pathogen of 1.2, 1.8 and 1.9 log CFU/cm$^2$, respectively. In a study conducted by Stopforth et al. *(226)* spraying 0.02% peroxyacetic acid on beef tissues resulted in *E. coli* O157:H7 reductions of 1.1 log CFU/cm$^2$. After application of 0.02% peroxyacetic acid, Gill and Badoni *(113)* observed reductions of <1.0 log CFU/ cm$^2$ on APC and coliforms on chilled beef carcasses.

L-lactic acid is one of the most commonly used organic acids as an antimicrobial intervention on beef carcasses at different steps of the slaughter process. It has been implemented as pre-evisceration, post-evisceration and post-chilling sprays in commercial facilities, based on the substantial number of studies that support its efficacy. Early studies on the effect of this organic acid on meat decontamination were performed in the late 1970s and early 1980s, with reports showing instant reductions of

1.5-2.0 log CFU/cm$^2$ on APC after application of 1, 1.2 and 1.5% lactic acid on beef, veal and pork, respectively *(221, 222)*. More studies were later conducted to evaluate the efficacy of lactic acid to reduce enteric pathogens on beef carcasses using different application parameters. Using a model cabinet to spray lactic acid solutions at concentrations of 1, 3 and 5% on beef carcass tissues inoculated with 5 log CFU/cm$^2$ of *E. coli* O157:H7, Cutter and Siragusa *(68)* observed reductions of 1.0, 1.8 and 2.6 log cycles. Hardin et al. *(125)* applied 2% L-lactic acid at 55°C for 11 s on the surface of beef carcasses inoculated with approximately 5 log CFU/cm$^2$ of *E. coli* O157:H7 and *S.* Typhimurium using a fecal slurry as inoculation vehicle. Maximal reductions obtained were 1.5 and 2.6 log CFU/cm$^2$ for *E. coli* O157:H7 and *S.* Typhimurium, respectively. Under similar experimental conditions, Castillo et al. *(44)* reported that initial counts of *S.* Typhimurium and *E. coli* O157:H7 of 5 log CFU/cm$^2$ were reduced >2.6 and 2.2 log cycles, respectively, while APC, *Enterobacteriaceae,* coliforms and *E. coli* populations were reduced by 3.0, 2.6, 2.7 and >2.6 log CFU/cm$^2$, respectively. In another study conducted by Castillo et al. *(48)* a treatment consisting of 4% L-lactic acid at 55°C applied for 15 s on chilled beef carcasses reduced *E. coli* O157:H7 and *S.* Typhimurium in 1.9 and 2.4 log CFU/cm$^2$. The approximate concentration of the pathogens on the carcass surface before the application of the lactic acid treatment was approximately 3.8 and 4.3 log CFU/cm$^2$, respectively. In a study conducted by Gill and Badoni *(113)*, APC and coliforms naturally present on the surface of chilled beef carcasses were reduced in >2.0 and >1.5 log cycles, respectively, after treatment with 4% lactic acid applied at 7°C.

Several studies were later developed to evaluate the efficacy of lactic acid solutions to reduce microbial populations on beef carcasses under commercial slaughter operation conditions. Gill and Landers *(114)* evaluated the application of 2% lactic acid at 25°C using an automated cabinet at a rate of about 35 L/min as a pre-evisceration intervention at four beef packing plants. The treatment only produced reductions of <1.0 log CFU/cm$^2$ on APC and the apparent inefficacy was attributed to the poor coverage of the acid spray on the carcass sides. Dormedy et al. *(85)* validated the efficacy of carcass washes with 2% lactic acid (no more data about the treatment parameters were given) in a large slaughter facility. The intervention produced reductions of 0.9, 0.8 and 1.1 log CFU/cm$^2$ in mesophilic, coliforms and *E. coli* counts, respectively. A study conducted by Bosilevac et al. *(22)* in a large beef processing plant showed reductions of 1.6 and 1.0 log CFU/100 cm$^2$ on APC and *Enterobacteriaceae*, respectively, after the application of 2% lactic acid at 42°C using an automated cabinet. Castillo et al. *(47)* performed an in-plant evaluation of a treatment consisting of spraying 500 ml of 4% L-lactic acid at 55°C for 35 s on chilled beef carcasses. The treatment reduced APC by 3.0 to 3.2 log cycles from an initial mean count of 4.5 log CFU/100 cm$^2$. The initial counts of coliforms and *E. coli* ranged between <1.4 to 3.0 CFU/100 cm$^2$ and were consistently reduced to undetectable levels.

Lactic acid has been also proposed for decontamination of beef trimmings used for ground beef production. Reductions of 1.1, 1.8 and 1.5 log cycles in *E. coli* O157:H7, *S.* Typhimurium and APC, respectively, were obtained by immersion of beef trimmings in hot water at 95°C and subsequent immersion in 2% lactic acid at 55°C

*(94)*. The effect of lactic acid on the reduction of gram positive pathogens on beef carcass surfaces has been also studied. *L. monocytogenes* was reduced by 0.8, 1.4 and 2.7 log CFU/cm$^2$ after application of hot water (75°C), 2% lactic acid (55°C for 30 s) and a combination of both treatments, respectively *(142)*.

**Validation of interventions for pathogen reduction in beef slaughter operations.** According to the HACCP principles and regulations, slaughter establishments must implement verification procedures to confirm the HACCP plan is working effectively. Verification consist of those activities, other than monitoring that determine the validity of the HACCP plan and that the system is operating according to the plan *(225)*. Verification procedures include validation, which is defined as that element of verification focused on collecting and evaluating scientific and technical information to determine if the HACCP plan is effectively controlling the hazards *(177)*. An initial validation of the HACCP plan must be conducted; which consist of a review of the hazard analysis by the HACCP team to confirm that all hazards were identified and the implemented control measures are appropriate to control those hazards. Subsequent validation procedures must be periodically conducted or whenever a change in the process is introduced. Scientific studies that support the control measures adopted, as well as in-plant evaluations of control measures, provide useful information for validation procedures *(143)*.

According to these principles and regulations, the antimicrobial interventions implemented as control measures for pathogen reduction on beef carcasses must be

validated to demonstrate efficacy under in-plant operation conditions. Validation procedures for antimicrobial interventions are commonly conducted using indicator groups such as APC, coliforms, generic *E. coli*, yeast and molds *(45, 76, 85, 113, 137)*. Within these indicator groups, coliforms and *E. coli* provide an estimation of the reduction on microbial populations ecologically related to enteric pathogens such as *E. coli* O157:H7 and *Salmonella*. However, these indicators may have limitations in predicting *E. coli* O157:H7 reductions due to the unusual acid resistance this pathogen has demonstrated *(81)*. It has been demonstrated that coliforms from hay-fed animals are more acid sensitive than coliforms from grain-fed cattle, while *E. coli* O157:H7 is acid resistant, regardless of cattle diet *(121)*. Therefore, the use of coliforms or *E. coli* as indicators to validate interventions based on the application of organic acids for reducing *E. coli* O157:H7 may lead to an overestimation of the intervention efficacy. The identification of nonpathogenic microorganisms with similar characteristics to *E. coli* O157:H7 may provide a useful tool for the validation of antimicrobial interventions on beef carcasses as part of the HACCP plans.

**Surrogate organisms.** Surrogates are nonpathogenic microorganisms that respond to a particular treatment in a manner equivalent to a pathogenic microorganism *(103)*. Surrogate microorganisms have growth characteristics and resistance properties similar to those of their target pathogens. For this reason, surrogates are used to validate the efficacy of control measures in food systems, and contrary to their target pathogens,

they can be introduced in commercial processing facilities, where pathogens cannot be used due to safety concerns.

*Characteristics of surrogates.* Surrogate organisms must show certain desirable characteristics to be useful for validation studies, for example, they should have similar growth and attachment characteristics, as well as similar susceptibility to injury and inactivation kinetics to the target pathogen when exposed to processing parameters. Additionally, surrogate organisms should possess certain attributes that offer advantages for their isolation and enumeration during the validation studies: they should have stable and consistent growth characteristics, should provide high-density populations, should be easily differentiated from other microbiota, and should be easily enumerated using rapid, sensitive and inexpensive methods *(30)*.

Sporulated bacteria have been commonly used as surrogates to validate thermal processing treatments in canned foods, while viruses have a long history of utilization as surrogates to verify the efficiency of water decontamination treatments. *Clostridium sporogenes* and *Bacillus stearothermophilus* have been used as surrogates in the low-acid canning industry to validate the destruction of *C. botulinum* spores *(103)*. *C. perfringens,* coliphages and feline calicivirus have been used as surrogates for virus and parasites in the evaluation of decontamination treatments for drinking water and wastewater *(191, 230)*, and in survival and persistence studies in mussels *(128)*, strawberries, lettuce and ham *(165)*. Coliphages have also been studied as potential surrogates for human enteric viruses in the evaluation of high pressure treatments *(122)*,

and Rotavirus virus-like particles have been proposed as surrogates for the evaluation of the persistence of Rotavirus in shellfish-growing waters *(156)*.

*L. innocua* has been used as surrogate for *L. monocytogenes* to evaluate the inactivation effect of ultrahigh-pressure treatments in milk and orange juice *(25)* and also in the evaluation of high hydrostatic pressure and pulsed electric fields treatments for liquid whole egg *(34, 192)*. *B. amyloliquefaciens* has been proposed as surrogate for *C. botulinum* in the validation of thermal and high-pressure treatments for mashed carrots *(163)* and egg patty mince *(197)*, while *Geobacillus stearothermophilus* was used to validate the commercial sterilization of sweet-potato purees using a continuous-flow microwave processing *(26)*. *Enterococcus faecium* and *Pediococcus* spp. were proposed as surrogates for *L. monocytogenes* and *Salmonella* to validate thermal processing of ground beef *(159)*. Nonpathogenic *E. coli* strains have been proposed as surrogates for *E. coli* O157:H7 in the evaluation of antimicrobial treatments on beef carcasses *(164)*; as well as to conduct studies involving attachment and recoverability on chilled produce *(141)*, and to evaluate washing and sanitizing treatments on fresh fruits and vegetables *(90)*. However, the evaluation and use of surrogates for *E. coli* O157:H7 and *Salmonella* in validation studies is still limited.

*Surrogate organisms to validate interventions for pathogen reduction on beef carcasses*. In the validation of the efficacy of antimicrobial interventions to reduce enteric pathogens on beef carcasses, the utilization of surrogate organisms show several potential advantages: a) they can represent the behavior of the target pathogens, b) they can be easily recovered by using specific markers, c) they can be inoculated at high

concentrations and therefore, reductions can be tracked easier and d) they can be introduced to commercial slaughter facilities where pathogens cannot be used due to safety concerns *(30, 102)*.

The identification of appropriate surrogates for the validation of antimicrobial interventions on beef carcasses is a process that requires several steps: the isolation of potential surrogates from similar environments to those of target pathogens; the comparison of growth, attachment, and resistance properties between potential surrogates and target pathogens; the selection of a particular antimicrobial intervention to be validated; the selection of inoculation and enumeration methods for the validation procedure, and the comparison in the response of potential surrogates and target pathogens to the intervention under laboratory controlled conditions and during processing operations.

Previous studies to identify surrogates for *E. coli* O157:H7 for the verification of the effectiveness of antimicrobial treatments on beef carcasses have been conducted. Nonpathogenic *E. coli* strains isolated from cattle hides were identified as potential surrogates for *E. coli* O157:H7 because they showed similar heat resistance at 55 and 65°C in phosphate buffered saline and similar reductions on beef carcass tissues when they were treated with hot water and 2% L-lactic acid under laboratory controlled studies *(164)*. The identification of surrogates to validate antimicrobial interventions commonly used for pathogen reductions on beef carcasses, such as hot water washes and organic acid sprays, is a process that requires the isolation of nonpathogenic enteric bacteria closely related to the target pathogens, the evaluation of their attachment properties to

beef tissues, as well as the evaluation of their resistance to acid and heat. Additionally, the response of the potential surrogates to the hot water washes and lactic acid treatments on beef carcass surfaces needs to be evaluated under controlled conditions in the laboratory and also during in-plant operation conditions, in order to extrapolate the results obtained in laboratory studies to the actual conditions that exist on a carcass surface during commercial slaughter operations.

# MATERIALS AND METHODS

**Bacterial cultures.** Fourteen total microorganisms were included in this study: four *E. coli* biotype I, five *E. coli* O157:H7 and five *Salmonella* strains. The source and the main characteristics of these organisms are described below.

*Fluorescent protein-marked* E. coli *biotype I strains.* Four *E. coli* biotype I strains identified as #1, 3, 14 and 66 were used as potential surrogates. These strains were previously isolated from cattle hides and proposed as potential surrogates for *E. coli* O157:H7 based on collaborative studies conducted by Texas A&M University (College Station, TX) and Iowa State University (Ames, IA). Preliminary results indicated that these strains exhibited similar acid resistance at pH 3.0, 4.0 and 5.0, as well as similar thermal resistance at 55 and 65˚C, when compared to *E. coli* O157:H7 strains *(161, 164)*. These potential surrogates were sent to the *E. coli* Reference Center (Penn State University, University Park, PA) for determining the presence of genes encoding for virulence attributes, including: (i) STa, heat stable toxin a; (ii) Stx1, Shiga toxin type 1; (iii) Stx2, Shiga toxin type 2; (iv) CNF1, cytotoxic necrotizing factor 1; (v) CNF2, cytotoxic necrotizing factor 2; (vi) K99, fimbrial adhesion factor; (vi) *eae,* intimin-gamma; (vii) F1845, fimbriae; and (viii) CS31A, fimbriae. Virulence attributes were tested using the polymerase-chain-reaction (PCR) according to the procedure described by DebRoy and Maddox *(74).* All strains tested negative for the virulence attributes and were considered as nonpathogenic, a necessary attribute for surrogate organisms.

The nonpathogenic *E. coli* biotype I strains were later transformed to express fluorescent proteins and ampicillin resistance using the plasmid vectors pDsRed-Express, pEGFP and pEYFP (Clontech Laboratories Inc., Mountain View, CA). The pDsRed-Express is a prokaryotic expression vector that encodes DsRed-Express, a variant of *Discosoma* sp. red fluorescent protein (DsRed). The pEGFP vector carries a red-shifted variant of the *Aequorea victoria* green fluorescent protein (GFP) that has been optimized for brighter fluorescence and higher expression. The pEYFP vector encodes an enhanced yellow-green variant of *A. victoria* green fluorescent-protein (EYFP). Fluorescent proteins are expressed from the *lac* promoter and a pUC plasmid backbone provides a high-copy number origin of replication and an ampicillin resistance gene for propagation and selection of *E. coli*. Prior to the transformation procedure, electrocompetent cells of each strain were obtained by culturing in 9 ml of Luria Bertani broth Lennox (LB, Fisher Scientific, Fair Lawn, NJ) and incubating at 35ºC for 18 h. One ml of each culture was transferred to 100 ml of fresh LB and grown at 35ºC at 250 rpm in a Classic C76 water bath shaker (New Brunswick Scientific, Edison, NJ) for approximately 3 h until the optical density ($OD_{600}$) was 0.6-0.7. To harvest the cells, 50 ml of each culture were transferred into pre-chilled Falcon™ conical centrifuge tubes (Becton Dickinson and Co., Franklin Lakes, NJ), cooled in an ice-water bath for 10 min and centrifuged at 1,620 × *g* for 15 min. The supernatant was decanted and the pellet gently resuspended in 1 ml of pre-chilled 10% glycerol (Sigma-Aldrich Inc., St. Louis, MO) using a micropipette and a sterile pre-chilled tip. The cell suspension was resuspended in 25 ml of pre-chilled 10% glycerol and centrifuged again at 1,620 × *g* for

15 min. This procedure was repeated two more times and the final pellet was gently resuspended in 1 ml of pre-chilled 10% glycerol. The electrocompetent cells were dispensed in 100-µl aliquots in sterile pre-chilled microcentrifuge tubes using pre-chilled tips. The tubes were placed in ice-water bath for 15 min and then stored at -80ºC for further transformation procedures.

For transformation, both electrocompetent cells and plasmid vectors were defrosted in an ice-water bath for 15 min. Using pre-chilled pipette tips, 1 µl of each plasmid DNA was transferred to individual pre-chilled 0.1-cm gap Gene Pulser® Cuvettes (Bio-Rad Laboratories, Inc., Hercules, CA) and combined with 100 µl of the correspondent electrocompetent *E. coli* cells. Cells were electroporated at a voltage of 1.8 kV (E = 18 kV/cm) using a MicroPulser™ Electroporator Apparatus (Bio-Rad Laboratories, Inc.) *(20, 170)*. The cuvette was rapidly removed from the chamber and 1 ml of pre-warmed LB was immediately added. The cell suspension was incubated at 37°C for 1 h and 100 µl were surface plated on tryptic soy agar (TSA, BD Diagnostic Systems, Sparks, MD) supplemented with ampicillin (100 µl/ml, Sigma-Aldrich, Inc.) (TSA+Amp). Two decimal dilutions were performed from the remaining cell suspension using 0.1% peptone water (Bacto™ Peptone, BD Diagnostic Systems, Sparks, MD) (PW) and 100 µl of each dilution were surface plated on TSA+Amp. All plates were incubated at 35ºC for 24 h and colonies examined under UV light (365 nm). Those colonies with the stronger fluorescence were streaked on TSA+Amp plates containing 100mM isopropyl β-D-thiogalactoside (IPTG; Novagen EMB Biosciences, Inc., Madison, WI) and incubated at 37ºC for 24 h. Ten colonies from each strain were

transferred three consecutive times to select for strong and stable fluorescence. The transformed strains were identified as *E. coli* RFP-1, GFP-3, YFP-14 and YFP-66 and the characteristic of the fluorescent proteins expressed by each strain are presented in Table 3.

E. coli *O157:H7 strains.* Four *E. coli* O157:H7 strains identified as #2, 26, 36 and 38 were selected as target pathogens for comparison against the potential surrogates. These strains were selected from a collection of isolates previously obtained from cattle fecal samples, as part of a research conducted at the Texas A&M University Food Microbiology Laboratory (College Station, TX) *(214)*. Briefly, fecal samples from steers and heifers from two feeding operations in the Southern region of the U.S. were obtained rectally upon arrival of cattle at the feedlot, after approximately 70 days on feed, and before transport to the harvesting facility. BBL CultureSwabs™ (Becton Dickinson and Co., Franklin Lakes, NJ) were inserted into the anus of the animal and twisted to make contact with the sides of the rectum. The swabs were placed in the Cary Blair medium included in the collection system and transported in insulated coolers to the laboratory for isolation of *E. coli* O157:H7 *(238)*. Forty two *E. coli* O157:H7 isolates obtained as part of that study were used to select four strains that could function as target pathogens. The 42 isolates were sent to the *E. coli* Reference Center to be tested for the presence of genes encoding for virulence attributes by the polymerase-chain-reaction (PCR) according to the protocols described by DebRoy and Maddox *(74)*. The isolates were also subjected to molecular subtyping to determine relatedness between strains by pulse field gel electrophoresis (PFGE) following the procedure described by Gautom *(109)*.

TABLE 3. *Plasmid vectors used for transformation of* E. coli *biotype I strains and properties of the fluorescent proteins expressed*

| Microorganism[a] | Plasmid vector[b] | Fluorescent protein | Excitation maxima (nm) | Emission maxima (nm) |
|---|---|---|---|---|
| *E. coli* RFP-1 | pDsRed-Express | Red (tetrameric) | 557 | 579 |
| *E. coli* GFP-3 | pEGFP | Green (monomeric) | 488 | 507 |
| *E. coli* YFP-14<br>*E. coli* YFP-66 | pEYFP | Yellow (monomeric) | 513 | 527 |

[a] Identification name for the *E. coli* biotype I strains after transformation
[b] Plasmid vectors were introduced by electroporation *(20, 170)*

The *E. coli* O157:H7 strains #2, 26, 38 and 38 were selected as target pathogens according to the following criteria: a) they possess virulence factors for Shiga toxin production (*stx1* and/or *stx2*) and for intimin (*eae*), b) they show <70% genetic similarity, according the dendrogram constructed from the PFGE profiles (Figure 1), and c) they exhibited acid resistance at pH 3.5 when a screening acid challenge was performed in phosphate buffered saline (PBS; EMD Biosciences, Inc., San Diego, CA) at 37°C (see more details about this procedure in the acid resistance assay of the materials and methods section). Additionally, *E. coli* O157:H7 ATCC® 43895 strain EDL933 was also included as a target pathogen for comparison against the potential surrogates; this strain was originally isolated from raw hamburger meat implicated in a hemorrhagic colitis outbreak *(252).*

Salmonella *strains*. Four *Salmonella* strains corresponding to serotypes Agona, Anatum, Montevideo and Typhimurium from the Texas A&M University Food Bacteriology Laboratory collection were selected as target pathogens for comparison against the potential surrogates. These serotypes represent some of the most common serotypes isolated from cattle and raw meat according to CDC and USDA reports *(54, 240). S.* Senftenberg ATCC® 43845 strain 775W was included in the study due to its high heat resistance, comparing to other *Salmonella* serotypes *(179).*

All strains were maintained at -80ºC in cryocare vials (Key Scientific Products, Round Rock, TX) and stock working cultures were prepared by transferring one bead from frozen cryocare vials to TSA or TSA+Amp slants for propagation of pathogenic or

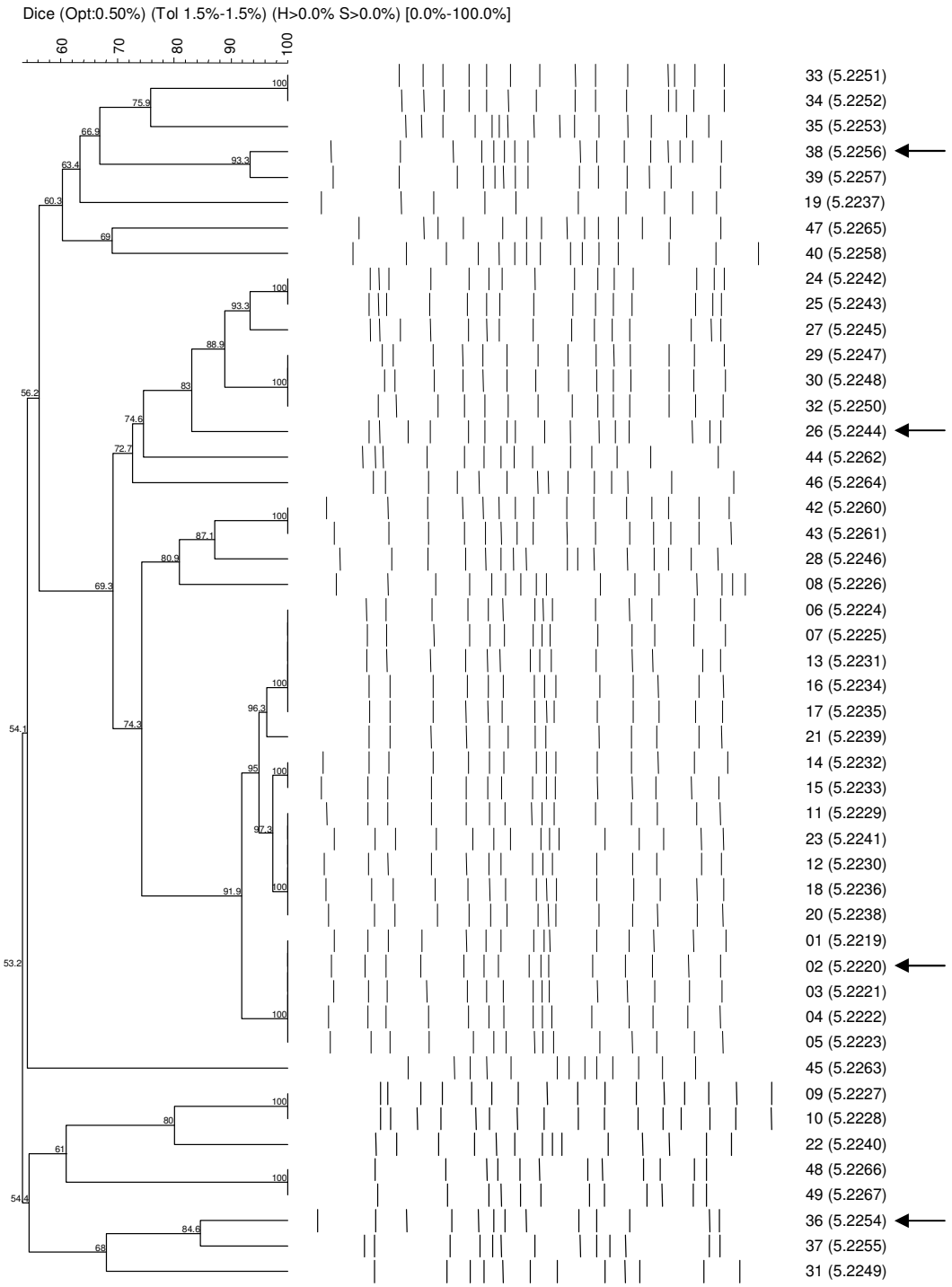


FIGURE 1. *Dendrogram based on PFGE profiles of* E. coli *O157:H7 isolates. The arrows indicate those isolates selected as target pathogens for comparison studies against potential surrogates.*

fluorescent protein-marked strains, respectively. Slants were incubated at 35ºC overnight and stock cultures were kept at room temperature for 2-3 weeks.

**Evaluation and comparison of growth characteristics and resistance properties among potential surrogates and target pathogens.** The growth characteristics, as well as the resistance properties to acid, heat and low temperatures among fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains were evaluated and compared.

*Growth characteristics.* The growth parameters of parent and fluorescent protein-marked *E. coli* strains were studied to determine if fluorescent protein expression had affected the growth characteristics of transformed cultures. In a separate experiment, the growth parameters of *E. coli* O157:H7 and *Salmonella* strains were compared to those of fluorescent protein-marked *E. coli* strains. For both experiments, each strain was individually cultured in tryptic soy broth (TSB, BD Diagnostic Systems) and incubated at 37°C for 18 h. Dilutions were made for each culture using 0.1% PW and 0.1 ml of the dilution 1:10,000 were transferred to tubes containing 10 ml of fresh TSB to achieve an initial concentration of 3 log CFU/ml. The inoculated tubes were incubated at 37°C in a constant temperature water bath (Magni Whirl, Blue M, Blue Island, IL). Over a 12-h period, one tube from each strain was removed from the water bath each hour for enumeration. Appropriate decimal dilutions performed in 0.1% PW were plated on TSA using a Whitley automated spiral plater (DW Scientific, Frederick, MD). All plates were

incubated at 37°C and after 24 h the colonies were enumerated using a Protos counter (Synoptics Ltd., Frederick, MD). Each growth curve was performed in triplicate.

Growth data (log CFU/ml) were plotted as a function of time and growth parameters such as initial population ($N_0$), maximum population density ($N_{max}$), lag phase time (t-lag), doubling time (t-d) and maximum specific growth rate (μmax) were estimated using the MicroFit software *(132).* The general linear model procedure of the Statistical Analysis System (SAS) (SAS Institute, Inc., Cary, NC) was used for data analysis *(212).* Multiple comparison procedures were conducted to determine differences among means when comparing growth parameters between parent and transformed *E. coli* strains, and also between pathogens and potential surrogates. Additionally, the biochemical profiles of both parent and fluorescent-protein marked *E. coli* strains were determined using the Vitek® System (bioMerieux, Inc., Durham, NC) and compared to verify that no major characteristics were affected by the transformation procedure.

*Acid resistance.* The acid resistance of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains was determined and compared. Each strain was individually cultured in TSB at 37ºC for 18 h and 0.1-ml aliquots of these fresh cultures were individually transferred to 10-ml TSB tubes supplemented with 1% glucose (TSB+G) to induce an acid tolerance response according to the procedure described by Buchannan and Edelson *(28).* After incubation at 37°C for 18 h, each acid-adapted culture was transferred to Falcon™ conical centrifuge tubes and cells were harvested by centrifugation at 1,620 × *g* for 15 min. The supernatant was discarded and the pellet resuspended in 10 ml of PBS (pH 7.4). Each cell suspension was centrifuged again

(1,620 × *g* for 15 min) and the procedure was repeated two more times. The final pellets were resuspended in 10 ml of PBS. Aliquots of 0.1 ml of each cell suspension were individually transferred into glass tubes (12 × 75 mm) containing 10 ml of pre-warmed (37°C) PBS at pH values of 2.5, 3.0 and 3.5. The pH of the PBS was previously adjusted with 88% L-lactic acid (Purac Inc., Arlington Heights, IL) using a pH meter (Orion 230A, Thermo Electro Co., Beverly, MA). All tubes were incubated at 37ºC in a constant temperature water bath (Magni Whirl, Blue M) with the water level at least 1 cm above the level of the cell suspension in each tube. One tube of each strain was removed from the water bath immediately after inoculation and every 0.5, 1.0, 1.5 and 2.0 h for enumeration of survivors. Enumeration was performed by plating appropriate decimal dilutions on TSA using the spiral plating technique. Colonies were enumerated after incubation of plates at 37°C for 24-36 h. Experiments were conducted in triplicate.

Log reduction (CFU/ml) values were calculated by subtracting the log count (CFU/ml) of each microorganism after each exposure time from the initial log count obtained at time zero. The general linear model procedure of SAS was used for data analysis *(212).* Multiple comparison procedures were conducted to determine differences among means when comparing log reductions between pathogens and potential surrogates.

*Thermal resistance.* The thermal resistance of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains was determined and compared. Each strain was individually cultured in TSB at 37°C for 18 h. Aliquots of 0.1 ml of each culture were individually transferred to 10 ml of TSB+G and incubated at 37°C for 18 h. Each culture

was then transferred to Falcon™ conical centrifuge tubes and the cells were harvested by centrifugation at 1,620 × *g* for 15 min. The supernatant was discarded and the pellet resuspended in 10 ml of PBS (pH 7.4). The cell suspension was centrifuged again (1,620 × *g* for 15 min) and the procedure was repeated two additional times. The final pellets were resuspended in 10 ml of PBS and 50 µl of each cell suspension were individually inoculated into capillary tubes (100 × 1.0 mm, Kontes Glass Co., Vineland, NJ). The capillary tubes were flame-sealed and submerged in a water bath (New Brunswick Scientific Co., Inc., Edison, NJ) at 55, 60 and 65 ± 0.5°C. At pre-determined time intervals, one capillary tube of each microorganism was removed and dipped in pre-chilled 70% ethanol for 30 s and then rinsed in pre-chilled sterile water in an ice-water bath. Each capillary tube was transferred into a Falcon™ conic centrifuge tube containing 10 ml of PBS and crushed using a sterile glass rod. Decimal dilutions were performed in 0.1% PW as necessary for the enumeration of survivors on TSA using the spiral plating technique. All plates were incubated at 37°C and colonies counted after 24-36 h.

The survivors data (log CFU/ml) were plotted as a function of time and D-values were calculated as the reciprocal of the slope obtained by linear regression using Microsoft®Excel *(169)*. Log D-values were plotted as a function of temperature, and *z*-values for each microorganism were calculated as the reciprocal of the slope obtained by linear regression. Each experiment was performed in triplicate. The general linear model procedure of SAS was used for data analysis *(212)*. Multiple comparison procedures

were conducted to determine differences among means when comparing D- and z-values between pathogens and potential surrogates.

*Resistance to low temperatures.* The resistance properties of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains to low temperatures was investigated and compared. Each strain was individually cultured in TSB at 37°C for 18 h. Aliquots of 0.1 ml of each culture were individually transferred to 10 ml of TSB and TSB+G and incubated at 37°C for 18 h. Each culture was then transferred to Falcon™ conical centrifuge tubes and the cells were harvested by centrifugation at 1,620 × *g* for 15 min. The supernatant was discarded and the pellet resuspended in 10 ml of PBS (pH 7.4). The cell suspension was centrifuged again (1,620 × *g* for 15 min) and the procedure was repeated two additional times. The final pellets were resuspended in 100 ml of PBS and 1-ml aliquots of each bacterial suspension were individually transferred to sterile microcentrifuge tubes. Fifty percent of the tubes (n=196) were stored at 4 ± 0.5ºC (refrigeration) and the remaining 50% (n=196) at -18 ± 0.5ºC (freezing). After 0, 7, 14, 21, 28, 60 and 90 d of storage, one tube of each culture was removed from refrigerated and frozen storage for enumeration of survivors. Frozen cultures were defrosted in ice water for 30 min before enumeration. Decimal dilutions were performed in 0.1% PW and plated on TSA using the spiral plating technique. Plates were incubated at 37ºC for 24 h before colonies were enumerated. The experiment was conducted in duplicate.

Log reductions (CFU/ml) values were calculated by subtracting the log count (CFU/ml) of each microorganism after each storage time from the initial log count obtained at day zero. The general linear model procedure of SAS was used for data

analysis *(212)*. Multiple comparison procedures were conducted to determine differences among means when comparing log reductions between microorganisms at different temperatures and growth conditions.

**Evaluation and comparison of cell surface hydrophobicity and attachment properties among potential surrogates and target pathogens.** The cell surface hydrophobicity and the attachment properties to beef carcass surfaces among fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains was investigated and compared.

*Cell surface hydrophobicity.* The cell surface hydrophobicity of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains was determined using the semi-quantitative bacterial adhesion to hydrocarbons assay (BATH) as described by Sweet et al. *(228)*. Each strain was individually cultured in TSB at 37°C for 18 h. Aliquots of 0.1 ml of each culture were individually transferred to 10 ml of TSB and TSB+G and incubated at 37°C for 18 h. Each culture was then transferred to Falcon™ conical centrifuge tubes and the cells were harvested by centrifugation at 1,620 × *g* for 15 min. The supernatant was discarded and the pellet resuspended in 10 ml of PBS (pH 7.4). The cell suspension was centrifuged again (1,620 × *g* for 15 min) and the procedure was repeated two additional times. The final pellets were resuspended in PBS to an approximate absorbance of 1.0. For each strain, triplicate test tubes containing 4 ml of the washed cells were prepared. A volume of 1 ml of *p*-xylene (Sigma-Aldrich, Inc.) was added to two bacterial assay tubes and the tube without *p*-xylene was used as control.

Tubes were allowed to equilibrate at 37°C for 10 min in a water bath (New Brunswick Scientific Co., Inc.), vortex mixed and then incubated at 37°C for 30 min in the water bath. After incubation, the aqueous layer from the assay tubes was transferred to clean tubes and *p*-xylene residues were removed by mixing in a vortex at high speed for 2 min. From the bottom of each tube, 1-ml aliquots of the bacterial suspensions were transferred to VWR® disposable cuvettes (VWR International, West Chester, PA). Optical density was measured at 520 nm using PBS as blank for calibration in a BioMate™ 3 Spectrophotometer (Thermo Electron Co., Pittsford, NY). The experiment was repeated in two different occasions.

The absorbance ratio of the bacterial assay tubes ($A_b$) to the bacterial control ($A_c$) was calculated and expressed as bacterial adhesion to hydrocarbon: %BATH = ($A_c - A_b$)/$A_c \times 100$. The general linear model procedure of SAS was used for data analysis *(212)*. Multiple comparison procedures were conducted to determine differences among means when comparing BATH values between potential surrogates and pathogens.

*Attachment to beef carcass surfaces.* The attachment properties to carcass surfaces of *E. coli* O157:H7, *Salmonella* and fluorescent protein-marked *E. coli* strains were investigated and compared. For this study, rifampicin-resistant mutants derived from parent strains of *E. coli* O157:H7 #2, 26, 36, 38, 43895, and from *S.* Agona, Anatum, Montevideo, Typhimurium and Senftenberg were obtained by the procedure described by Kaspar and Tamplin *(139)*. Each rifampicin-resistant pathogen as well as each fluorescent protein-marked *E. coli* strain was individually cultured in TSB at 37°C for 18 h for two consecutive days. A bacterial cocktail was prepared by mixing equal

volumes of each culture and the final concentration of each organism in the cocktail was approximately 8 log CFU/ml. To prepare the bacterial inoculum for the carcass surfaces, bovine feces were randomly collected from cattle in holding pens at the Texas A&M University ASTREC Beef Center (College Station, TX) at each day of the study. Feces were dispensed in 10-g portions to individual stomacher plastic bags. Ten ml of the bacterial cocktail containing the rifampicin-resistant pathogens and fluorescent protein-marked *E. coli* surrogates were transferred to each bag of feces and the mixture was hand-kneaded for 1 min. The final concentration of each organism in the fecal slurry was approximately 8 log CFU/g. A preliminary study was conducted to verify that the fluorescent protein-marked *E. coli* strains maintain stable characteristics when they are inoculated in bovine fecal slurries. Concentration of each strain and fluorescence properties were stable in fecal slurries after 8 h of storage at room temperature. Previous reports have shown that rifampicin-resistant mutants of *E. coli* O157:H7 and *S.* Typhimurium were also stable when inoculated in fecal slurries *(44)*.

Cattle typical of those entering the U.S. meat supply were transported to the Texas A&M University Rosenthal Meat Science and Technology Center (RMSTC, College Station, TX) abattoir where they were slaughtered and dressed following USDA-FSIS regulated procedures *(234, 235)*. At each day of the study, the outside round, brisket and clod of one carcass side were removed subsequent to carcass splitting and before washing. The carcass regions were placed in one insulated cooler and transported to the Food Microbiology Laboratory located in the adjacent building. Immediately upon arrival, a 400-$cm^2$ area of each carcass region was inoculated with the

fecal slurry using a sterile spatula. Thirty minutes after inoculation, loose and strong attachment of cells was determined.

To enumerate loosely attached cells, three composite samples were collected from the inoculated carcass surface region. Each composite sample consisted of three 10-$cm^2$ pieces excised from the inoculated carcass surface using a sterile surgical blade. One of the composite samples was used as a positive control for enumeration of the total number of microorganisms inoculated on the carcass surface regions. The other two composite samples were placed into polypropylene wide-mouth bottles containing 99 ml of 0.1% PW and gently mixed by 15 inversion movements. Loosely attached cells in the peptone rinse were enumerated. For enumeration of strongly attached cells, the excised samples were removed from each plastic bottle, transferred to stomacher bags with 99 ml of sterile 0.1% PW and pummeled using a stomacher lab blender (A.J. Seward, London, UK) for 2 min. Strongly attached cells in the peptone rinse were enumerated. Rifampicin-resistant pathogens were enumerated on lactose-sulfite-phenol red-rifampicin (LSPR) agar *(44)*, and fluorescent protein-marked potential surrogates were enumerated on TSA+Amp. The experiment was repeated on three different occasions. The proportion of the total bacterial population which is physically attached to the surface ($S_R$) was calculated as follows: $S_R$ = (strongly attached cells)/(strongly attached cells + loosely attached cells) *(79)*. The general linear model procedure of SAS was used for data analysis *(212)*. Multiple comparison procedures were conducted to determine differences among means when comparing loosely and strongly attached cells of pathogens and potential surrogates to carcass surfaces as well as $S_R$ values.

**Evaluation and comparison of the response of potential surrogates and target pathogens to interventions on beef carcasses.** The response of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains to interventions commonly used in beef slaughter operations, such as hot water washes and lactic acid sprays, was investigated and compared.

*Hot water interventions.* Two rifampicin-resistant mutants derived from parent strains of *S.* Typhimurium and *E. coli* O157:H7 were used as marker pathogens while the fluorescent protein-marked *E. coli* biotype I strains were used as marker surrogates. Each microorganism was individually cultured in TSB at 35°C for 18 h for two consecutive days to obtain stationary phase cultures. A bacterial cocktail was prepared by mixing 1 ml of each culture with 94 ml of 0.1% PW. To prepare the bacterial inoculum, bovine feces were randomly collected from cattle in holding pens at the Texas A&M ASTREC Beef Center at each day of the study and dispensed in 20-g portions to individual stomacher plastic bags. Twenty ml of the bacterial cocktail containing the rifampicin-resistant pathogens and fluorescent protein-marked *E. coli* surrogates were transferred to each bag of feces and the mixture was hand-kneaded for 1 min. The final concentration of each organism in the fecal slurry was approximately 7 log CFU/g.

On each day of the study, the outside rounds from three beef carcass sides were removed subsequent to carcass splitting and before washing, as previously described. The outside rounds were placed in insulated coolers and transported to the Texas A&M University Food Microbiology Laboratory (located in the adjacent building) to be inoculated and treated. Immediately upon arrival, each outside round was randomly

assigned to a hot water treatment and the entire surface was inoculated with the fecal slurry using a sterile spatula. The initial concentration of each organism on the meat surface was approximately 6 log CFU/$cm^2$.

After inoculation, a water wash (25°C) was applied to each outside round using a polyethylene hand sprayer (Ortho® Heavy Duty Sprayer, The Fountainhead Group, Inc., New York Mills, NY)) for 150 s to remove gross fecal contamination. Each outside round was then sectioned into five pieces of approximately 300 $cm^2$. Each inoculated piece was randomly assigned to receive the hot water treatment. Treatments evaluated were: a) hot water at 76.3 ± 1.1ºC for 0, 2, 4, 6, 8 and 10 s; b) hot water at 86.0 ± 0.2ºC for 0, 2, 3, 5, 7 and 9 s and c) hot water at 97.1 ± 0.9ºC for 0, 1, 2, 3, 4 and 5 s. The treatments were applied in a model spray cabinet (Chad Company, Lenexa, KS) using a flat spray nozzle at a pressure of 30 lb/$in^2$ from a distance of approximately 10 cm. The temperature of the water at the source and at the nozzle opening was measured using type K thermocouples connected to a total-range digital thermometer (VWR International, West Chester, PA). The temperature of the carcass surface during the treatment was measured with a type K thermocouple inserted just below the meat surface and connected to the same type of thermometer previously described. The experiment was replicated three times. From each treatment, two composite samples were collected from the treated carcass surface. Each composite sample consisted of two 10-$cm^2$ pieces excised with a sterile surgical blade. The composite samples were placed into stomacher bags and pummeled with 99 ml of 0.1% PW in a stomacher for 1 min. Appropriate decimal dilutions were plated on LSPR for enumeration of *E. coli* O157:H7 and

*S.* Typhimurium *(45)*, and on TSA+Amp for enumeration of fluorescent protein-marked *E. coli* surrogates. All plates were incubated at 37ºC and enumerated after 24 to 36 h.

The survivor data (log CFU/cm$^2$) from each treatment were plotted as a function of time and D-values calculated as the reciprocal of the slope obtained by linear regression using Microsoft® Excel *(169)*. Log D-values were plotted as a function of temperature and the curve fitted by linear regression. Theoretical D-values at different temperatures were obtained from the regression equations and the time required to achieve a 5-log reduction (TD5) for pathogens and surrogates was calculated. The general linear model procedure of SAS was used for data analysis. Multiple comparison procedures were conducted to determine significant differences when comparing D-values among organisms and treatments *(212)*.

*Lactic acid interventions.* Rifampicin-resistant mutants derived from parent strains of *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895 and also from *Salmonella* Agona, Anatum, Montevideo, Typhimurium and Senftenberg were used as marker pathogens. The fluorescent protein-marked *E. coli* biotype I strains were used as marker surrogates. Each microorganism was individually cultured in TSB at 35°C for 18 h for two consecutive days to obtain stationary phase cultures. A bacterial cocktail was prepared by mixing 1 ml of each culture with 86 ml of 0.1% PW. To prepare the bacterial inoculum, bovine feces were randomly collected from cattle in holding pens at the Texas A&M University Beef Center at each day of the study and dispensed in 20-g portions to individual stomacher plastic bags. Twenty ml of the bacterial cocktail containing the rifampicin-resistant pathogens and fluorescent protein-marked *E. coli*

surrogates were transferred to each bag of feces and the mixture was hand-kneaded for 1 min. The final concentration of each organism in the fecal slurry was approximately 7 log CFU/g.

On each day of the study, the outside rounds from two carcass sides were removed subsequent to carcass splitting and before washing, as previously described. The outside rounds were placed in insulated coolers and transported to the Texas A&M University Food Microbiology Laboratory to be inoculated and treated. After inoculation, a water wash (25°C) was applied to each outside round using a polyethylene hand sprayer for 150 s to remove gross fecal contamination. Each outside round was then sectioned into four pieces of approximately 300 $cm^2$. Each inoculated piece was randomly assigned to receive a lactic acid treatment. Treatments evaluated were: a) 2% L-lactic acid at 25ºC for 0, 2, 4, 6 and 8 s and b) 2% L-lactic acid at 55ºC for 0, 2, 4, 6 and 8 s. The treatments were applied in a model spray cabinet (Chad Company) using a flat spray nozzle at a pressure of 30 $lb/in^2$ from a distance of approximately 15 cm. Carcass surface pH was determined after application of each treatment. From each treatment, two composite samples were collected from the treated carcass surface. Each composite sample consisted of two 10-$cm^2$ pieces excised with a sterile surgical blade. The composite samples were placed into stomacher bags and pummeled with 99 ml of 0.1% PW in a stomacher for 1 min. Appropriate decimal dilutions were plated on LSPR for enumeration of *E. coli* O157:H7 and *Salmonella*, and on TSA+Amp for enumeration of fluorescent protein-marked *E. coli* surrogates. All plates were incubated at 37ºC and enumerated after 24-36 h. Experiments were performed in triplicate.

Reductions (log CFU/cm$^2$) of surrogates and pathogens after each treatment were calculated and compared among microorganisms. The general linear model procedure of SAS was used for data analysis. Multiple comparison procedures were conducted to determine significant differences for mean log reductions among organisms and treatments *(212)*.

**Validation of interventions in commercial beef slaughter establishments using fluorescent protein-marked *E. coli* strains as surrogates for enteric pathogens.** To evaluate the performance of the potential surrogates in validation studies at commercial food processing environments, six small beef slaughter establishments located in the State of Texas were selected to conduct this study. At each establishment, six beef carcasses from cattle harvested and dressed following USDA-FSIS-regulated commercial procedures *(234, 235)* were utilized to evaluate the efficacy of the antimicrobial intervention(s) implemented as part of their HACCP Plans. After trimming of carcasses and prior to the water wash, the neck region of each carcass side was inoculated with a fecal slurry. One carcass side ("A") was inoculated with a fecal slurry containing the fluorescent protein-marked *E. coli* surrogates. The other side ("B") was inoculated with a fecal slurry that did not contain the surrogates and it was used to evaluate the effect of interventions on traditional indicator organisms naturally present in feces, such as APC, coliforms and *E. coli* biotype I.

To prepare the fecal slurries, feces were randomly collected from cattle in the holding pens each slaughter day and dispensed in 10-g portions in twelve stomacher

bags. In six bags, 10 ml of a bacterial cocktail containing the fluorescent protein-marked surrogates previously cultured in TSB for 18-24 h were added. The final concentration of each fluorescent protein-marked *E. coli* strain in each fecal slurry was approximately 7 log CFU/g. For the remaining bags, 10 ml of sterile 0.1% PW were added. The fecal slurries were spread on the surface of the neck area of the correspondent carcass side and a water wash at room temperature was applied for 90 s using a hand-held polyethylene sprayer, until visible feces were removed. Excised samples (10-$cm^2$ × 2-mm thick) were collected from the inoculated neck area of each carcass side before and after the carcass sides were water washed and after the application of L-lactic acid sprays implemented as antimicrobial interventions.

After the sampling process was concluded, the inoculated neck area of each carcass side was trimmed and discarded as inedible. All samples were placed inside stomacher bags, packed into insulated coolers (32×61 cm, Igloo Products Corp., TX) with ice packs (Polyfoam Packers Corp., Wheeling, IL) and transported to the Texas A&M Food Microbiology Laboratory for analysis. Additional information was collected at each slaughter establishment, including pH and carcass surface temperature before and after water washes and interventions, temperature and application time of water washes, type, concentration and application time of chemical solutions. For microbiological analysis, each sample was pummeled with 99 ml of 0.1% PW in a stomacher lab blender for 1 min. Fluorescent protein-marked *E. coli* surrogates were enumerated by plating appropriate dilutions on TSA+Amp while APC, *E. coli* and

coliforms were enumerated on 3M™ Petrifilm™ Aerobic Count and 3M™ Petrifilm™ *E.coli*/Coliforms Count plates, respectively (3M, St. Paul, MN).

Log reductions (CFU/cm$^2$) for each microbial group were calculated by subtracting the log counts (CFU/cm$^2$) obtained after water washes and after antimicrobial interventions from the initial log count (CFU/cm$^2$) obtained before any treatment was applied. The general linear model procedure of SAS was used for data analysis *(212)*. Multiple comparison procedures were conducted to determine differences among means when comparing log reductions of microbial populations.

# RESULTS AND DISCUSSION

## Growth characteristics of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains.

Growth of surrogate organisms must be equivalent or slightly greater but not less than target pathogens, in order to be useful in predicting the behavior of pathogens during processing or in analytical situations *(102)*. It has been previously suggested that fluorescent protein-marked microorganisms may have slower growth rates after transformation with high-copy plasmids and this may compromise their value as surrogate organisms *(190)*. Rang et al. *(198)* reported that the production of a green fluorescent protein (GFP) had a fitness cost in enteric bacteria transformed with a high-copy plasmid (pGEN91) by increasing their doubling time; the effect was dose-dependent (increased with the amount of GFP expressed) and it was variable across different bacterial strains. Lissemore et al. *(154)* observed poor growth in *E. coli* strains expressing GFP in liquid cultures, but the effect was not seen when the same strains were grown in agar. However, other authors have reported no adverse effects in growth characteristics of microorganisms expressing GFP after transformation with different types of plasmid vectors. Growth rates and motility of *Pseudomonas aeruginosa* and *P. fluorescens* with and without GFP-expressing plasmids were identical, suggesting that there is not a significant burden to the cells carrying these plasmids *(21)*. According to Vialette et al. *(246)* the transformation of *E. coli* O157:H7 strains with the pBAD-GFPuv plasmid vector had negligible effect on the growth capacities of the transformed strains. When *E. coli* and *Salmonella* strains were transformed using the suicide vector pAG408

to express a green fluorescent protein, Noah et al. *(181)* found no major differences in growth kinetics among the parent and transformed strains.

When growth parameters were compared between parent and fluorescent protein-marked *E. coli* strains proposed as surrogates in this study, no differences were observed in the maximum specific growth rate and doubling time ($P>0.05$), indicating that expression of fluorescent proteins did not resulted in a fitness cost for the transformed organisms (Table 4). A significant increase in the lag time was observed only between *E. coli* parent #66 and the corresponding transformed strain ($P<0.05$). On the other hand, the biochemical profiles of parent and transformed *E. coli* strains showed no major differences, suggesting that the insertion of the plasmids did not produced additional phenotypical changes. Other studies have also reported that pGFP-bearing *E. coli* strains were indistinguishable from their parent strains in biochemical, immunological and multiple PCR tests *(105)*.

After comparing growth parameters between fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* strains, it was observed that the initial population, maximum population density and lag time were not different ($P>0.05$) (Table 5). However, *S*. Montevideo and *S*. Typhimurium showed lower growth rate and doubling time than the fluorescent protein-marked *E. coli* strains ($P<0.05$). Those differences may constitute a limitation for the use of these fluorescent protein-marked *E. coli* strains as surrogates for *Salmonella* in studies involving growth measurements; but may not represent a limitation for using the fluorescent protein-marked *E. coli* strains as surrogates in the validation of antimicrobial interventions on beef carcasses, since the

TABLE 4. *Growth parameters for parent and fluorescent protein-marked* E. coli *strains in tryptic soy broth at 37ºC*

| | Mean[a] ± SD[b] | | | | |
|---|---|---|---|---|---|
| Microorganism | $N_0$ (log CFU/ml) | $N_{max}$ (log CFU/ml) | μmax ($h^{-1}$) | t-lag (h) | t-d (h) |
| *E. coli* parent-1 | 3.5 ± 0.2 AB[c] | 9.5 ± 0.2 A | 2.1 ± 0.2 A | 0.3 ± 0.3 AB | 0.33 ± 0.03 A |
| *E. coli* RFP-1 | 3.2 ± 0.2 AB | 9.3 ± 0.1 A | 2.0 ± 0.2 A | 0.9 ± 0.1 AB | 0.35 ± 0.03 A |
| *E. coli* parent-3 | 3.4 ± 0.1 AB | 9.5 ± 0.2 A | 2.0 ± 0.1 A | 0.0 ± 0.5 B | 0.34 ± 0.02 A |
| *E. coli* GFP-3 | 3.5 ± 0.3 AB | 9.4 ± 0.2 A | 2.2 ± 0.1 A | 0.5 ± 0.6 AB | 0.32 ± 0.02 A |
| *E. coli* parent-14 | 3.6 ± 0.1 A | 9.4 ± 0.2 A | 2.2 ± 0.2 A | 0.4 ± 0.4 AB | 0.32 ± 0.03 A |
| *E. coli* YFP-14 | 3.6 ± 0.1 A | 9.4 ± 0.1 A | 2.4 ± 0.2 A | 0.7 ± 0.5 AB | 0.30 ± 0.03 A |
| *E. coli* parent-66 | 3.5 ± 0.0 AB | 9.5 ± 0.1 A | 2.2 ± 0.6 A | 0.1 ± 0.3 B | 0.32 ± 0.04 A |
| *E. coli* YFP-66 | 2.9 ± 0.6 B | 9.4 ± 0.1 A | 2.2 ± 0.1 A | 1.4 ± 0.3 A | 0.32 ± 0.02 A |

$N_0$ = initial bacterial cell density; $N_{max}$ = final bacterial cell density; μmax = maximum specific growth rate; t-lag = lag time; t-d = doubling time

[a] Mean values were obtained from three independent replicates

[b] Standard deviation

[c] Means in the same column with the same letter (ABC) are not different ($P$>0.05)

TABLE 5. *Growth parameters for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *strains in tryptic soy broth at 37ºC*

| | Mean[a] ± SD[b] | | | | |
|---|---|---|---|---|---|
| Microorganism | $N_0$ (log CFU/ml) | $N_{max}$ (log CFU/ml) | μmax ($h^{-1}$) | t-lag (h) | t-d (h) |
| *E. coli* RFP-1 | 3.0 ± 0.2 A[c] | 9.1 ± 0.1 A | 2.7 ± 0.2 A | 0.9 ± 0.4 A | 0.26 ± 0.02 A |
| *E. coli* GFP-3 | 3.1 ± 0.1 A | 9.1 ± 0.2 A | 2.6 ± 0.2 AB | 0.7 ± 0.4 A | 0.27 ± 0.03 A |
| *E. coli* YFP-14 | 3.2 ± 0.0 A | 9.3 ± 0.1 A | 2.7 ± 0.1 A | 0.9 ± 0.2 A | 0.25 ± 0.01 A |
| *E. coli* YFP-66 | 3.0 ± 0.2 A | 9.1 ± 0.1 A | 2.9 ± 0.4 A | 1.0 ± 0.2 A | 0.25 ± 0.03 A |
| *E. coli* O157:H7-2 | 3.2 ± 0.1 A | 9.2 ± 0.1 A | 2.7 ± 0.3 A | 1.4 ± 0.3 A | 0.26 ± 0.03 A |
| *E. coli* O157:H7-26 | 3.1 ± 0.1 A | 9.3 ± 0.1 A | 2.7 ± 0.2 A | 1.2 ± 0.4 A | 0.26 ± 0.02 A |
| *E. coli* O157:H7-36 | 3.2 ± 0.1 A | 9.2 ± 0.1 A | 2.6 ± 0.2 AB | 1.4 ± 0.4 A | 0.27 ± 0.03 A |
| *E. coli* O157:H7-38 | 2.9 ± 0.1 A | 9.1 ± 0.1 A | 2.6 ± 0.1 AB | 1.3 ± 0.1 A | 0.27 ± 0.02 A |
| *E. coli* O157:H7-43895 | 3.0 ± 0.1 A | 9.2 ± 0.1 A | 2.5 ± 0.1 ABC | 1.4 ± 0.2 A | 0.28 ± 0.02 A |
| *S.* Agona | 3.2 ± 0.1 A | 9.2 ± 0.1 A | 2.6 ± 0.2 AB | 1.2 ± 0.1 A | 0.27 ± 0.02 A |
| *S.* Anatum | 3.0 ± 0.3 A | 9.2 ± 0.1 A | 2.5 ± 0.3 ABC | 1.1 ± 0.2 A | 0.28 ± 0.03 A |
| *S.* Montevideo | 3.2 ± 0.4 A | 9.2 ± 0.2 A | 2.0 ± 0.1 BC | 1.0 ± 0.3 A | 0.35 ± 0.02 B |
| *S.* Typhimurium | 2.9 ± 0.2 A | 9.0 ± 0.2 A | 1.9 ± 0.1 C | 0.6 ± 0.3 A | 0.36 ± 0.02 B |
| *S.* Senftenberg | 3.4 ± 0.6 A | 9.1 ± 0.1 A | 2.3 ± 0.1 ABC | 1.2 ± 0.1 A | 0.30 ± 0.01 AB |

$N_0$ = initial bacterial cell density; $N_{max}$ = final bacterial cell density; μmax = maximum specific growth rate; t-lag = lag time; t-d = doubling time

[a] Mean values were obtained from three independent replicates

[b] Standard deviation

[c] Means in the same column with the same letter (ABC) are not different ($P$>0.05)

pathogenic strains multiply at a slower rate and, more importantly, since microbial growth on carcasses is not expected during the slaughter/chill process. In general, the potential surrogates showed similar growth parameters to *E. coli* O157:H7 and some *Salmonella* strains, which is an important attribute when considering the identification of appropriate surrogate organisms.

**Acid resistance of fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* strains.** Surrogate organisms must have similar behavior and susceptibility to injury to that of target pathogens when exposed to processing parameters *(30, 102)*. Chemical decontamination treatments implemented in beef slaughter operations frequently include the application of organic acids such as lactic acid, which has demonstrated to be effective in reducing *E. coli* O157:H7 and *Salmonella* populations on the surface of beef carcasses *(35, 44, 48, 67, 94, 226)*. Since *E. coli* O157:H7 has shown a strong acid resistance *(13, 65, 81)* and ability to survive in acidic foods *(115, 171, 250, 258)*, surrogate organisms for this target pathogen must have similar acid resistance. The acid resistance of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains was compared in PBS acidified with L-lactic acid at pH values of 2.5, 3.0 and 3.5. This pH range was selected based on the expected carcass surface pH after application of 2% L-lactic acid, according to previous reports *(125)*. All cultures were grown to stationary phase in TSB+G to induce their acid tolerance. It has been demonstrated that *E. coli* cultures grown in the presence of glucose reduce the pH in the medium and induce a pH-dependent acid tolerance response (ATR) *(28)*. When the cells

reach the stationary phase, other acid resistance pH-independent systems (AR1, AR2 and AR3) are triggered to protect them from acid stress *(147, 202)*. Cells that have reached stationary-phase may also develop a pH-dependent acid resistance which further increases their AR systems *(147)*.

Inducing an acid adaptation response before conducting resistance challenges is important because it has been widely documented that acid adaptation induces cross-protection against subsequent environmental stresses in gram positive and gram negative bacteria. For example, Goodson and Rowbury *(117)* showed that growing *E. coli* at a sub-lethal pH value induced a habituation response that allowed the microorganism to subsequently grow at low pH values that were lethal to non-habituated cells. Buchanan and Edelson *(27, 28)* reported that culturing enterohemorrhagic *E. coli* in the presence of glucose reduced the pH in the medium during the growth of the microorganism, making the cells more resistant to subsequent exposure to acidic conditions. Leyer and Jonhson *(149)* found that acid adaptation in *Salmonella* Typhimurium induces cross-protection against subsequent stresses including heat, salt, activated lactoperoxidase system and to surface-active agents such as crystal violet and polymyxin B. Lou and Yousef *(157)* found that adaptation to sublethal environmental stresses including low concentrations of acids, ethanol, hydrogen peroxide and salt, protects *L. monocytogenes* against lethal preservation factors.

It is also important to consider that enteric pathogens such as *E. coli* O157:H7 and *Salmonella* are exposed to reduced pH conditions and to the presence of volatile fatty acids (VFA) in the intestinal tract of cattle. This exposure to mild acidic conditions

has demonstrated to increases their acid resistance. Diez-Gonzalez et al. *(80)* reported that *E. coli* recovered from the feces of grain-fed cattle were 1,000-fold more resistant to an acid shock that simulated passage through the human stomach, than were *E. coli* from cattle fed only hay. Subsequent studies showed that pH and concentration of VFA in the intestinal tract is affected by cattle diet, with high grain diets resulting in an increased fermentation process, higher VFA production and more acidic pH. Fu et al. *(106)* studied the effect of dietary shifts on the fecal VFA concentration, log population and acid resistance of *E. coli* in steers; the authors showed that recovery of acid-resistant *E. coli* was higher in grain fed animals and was more dependent on the VFA concentration, particularly acetate and butyrate, rather than on pH.

Log reductions (CFU/ml) after 0.5, 1.0, 1.5 and 2.0 h of exposure to acidic conditions at pH 2.5, 3.0 and 3.5 were calculated and compared among the fourteen microorganisms included in this study. Regardless of the pH, the reductions observed for the fluorescent protein-marked *E. coli* strains were not different to ($P>0.05$), or were lower than ($P<0.05$) those reductions observed for *E. coli* O157:H7 and *Salmonella* strains (Tables 6 to 8). At pH 3.5, reductions ranged from -0.5 to 1.6, -0.5 to 2.8, 0.4 to 3.6 and 0.8 to 3.2 log CFU/ml after 0.5, 1.0, 1.5 and 2.0 h of exposure, respectively. *S.* Typhimurium showed significantly higher reductions ($P<0.05$) in most situations (Table 6). At pH 3.0, observed reductions ranged from 0.1 to 4.5, 1.9 to 4.9, 1.9 to 5.0 and from 2.7 to 4.9 log CFU/ml after 0.5, 1.0, 1.5 and 2.0 h, respectively. *S.* Agona, Anatum, Typhimurium and Senftenberg showed significantly higher reductions than the

TABLE 6. *Mean reductions (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli*,* E. coli *O157:H7 and* Salmonella *strains after exposure to phosphate buffered saline acidified with lactic acid at pH 3.5*

| | Mean reduction[a] ± SD[b] | | | |
|---|---|---|---|---|
| | Exposure time (h) | | | |
| Microorganism | 0.5 | 1.0 | 1.5 | 2.0 |
| *E. coli* RFP-1 | -0.5 ± 1.0 C[c] | 0.2 ± 0.4 CDE | 0.4 ± 0.4 E | 1.1 ± 0.5 CD |
| *E. coli* GFP-3 | 0.2 ± 0.3 ABC | 0.7 ± 0.2 BCDE | 1.0 ± 0.6 BCDE | 1.3 ± 0.7 BCD |
| *E. coli* YFP-14 | 0.1 ± 0.3 ABC | 0.9 ± 1.1 ABCDE | 1.1 ± 1.1 BCDE | 1.9 ± 0.9 ABCD |
| *E. coli* YFP-66 | -0.4 ± 0.4 C | -0.5 ± 0.6 E | 0.7 ± 0.6 DE | 2.0 ± 0.6 ABCD |
| *E. coli* O157:H7-2 | 0.1 ± 0.1 ABC | 0.4 ± 0.4 CDE | 0.5 ± 0.7 DE | 0.9 ± 1.1 D |
| *E. coli* O157:H7-26 | -0.1 ± 1.6 BC | -0.1 ± 0.8 DE | 0.5 ± 1.1 DE | 0.8 ± 0.7 D |
| *E. coli* O157:H7-36 | 0.0 ± 0.4 BC | 0.1 ± 0.6 CDE | 0.7 ± 0.5 CDE | 1.1 ± 0.7 CD |
| *E. coli* O157:H7-38 | 0.0 ± 0.1 BC | 1.6 ± 0.4 ABCD | 2.4 ± 0.6 AB | 2.8 ± 0.4 ABC |
| *E. coli* O157:H7-43895 | -0.2 ± 0.4 BC | 0.8 ± 0.9 BCDE | 2.0 ± 0.6 BCD | 2.2 ± 1.0 ABCD |
| *S.* Agona | 0.6 ± 0.3 ABC | 2.0 ± 1.1 ABC | 2.3 ± 0.4 ABC | 2.1 ± 0.5 ABCD |
| *S.* Anatum | 1.3 ± 0.6 AB | 2.5 ± 0.4 AB | 2.5 ± 0.1 AB | 3.0 ± 0.4 AB |
| *S.* Montevideo | 0.5 ± 0.6 ABC | 1.7 ± 1.0 ABCD | 1.6 ± 0.4 BCDE | 1.6 ± 0.4 ABCD |
| *S.* Typhimurium | 1.6 ± 0.7 A | 2.8 ± 0.2 A | 3.6 ± 0.1 A | 3.2 ± 0.4 A |
| *S.* Senftenberg | 1.0 ± 1.0 ABC | 1.9 ± 0.6 ABC | 2.4 ± 0.6 AB | 2.5 ± 0.7 ABCD |

[a] Each value (log CFU/ml) represents the mean of three independent experiments
[b] Standard deviation
[c] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)

TABLE 7. *Mean reductions (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli*,* E. coli *O157:H7 and* Salmonella *strains after exposure to phosphate buffered saline acidified with lactic acid at pH 3.0*

| | Mean reduction[a] ± SD[b] | | | |
|---|---|---|---|---|
| | Exposure time (h) | | | |
| Microorganism | 0.5 | 1.0 | 1.5 | 2.0 |
| *E. coli* RFP-1 | 0.2 ± 0.7 D[c] | 3.6 ± 0.2 ABC | 3.7 ± 0.3 AB | 3.0 ± 1.1 A |
| *E. coli* GFP-3 | 0.6 ± 0.5 CD | 2.9 ± 0.5 ABC | 3.0 ± 0.4 AB | 3.2 ± 0.4 A |
| *E. coli* YFP-14 | 1.1 ± 0.6 BCD | 3.4 ± 0.2 ABC | 3.5 ± 0.2 AB | 3.9 ± 0.3 A |
| *E. coli* YFP-66 | 0.4 ± 0.5 D | 3.1 ± 0.2 ABC | 3.7 ± 1.0 AB | 3.2 ± 0.4 A |
| *E. coli* O157:H7-2 | 0.1 ± 0.1 D | 3.2 ± 0.8 ABC | 3.3 ± 0.7 AB | 3.1 ± 0.2 A |
| *E. coli* O157:H7-26 | 0.1 ± 1.7 D | 1.9 ± 1.5 C | 1.9 ± 1.3 B | 2.7 ± 1.4 A |
| *E. coli* O157:H7-36 | 1.2 ± 1.3 BCD | 2.5 ± 0.5 BC | 2.5 ± 0.2 AB | 3.1 ± 0.8 A |
| *E. coli* O157:H7-38 | 3.2 ± 0.5 ABC | 2.9 ± 1.0 ABC | 3.4 ± 0.4 AB | 3.3 ± 0.4 A |
| *E. coli* O157:H7-43895 | 3.1 ± 1.6 ABC | 3.3 ± 0.9 ABC | 2.8 ± 0.4 AB | 3.9 ± 0.9 A |
| *S.* Agona | 4.1 ± 0.7 A | 4.8 ± 0.3 AB | 4.4 ± 1.2 AB | 3.7 ± 1.1 A |
| *S.* Anatum | 4.0 ± 1.0 A | 4.0 ± 1.1 ABC | 3.9 ± 1.2 AB | 3.8 ± 1.2 A |
| *S.* Montevideo | 3.5 ± 0.6 AB | 3.7 ± 0.7 ABC | 3.9 ± 0.7 AB | 3.3 ± 0.6 A |
| *S.* Typhimurium | 4.5 ± 0.5 A | 4.2 ± 1.2 ABC | 4.5 ± 1.6 A | 4.4 ± 1.1 A |
| *S.* Senftenberg | 4.2 ± 0.5 A | 4.9 ± 0.5 A | 5.0 ± 0.6 A | 4.9 ± 0.6 A |

[a] Each value (log CFU/ml) represents the mean of three independent experiments
[b] Standard deviation
[c] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)

TABLE 8. *Mean reductions (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli*,* E. coli *O157:H7 and* Salmonella *strains after exposure to phosphate buffered saline acidified with lactic acid at pH 2.5*

| | Mean reduction[a] ± SD[b] | | | |
|---|---|---|---|---|
| | Exposure time (h) | | | |
| Microorganism | 0.5 | 1.0 | 1.5 | 2.0 |
| *E. coli* RFP-1 | 4.7 ± 0.3 AB[c] | 4.7 ± 0.3 BCD | 4.7 ± 0.3 BC | 4.7 ± 0.3 BCD |
| *E. coli* GFP-3 | 4.4 ± 0.4 AB | 5.1 ± 0.3 ABC | 5.7 ± 0.7 AB | 5.0 ± 0.2 ABCD |
| *E. coli* YFP-14 | 3.5 ± 0.3 B | 4.0 ± 1.3 CD | 5.5 ± 0.2 AB | 5.0 ± 0.2 ABCD |
| *E. coli* YFP-66 | 5.2 ± 0.8 B | 5.4 ± 0.3 ABC | 5.5 ± 0.9 AB | 5.6 ± 0.8 ABCD |
| *E. coli* O157:H7-2 | 3.4 ± 0.1 B | 3.3 ± 0.4 D | 4.5 ± 0.7 BC | 4.4 ± 1.1 CD |
| *E. coli* O157:H7-26 | 3.9 ± 1.6 AB | 4.2 ± 0.8 CD | 3.5 ± 1.1 C | 4.2 ± 0.7 D |
| *E. coli* O157:H7-36 | 4.0 ± 0.5 AB | 4.3 ± 0.3 CD | 3.6 ± 0.2 C | 4.5 ± 0.8 BCD |
| *E. coli* O157:H7-38 | 5.9 ± 0.2 A | 5.9 ± 0.2 AB | 5.9 ± 0.2 AB | 5.9 ± 0.2 ABC |
| *E. coli* O157:H7-43895 | 5.6 ± 0.5 AB | 6.0 ± 0.1 AB | 6.0 ± 0.1 AB | 6.0 ± 0.1 ABC |
| *S.* Agona | 5.9 ± 0.7 A | 5.9 ± 0.7 AB | 5.9 ± 0.7 AB | 5.9 ± 0.7 ABC |
| *S.* Anatum | 6.0 ± 0.5 A | 6.0 ± 0.5 AB | 6.0 ± 0.5 AB | 6.0 ± 0.5 AB |
| *S.* Montevideo | 6.1 ± 1.0 A | 6.4 ± 0.4 A | 6.4 ± 0.5 A | 6.4 ± 0.4 A |
| *S.* Typhimurium | 5.4 ± 0.1 AB | 5.4 ± 0.1 ABC | 5.4 ± 0.1 AB | 5.4 ± 0.1 ABCD |
| *S.* Senftenberg | 3.8 ± 1.6 AB | 4.9 ± 0.2 ABC | 4.9 ± 0.2 ABC | 4.9 ± 0.2 ABCD |

[a] Each value (log CFU/ml) represents the mean of three independent experiments
[b] Standard deviation
[c] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)

fluorescent protein-marked *E. coli* strains, *E. coli* O157:H7 #2 and 26, after 0.5 h of exposure ($P<0.05$), however as the exposure time increased, less differences in acid resistance were observed among organisms (Table 7). At pH 2.5, reductions ranged from 3.3 to 6.1, 3.3 to 6.4, 3.5 to 6.4 and 4.2 to 6.4 log CFU/ml after 0.5, 1.0, 1.5 and 2.0 h of exposure, respectively. At this pH value, *Salmonella* strains demonstrated significantly higher reductions than some fluorescent protein-marked and *E. coli* O157:H7 strains (Table 8).

Log reductions (CFU/ml) were also calculated for groups of microorganisms. The average reductions for the fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* groups at different times of exposure to acidic conditions are presented in Figures 2 to 4; while the average reductions for the three groups of microorganisms at all exposure times are presented in Table 9. According to these data, the acid resistance of the fluorescent protein-marked *E. coli* surrogates was not different from the acid resistance of *E. coli* O157:H7 ($P>0.05$), and was higher than the acid resistance of *Salmonella* serotypes ($P<0.05$) in most cases. It was also observed that reductions significantly increased as the pH was reduced ($P<0.05$) for the three groups of microorganisms.

In general, *Salmonella* strains showed the lowest acid resistance when compared to the fluorescent protein-marked *E. coli* and to some of the *E. coli* O157:H7 strains. This is in agreement with previous reports. Gorden and Small *(118)* studied the ability of *E. coli, Shigella* and *Salmonella* isolates to survive acidic conditions. When isolates were

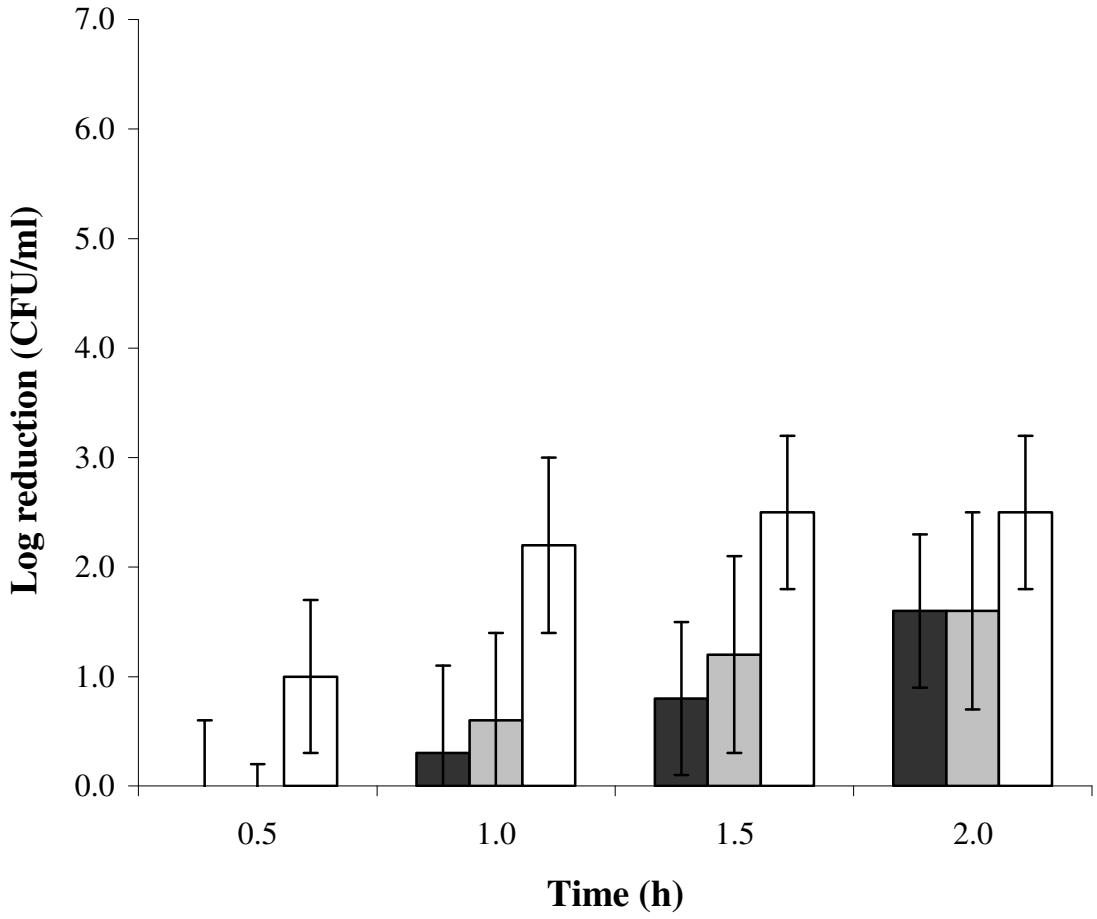


FIGURE 2. *Mean reductions (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli (■)*,* E. coli *O157:H7* (■) *and* Salmonella (□) *strains after exposure to phosphate buffered saline acidified with lactic acid to pH 3.5.*

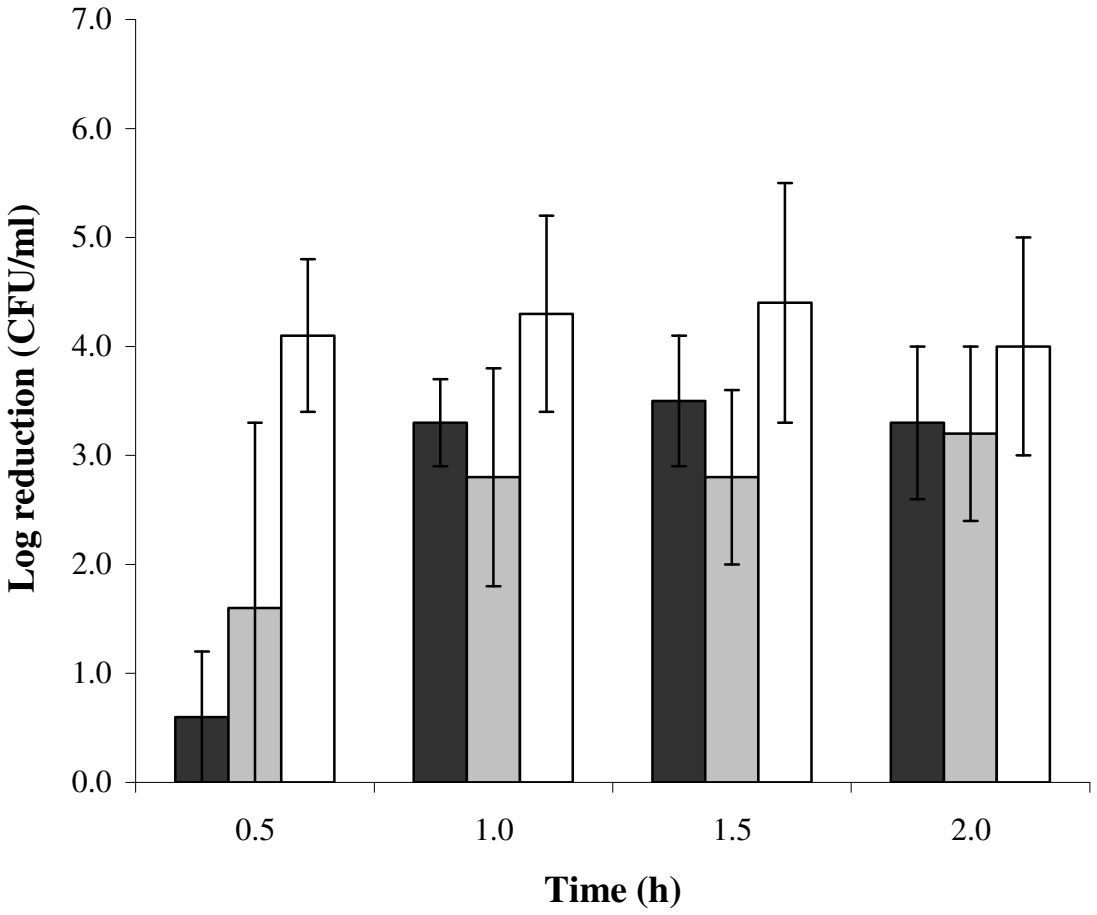


FIGURE 3. *Mean reductions (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli (■)*,* E. coli *O157:H7* (■) *and* Salmonella (□) *strains after exposure to phosphate buffered saline acidified with lactic acid to pH 3.0.*

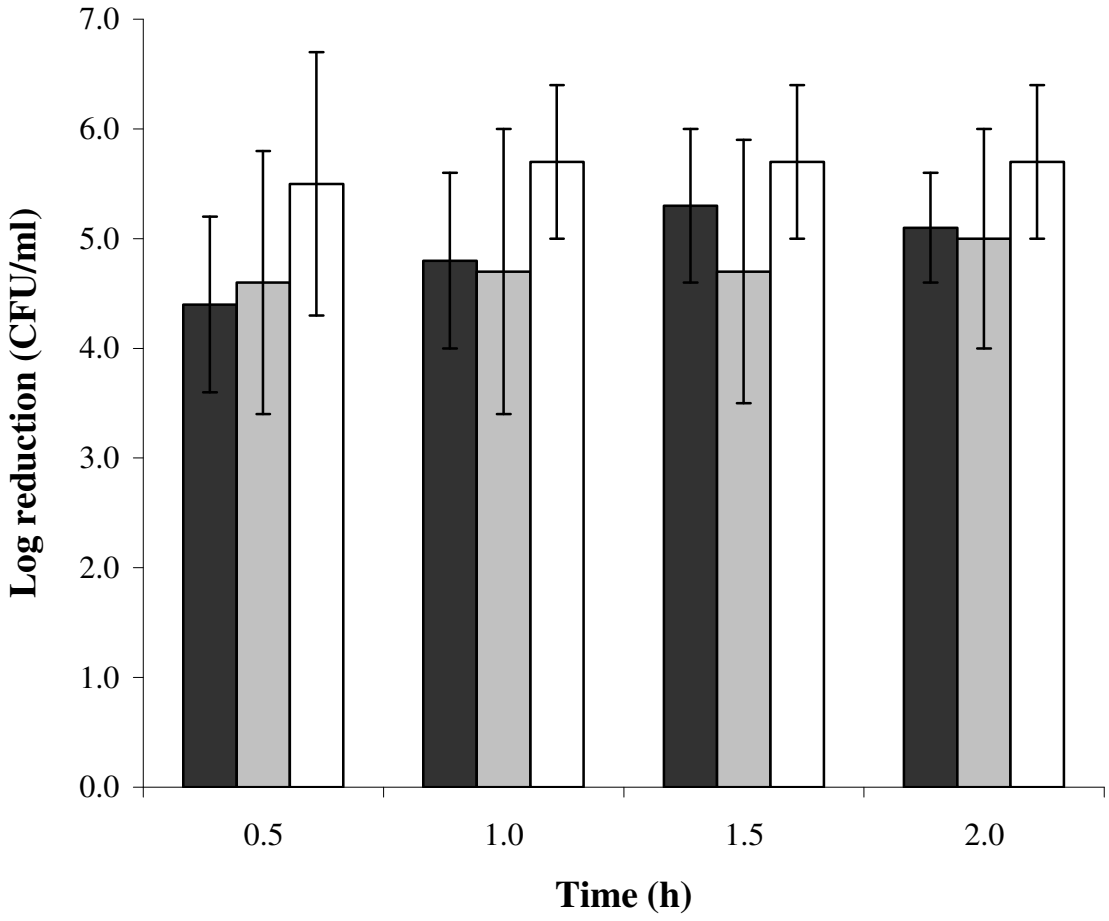


FIGURE 4. *Mean reductions (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli (■)*,* E. coli *O157:H7* (■) *and* Salmonella (□) *strains after exposure to phosphate buffered saline acidified with lactic acid to pH 2.5.*

TABLE 9. *Mean reductions*[a] *(log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli *surrogates,* E. coli *O157:H7 and* Salmonella *after exposure to phosphate buffered saline acidified with lactic acid*

| Microorganism | Mean ± SD[b] | | |
|---|---|---|---|
| | pH 3.5 | pH 3.0 | pH 2.5 |
| *E. coli* surrogates[c] | 0.6 ± 0.9 A[f] *x*[g] | 2.7 ± 1.3 A *y* | 4.9 ± 0.8 A *z* |
| *E. coli* O157:H7[d] | 0.8 ± 1.0 A *x* | 2.6 ± 1.3 A *y* | 4.7 ± 1.1 A *z* |
| *Salmonella*[e] | 2.0 ± 1.0 B *x* | 5.7 ± 0.8 B *y* | 5.7 ± 0.8 B *z* |

[a] Overall mean log reductions after 0.5, 1.0, 1.5 and 2.0 h exposure
[b] Standard deviation
[c] Includes fluorescent protein-marked *E. coli* strains RFP-1, GFP-3, YFP-14 and YFP-66
[d] Includes *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895
[e] Includes *Salmonella* serotypes Agona, Anatum, Montevideo and Typhimurium and Senftenberg
[f] Mean values in the same column with the same letter (ABC) are not different ($P>0.05$)
[g] Mean values in the same row with the same letter (*xyz*) are not different ($P>0.05$)

exposed to pH 2.5 for 2 h in LB broth acidified with HCl, most *Shigella* and *E. coli* O157:H7 isolates survived, whereas no *Salmonella* isolates survived. Samelis et al. *(211)* found a substantial difference in the acid tolerance of different pathogenic strains including *S.* Typhimurium DT104, *L. monocytogenes* and *E. coli* O157:H7; *E. coli* O157:H7 was the pathogen with the greatest acid resistance. Lin et al. *(153)* demonstrated that *E. coli* possesses several acid survival systems (AR) that were not demonstrable in *S*. Typhimurium. Their comparative analysis suggests that even when these microorganisms are evolutionarily close, they have developed different acid survival strategies.

Differences in acid resistance were also observed among *E. coli* O157:H7 strains at all pH values (Tables 6 to 8). Previous studies have also reported phenotypic variation in the acid resistance of EHEC strains and also among *E. coli* O157:H7 isolates. Benjamin and Datta *(13)* tested the ability of fourteen EHEC strains to survive in LB broth acidified to pH 2.5 and 3.0 with HCl after 2 h of exposure; all the strains survived the challenge, but the percentage of survival was different among them and ranged from 0.38 to 100% at pH 2.5 and from 26 to 100% at pH 3.0. Bergholz and Whittam *(15)* also reported differences in the acid resistance among 26 EHEC strains. *E. coli* O157:H7 strains showed an average survival rate twice as great as *E. coli* O126 and O111 strains in a model stomach system under conditions simulating the gastric environment. Buchanan and Edelson *(28)* found differences in the acid resistance among *E. coli* O157:H7 strains previously grown in the presence of glucose; reductions from <0.3 to >3.8 log CFU/ml in brain heart infusion broth (BHI) adjusted to pH 2.5 and 3.0 with HCl

were observed. The same authors reported in another study *(27)* that acid resistance varied substantially among *E. coli* O157:H7 strains, being dependent on the strain, the type of acidulant and the induction of a pH-dependent acid resistance previous to the acid challenges. Large et al. *(146)* found a notable variation in survival rates among 66 *E. coli* O157:H7 strains when exposed to pH 2.0 and 2.5 in minimal E medium supplemented with 0.4% glucose and acidified with HCl. McKellar and Knight *(167)* related the differences in acid resistance among *E. coli* O157:H7 isolates to their origin, reporting that outbreak isolates are more acid resistant than animal or food isolates. Duffy et al. *(88)* found variable acid resistance among 45 *E. coli* biotype I and 20 EHEC strains associated with foodborne outbreaks and reported that only two of the most acid resistant strains corresponded to EHEC.

Differences in acid resistance among *Salmonella* strains were observed only at pH 3.5 (Table 6). Previous reports have demostrated a significant difference in the acid resistance among *Salmonella* strains. When 37 strains of *S.* Typhimurium DT104 were exposed to an acid challenge in BHI at pH 2.5 for 1 and 2 h, a large variation was observed among strains *(16)*. The survival rate after 1 h ranged from 0.13 to 78% and after 2 h ranged from 0.0001% to 36%. The authors reported that human isolates were significantly more resistant than food isolates.

Due to the variability observed among the studied strains, we found that no single fluorescent-marked *E. coli* strain showed an acid resistance pattern that represented all pathogenic strains at all pH values. Other studies conducted to identify surrogates have also concluded that single strains are not able to mimic the behavior of

all pathogenic strains tested *(90, 164)*. However, when considered as a cocktail, the fluorescent protein-marked *E. coli* strains may provide a range of responses to acidic environments that may show them as appropriate surrogates for *E. coli* O157:H7 and *Salmonella* for the validation of interventions that relay in use of lactic acid to reduce enteric pathogens. Using mixtures of strains as surrogates for validation studies has been previously recommended *(30)*.

**Thermal resistance of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains.** Physical decontamination treatments implemented in beef slaughter operations commonly include the application of hot water washes or steam to increase the temperature of the carcass surface and inactivate enteric pathogens. Surrogate organisms intended for use in the validation of interventions that rely on heat, must demonstrate similar or slightly higher thermal resistance than the target pathogens. In this study, the thermal resistance of the fluorescent protein-marked *E. coli* strains proposed as surrogates was determined in PBS at 55, 60 and 65°C and compared to the thermal resistance of *E. coli* O157:H7 and *Salmonella* strains. As previously stated, enteric pathogens such as *E. coli* O157:H7 and *Salmonella* find a reduced pH environment in the intestinal tract of cattle which may trigger an acid adaptation response and induce a cross-protection against those antimicrobial interventions that rely on acid and heat to reduce pathogens from beef carcass surfaces. For this reason, before conducting the heat challenges, all cultures were individually grown in TSB+G to

provide a mild low pH during the growth and stationary phases, induce an acid adaptation response and produce a cross-protection against heat *(149, 166, 210, 216).*

The thermal inactivation curves obtained for each microorganism at 55, 60 and 65°C were fitted by linear regression and used to calculate their D-values. Each thermal challenge was performed in triplicate and three independent D-values were calculated for each organism at each temperature, and used for comparison purposes. The mean D-values obtained for each of the fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains are presented in Table 10.

In most cases, D-values observed for the fluorescent protein-marked *E. coli* strains were not significantly different ($P>0.05$) or were higher ($P<0.05$) than those D-values obtained for *E. coli* O157:H7 and *Salmonella* strains. $D_{55}$ values ranged from 25.3 ± 1.0 to 41.9 ± 10.3 min among fluorescent protein-marked *E. coli* strains, from 14.2 ± 1.3 to 21.3 ± 1.3 min for *E. coli* O157:H7, and from 4.6 ± 0.5 to 21.1 ± 1.0 min for *Salmonella* strains. $D_{55}$ values ranged from 4.6 ± 0.5 to 41.9 ± 10.3 min among all the microorganisms, which represents a nine-fold difference between the lowest and the highest D-value observed at this temperature. $D_{60}$ values ranged from 1.4 ± 0.0 to 1.8 ± 0.4 min among fluorescent protein-marked *E. coli*, from 1.0 ± 0.1 to 1.9 ± 0.2 min for *E. coli* O157:H7, and from 0.4 ± 0.0 to 2.2 ± 0.2 min for *Salmonella* strains. $D_{60}$ values ranged from 0.4 ± 0.0 to 1.9 ± 0.2 min among all the microorganisms, which correspond to a five-fold difference between the lowest and the highest D-value.

TABLE 10. *Decimal reduction time (D-value) for acid-adapted fluorescent protein-marked* E. coli*,* E. coli *O157:H7 and* Salmonella *strains in phosphate buffer saline at different temperatures*

| | D-value (min)[a] ± SD[b] | | | |
|---|---|---|---|---|
| Microorganism | 55°C | 60°C | 65°C | *z*-value (°C)[c] |
| *E. coli* RFP-1 | 28.0 ± 0.9 BC[d] | 1.4 ± 0.0 BCDE | 0.20 ± 0.06 A | 4.6 ± 0.3 AB |
| *E. coli* GFP-3 | 36.8 ± 8.4 AB | 1.6 ± 0.2 ABCD | 0.14 ± 0.01 AB | 4.2 ± 0.2 BC |
| *E. coli* YFP-14 | 25.3 ± 1.0 CD | 1.5 ± 0.1 BCDE | 0.11 ± 0.00 BCD | 4.2 ± 0.0 BC |
| *E. coli* YFP-66 | 41.9 ± 10.3 A | 1.8 ± 0.4 ABC | 0.11 ± 0.01 BC | 3.9 ± 0.1 C |
| *E. coli* O157:H7-2 | 21.3 ± 1.3 CDE | 1.9 ± 0.2 AB | 0.12 ± 0.01 BC | 4.4 ± 0.1 ABC |
| *E. coli* O157:H7-26 | 20.1 ± 0.9 CDE | 1.0 ± 0.1 EFGH | 0.10 ± 0.01 BCD | 4.4 ± 0.1 ABC |
| *E. coli* O157:H7-36 | 19.6 ± 2.1 CDE | 1.2 ± 0.3 CDEF | 0.10 ± 0.01 BCD | 4.4 ± 0.1 ABC |
| *E. coli* O157:H7-38 | 14.2 ± 1.3 DEF | 1.1 ± 0.1 DEFG | 0.10 ± 0.01 BCD | 4.7 ± 0.1 AB |
| *E. coli* O157:H7-43895 | 14.2 ± 0.9 DEF | 1.1 ± 0.0 DEFG | 0.11 ± 0.01 BCD | 4.7 ± 0.1 AB |
| *S.* Agona | 8.1 ± 1.8 F | 0.8 ± 0.1 FGHI | 0.07 ± 0.02 CDE | 4.8 ± 0.5 A |
| *S.* Anatum | 8.3 ± 1.2 F | 0.5 ± 0.1 HI | 0.06 ± 0.02 DE | 4.6 ± 0.1 AB |
| *S.* Montevideo | 10.4 ± 2.2 EF | 0.6 ± 0.1 GHI | 0.07 ± 0.02 CDE | 4.6 ± 0.4 AB |
| *S.* Typhimurium | 4.6 ± 0.5 F | 0.4 ± 0.0 I | 0.04 ± 0.00 E | 4.9 ± 0.2 A |
| *S.* Senftenberg | 21.1 ± 1.0 CDE | 2.2 ± 0.2 A | 0.19 ± 0.01 A | 4.9 ± 0.2 A |

[a] Decimal reduction time represents the mean of three independent experiments
[b] Standard deviation
[c] *z*-value represents the mean of three independent experiments ± standard deviation
[d] Mean values in the same column with the same letter (ABC) are not different ($P>0.05$)

The observed $D_{65}$ values among fluorescent protein-marked *E. coli* strains ranged from 0.11 ± 0.01 to 0.20 ± 0.06 min, from 0.10 ± 0.01 to 0.12 ± 0.01 min for *E. coli* O157:H7, and from 0.04 ± 0.00 to 0.19 ± 0.01 min for *Salmonella* strains. $D_{65}$ values ranged from 0.04 ± 0.00 to 0.20 ± 0.06 min among all the microorganisms, which represents a five-fold difference between the lowest and the highest D-value.

Differences in heat resistance among strains were observed at all temperatures. Previous studies have also reported phenotypic variation in the heat resistance among *E. coli* O157:H7 and *Salmonella* strains, and also among strains of the O157:H7 serotype. Whiting and Golden *(253)* found variation in the thermal resistance of seventeen *E. coli* O157:H7 strains. $D_{55}$ values in BHI broth ranged from 2.6 to 21.5 min (an eight-fold difference) and $D_{60}$ values from 0.69 to 2.13 min (a three-fold difference) among strains. Clavero et al. *(62)* compared the thermal inactivation of ten *E. coli* O157:H7 strains isolated from ground beef and bovine feces. $D_{54.4}$ values in BHI ranged from 17.4 to 35.4 min while $D_{65.6}$ ranged from 0.16 to 0.21 min. The differences observed were strain-dependent rather than source-dependent and were larger at 54.4 than at 65.6°C. Marshall et al. *(164)* compared the thermal resistance among nonpathogenic *E. coli* and *E. coli* O157:H7 strains in PBS at 55 and 65°C. A large variability in D-values among strains was observed, ranging from 53 to 325 min at 55°C and from 1.84 to 2.78 min at 65°C. However, in general, the thermal resistance for the nonpathogenic *E. coli* strains was not different from that of *E. coli* O157:H7 strains. The high D-values reported by these authors may be attributed to the testing conditions, since it has been demonstrated that thermal inactivation parameters are affected when using

large tubes instead of capillary tubes for the heat challenges *(61)*. Doyle and Mazzota *(87)* published a review of studies on the thermal resistance of *Salmonella*. A large variability was found in the D-values reported among *Salmonella* serotypes and among strains from the same serotype. Variation in heat resistance among microorganisms is affected by several factors including strain to strain variation, age of cells, pH, water activity, presence of additives, composition of the heating medium, and factors affecting the cells before the heat challenge such as growth temperature, acid adaptation, cold stress and starvation, among others *(84, 87, 95, 174, 216, 223)*.

*Salmonella* serotypes other than Senftenberg were the most heat sensitive organisms at all tested temperatures (Table 10). As expected, *S*. Senftenberg, which is considered an unusual heat resistant serotype *(87, 179)*, was the most heat resistant strain in the *Salmonella* group and showed no different ($P>0.05$) or lower ($P<0.05$) D-values than the fluorescent protein-marked *E. coli* strains at 55°C. As the temperature increased, *S.* Senftenberg was among the most resistant strains, along with the fluorescent protein-marked *E. coli* RFP-1 and GFP-3 strains. In general, the thermal resistance was: fluorescent protein-marked *E. coli* strains > *E. coli* O157:H7 strains > *Salmonella* (other than *S.* Senftenberg), and these findings are in agreement with previous reports. Eblen et al. *(90)* compared the thermal resistance of 15 nonpathogenic *E. coli* strains with that of two *E. coli* O157:H7 and two *Salmonella* strains (serotypes Poona and Montevideo) in 0.1% PW at 60°C. *E. coli* O157:H7 was more resistant than *Salmonella* serotypes, and five of the nonpathogenic *E. coli* strains had similar D-values to *E. coli* O157:H7 strains. Enache et al. *(95)* conducted thermal inactivation studies in grape juice concentrate and

found that *Salmonella* serotypes including Rubislaw, Gaminara, Hartford and Thompson, were more heat sensitive than *E. coli* O157:H7 strains. Mazzota *(166)* investigated the thermal inactivation of pathogens in apple, orange and white grape juices. Acid-adapted *E. coli* O157:H7 strains were more heat resistant at 56, 60 and 62°C than *Salmonella* and *L. monocytogenes* strains. Sharma et al. *(216)* reported higher thermal resistance for *E. coli* O157:H7 strains in cantaloupe and watermelon juices when compared to *S.* Poona and *S.* Saphra. Osaili et al. *(189)* reported significantly lower D-values for *E. coli* O157:H7 than for *Salmonella* in chicken-fried beef patties. However, *S.* Senftenberg was included in this study. Murphy at el. *(174)* also reported lower D-values for *E. coli* O157:H7 strains when compared to *Salmonella* in ground turkey but *S.* Senftenberg was also included in the inoculum.

Thermal inactivation curves were constructed for groups of microorganisms at 55, 60 and 65°C and fitted by linear regression (Figures 5 to 8). From the inactivation curves, D-values were calculated and compared among the fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* groups, except for *S.* Senftenberg which was individually included in the analysis (Table 11). According to these data, the mean D-value calculated for the fluorescent protein-marked *E. coli* surrogates was higher than the mean D-value obtained for *E. coli* O157:H7, *Salmonella* and *S.* Senftenberg at 55°C. At 60 and 65°C the fluorescent protein-marked *E. coli* surrogates showed a mean D-value higher than the mean D-value for *E. coli* O157:H7 and *Salmonella* groups. *S.* Senftenberg showed the highest mean D-values at 60 and 65°C.

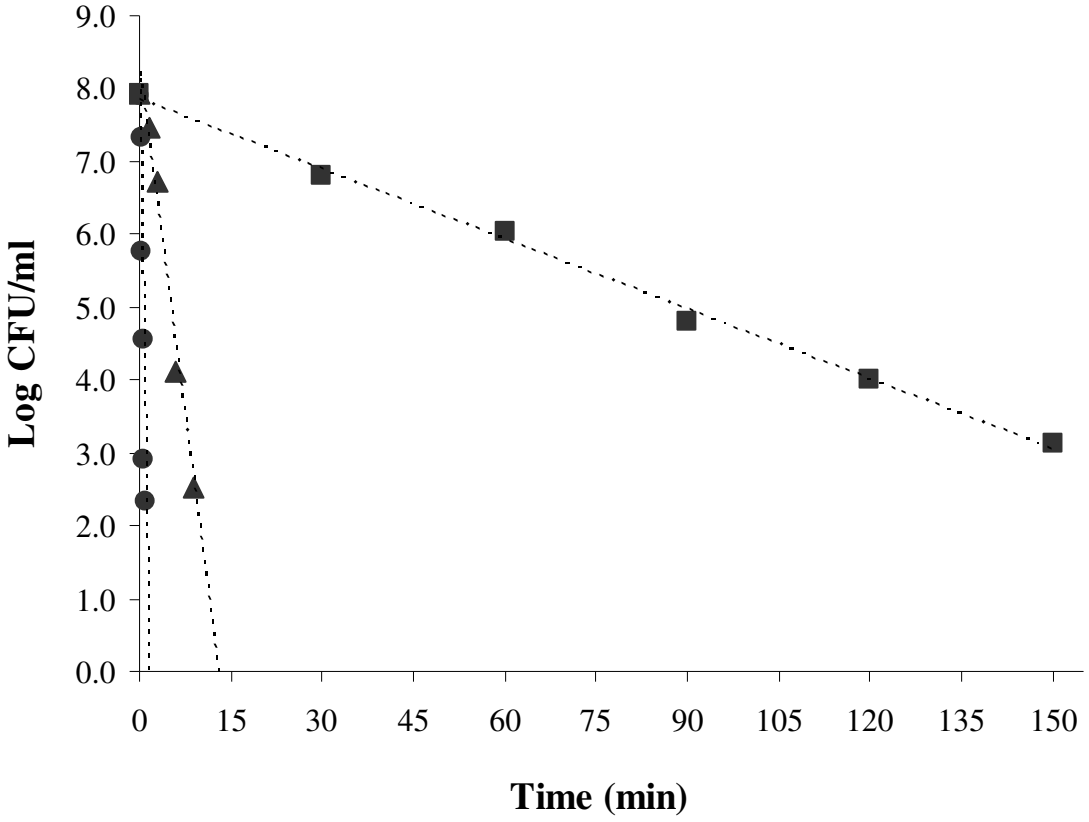


FIGURE 5. *Heat inactivation curves for acid-adapted fluorescent protein-marked* E. coli *strains at 55°C* (■)*, 60°C* (▲) *and 65°C* (●) *in phosphate buffered saline. Each data point represents the mean count of three replicates for the strains RFP-1, GFP-3, YFP-14 and YFP-66 (n=12). The dotted lines represent the linear regression of the survival curves at each temperature.*

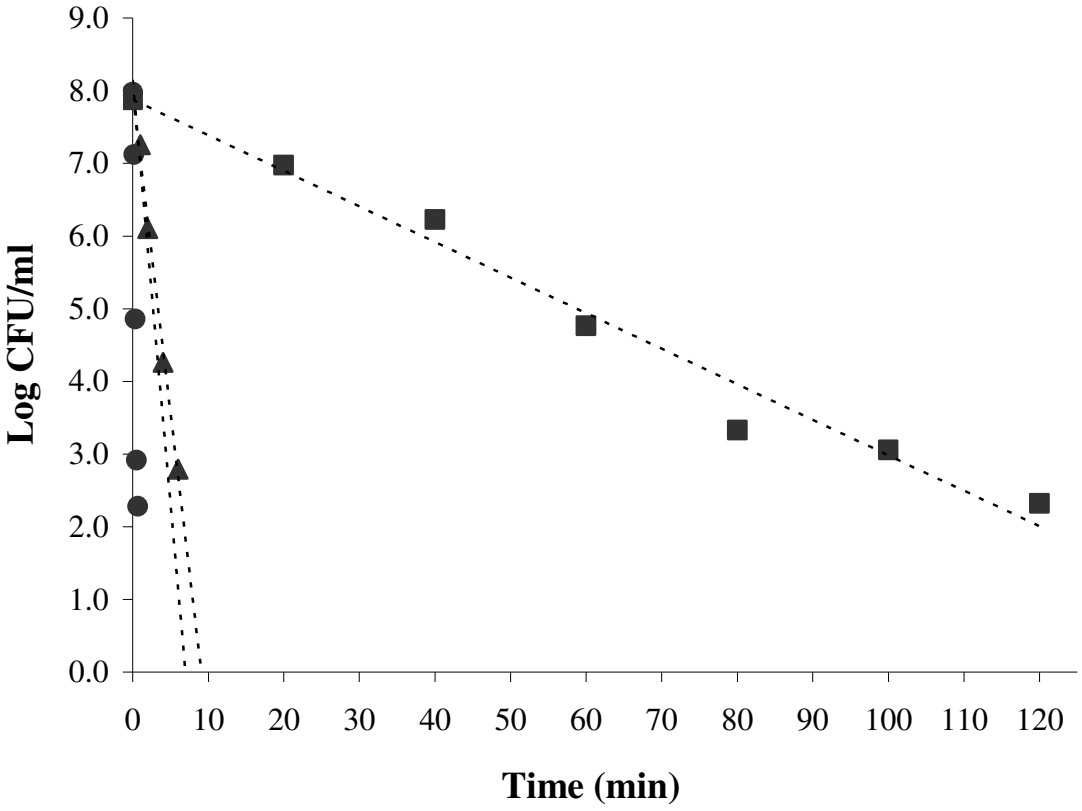


FIGURE 6. *Heat inactivation curves for acid-adapted* E. coli *O157:H7 strains at 55°C* (■)*, 60°C* (▲) *and 65°C* (●) *in phosphate buffered saline. Each data point represents the mean count of three replicates for strains #2, 26, 36, 38 and 43895 (n=15). The dotted lines represent the linear regression of the survival curves at each temperature.*

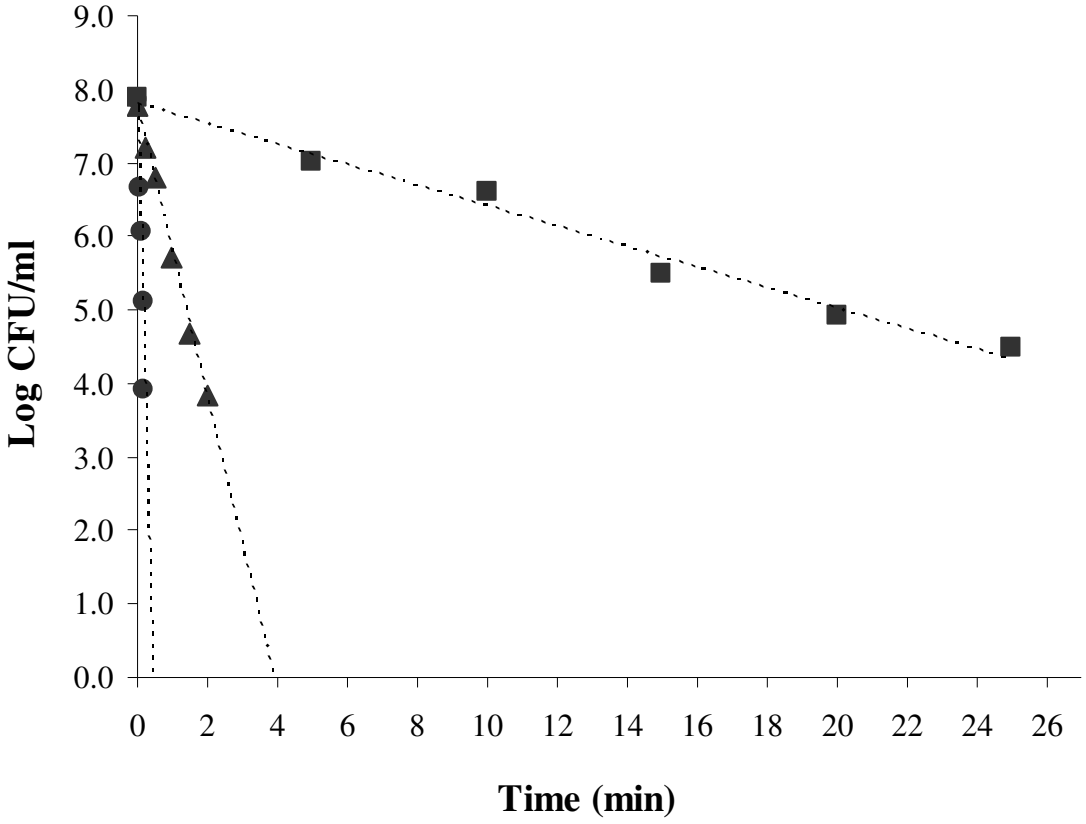


FIGURE 7. *Heat inactivation curves for acid-adapted* Salmonella *strains at 55°C* (■)*, 60°C* (▲) *and 65°C* (●) *in phosphate buffered saline. Each data point represents the mean count of three replicates for the serotypes Agona, Anatum, Montevideo and Typhimurium (n=12). The dotted lines represent the linear regression of the survival curves at each temperature.*

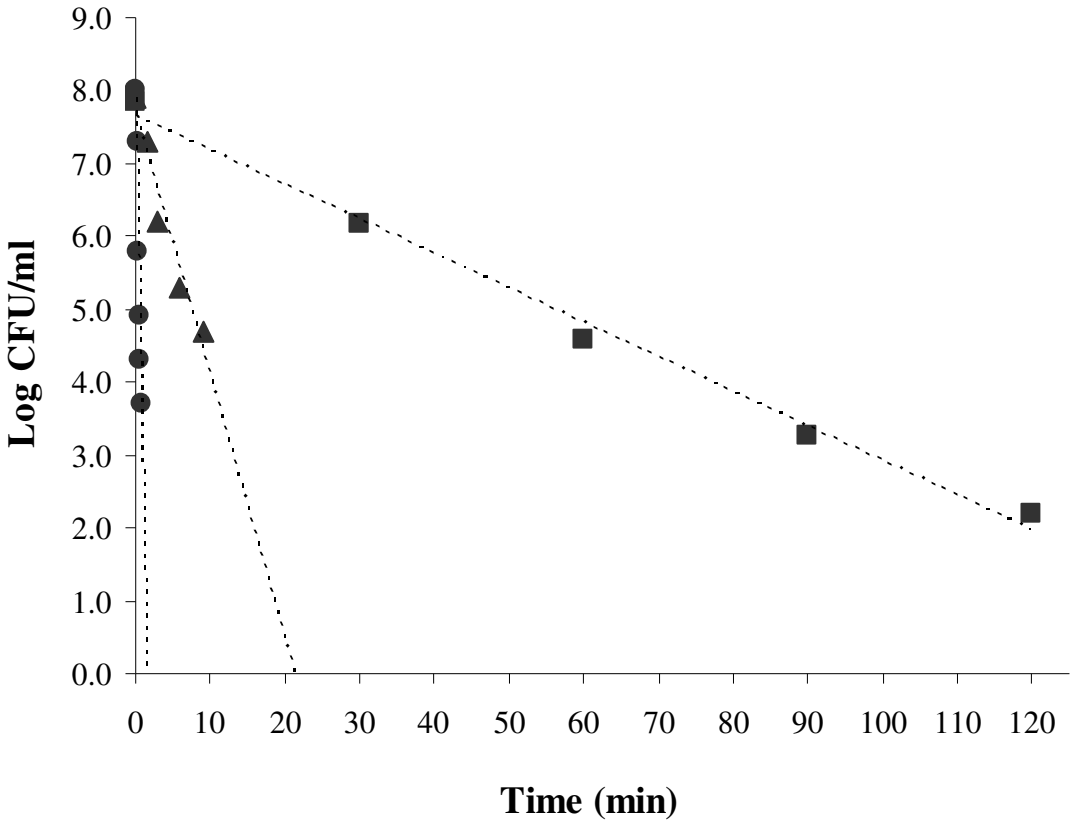


FIGURE 8. *Heat inactivation curves for acid-adapted* Salmonella *Senftenberg at 55°C* (■)*, 60°C* (▲) *and 65°C* (●) *in phosphate buffered saline. Each data point represent the mean of three replicates (n=3). The dotted lines represent the linear regression of the survival curves at each temperature.*

TABLE 11. *Decimal reduction time (D-value) for acid adapted fluorescent protein-marked* E. coli*,* E. coli *O157:H7 and* Salmonella *in phosphate buffer saline*

| Microorganism | Mean D-value[a] (min) ± SD[b] | | | Mean *z*-value (°C) ± SD |
|---|---|---|---|---|
| | 55°C | 60°C | 65°C | |
| *E. coli* surrogates[c] | 33.0 ± 9.0 A[f] | 1.59 ± 0.25 A | 0.14 ± 0.04 A | 4.2 ± 0.3 A |
| *E. coli* O157:H7[d] | 17.9 ± 3.4 B | 1.26 ± 0.37 B | 0.11 ± 0.01 B | 4.5 ± 0.3 A |
| *Salmonella*[e] | 7.9 ± 2.5 C | 0.55 ± 0.19 C | 0.06 ± 0.02 C | 4.7 ± 0.3 A |
| *S.* Senftenberg | 21.1 ± 1.0 B | 2.16 ± 0.24 D | 0.19 ± 0.01 D | 4.9 ± 0.2 B |

[a] D-values represents the mean of three independent experiments
[b] Standard deviation
[c] Includes fluorescent protein-marked *E. coli* strains RFP-1, GFP-3, YFP-14 and YFP-66
[d] Includes *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895
[e] Includes *Salmonella* serotypes Agona, Anatum, Montevideo and Typhimurium
[f] Mean values in the same column with the same letter (ABC) are not different ($P>0.05$)

Log D-values were plotted as a function of temperature and *z*-values for the fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains were calculated as the reciprocal of the slope obtained by linear regression. Variability was observed in the *z*-values among individual strains (Table 10). However, the fluorescent protein-marked *E. coli* strains showed no difference in *z*-values when compared to the different pathogenic strains. The *z*-values provide information about the response of a microorganism to changes in temperature, the greater the *z*-value, the greater the temperature change required to decrease the D-value of the microorganism.

The *z*-values were also averaged for groups of microorganisms and compared. Calculated *z*-values were not different among fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* other than *S.* Senftenberg ($P>0.05$), which was the organism with the highest *z*-value (Table 11). In Figure 9, the plot of log D-values versus temperature for the three groups of microorganisms shows similar heat lethality kinetics among the fluorescent protein-marked *E. coli* surrogates, *E. coli* O157:H7 and *Salmonella* groups, as the slopes are similar. The slope observed for *S.* Senftenberg is slightly different, but still very close to the slope observed for the surrogates group.

No single strain of fluorescent protein-marked *E. coli* showed a thermal resistance pattern that represented all strains of pathogens at all temperatures. However, surrogates showed a wide range of responses to heat in the range of 55 to 65°C, and in general, their thermal resistance was similar or higher than the thermal resistance observed for the pathogenic strains included in this study. Strain to strain variation is an inherent property of microorganisms that even under controlled conditions during

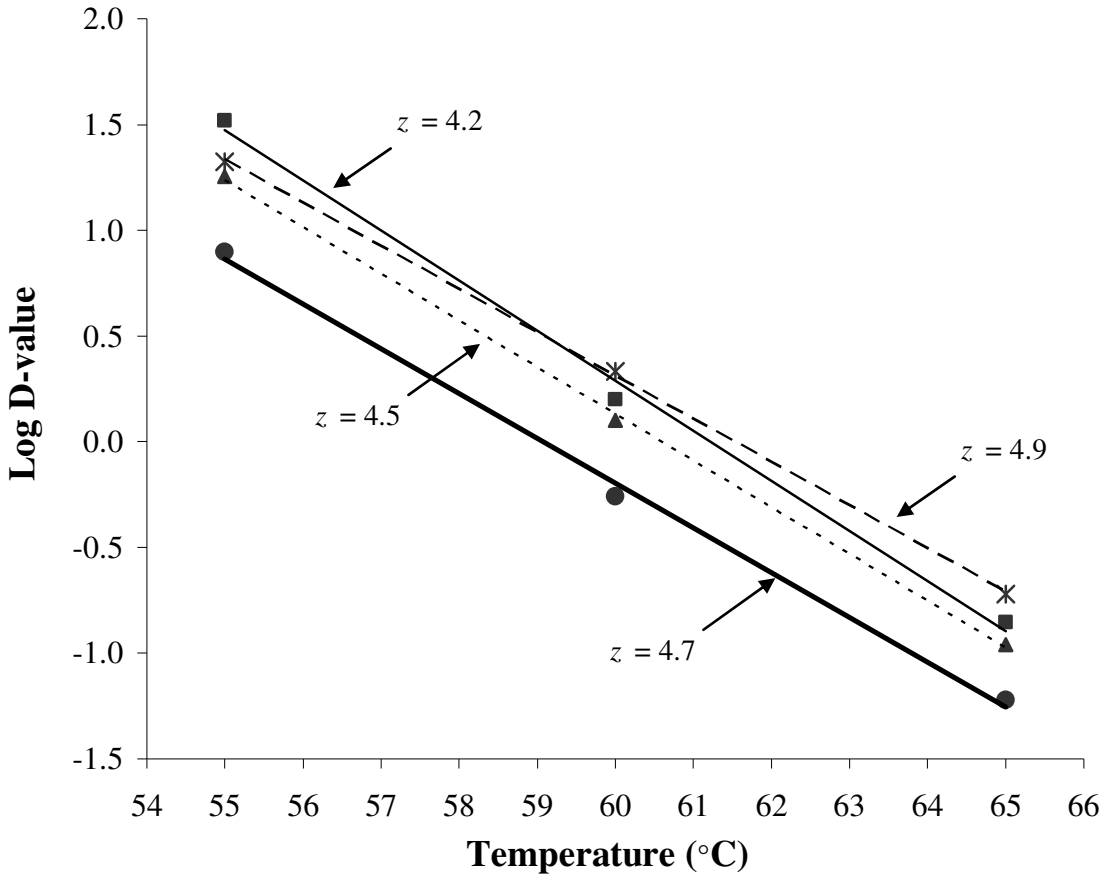


FIGURE 9. *Thermal death times (z-values) for fluorescent protein-marked* E. coli (■), E. coli *O157:H7* (▲), Salmonella (●) *and* S. *Senftenberg* (∗). *The fine solid line represents the fitted curve for the fluorescent protein-marked* E. coli; *the dotted line is the fitted curve for* E. coli *O157:H7, the heavy solid line is the fitted curve for* Salmonella *and the dashed line is the fitted curve for* S. *Senftenberg*.

experiments, it cannot be reduced *(253)* and it is the most important reason why a cocktail of strains should be used in validation studies *(30)*.

**Resistance to low temperatures of fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* strains.** In the process of selecting appropriate surrogate organisms for enteric pathogens, different properties must be evaluated for the potential surrogates and compared to those of target pathogens. The ability of surrogates and pathogens to survive under refrigeration and freezing temperatures should be similar if surrogates are intended to be utilized in validation studies for meat and meat products, because storage at low temperatures is a key factor in their preservation. In this study, the ability of fluorescent protein-marked *E. coli* strains proposed as potential surrogates to survive at low temperatures, was compared to that of *E. coli* O157:H7 and *Salmonella* strains after storage in PBS at refrigeration (4°C) and freezing (-18°C) conditions.

Each microorganism was individually grown in TSB and also in TSB+G, and then suspended in PBS and stored at 4 ± 0.5°C and -18 ± 0.5°C. The purpose of culturing the microorganisms in the presence of glucose (acid-adapted) or in absence of this fermentable carbohydrate (non acid-adapted) was to investigate if acid adaptation induces a cross-protection against cold stress produced by refrigeration and freezing temperatures for a prolonged period of time. As previously discussed, it has been demonstrated that acid adaptation induces cross-protection against subsequent exposure to different stresses including acid, heat, and presence of inhibitory compounds *(149)*.

The mean counts (log CFU/ml) for fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* strains after storage at 4°C and -18°C during 0, 7, 14, 21, 28, 60 and 90 d, are presented in Tables 12 to 15. In general, *Salmonella* strains appeared to be more sensitive to low temperatures, particularly *S.* Montevideo which in several cases was the serotype showing significantly lower counts than fluorescent protein-marked *E. coli* and *E. coli* O157:H7 strains ($P<0.05$). According to the results obtained, no single fluorescent-marked *E. coli* strain showed a survival pattern that mimics all pathogenic strains at 4°C or at -18°C. As discussed before, strain to strain variation in response to stress situations is an inherent property of microorganisms. Differences in the cryotolerance among *E. coli* strains and also among *Salmonella* strains have been related to the composition of the outer cell membrane, particularly to the composition of fatty acids as well as the type and amounts of proteins *(14, 32, 33)*. It is possible that variation observed in the sensitivity to cold stress among *E. coli* and *Salmonella* strains in this study are related to differences in their cell membrane structure, but the studies required to demonstrate it, are above the scope of this research.

The ability to survive at low temperatures was also compared for groups of microorganisms. The mean counts (log CFU/ml) obtained for acid-adapted and non acid-adapted fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* groups were compared after 0, 7, 14, 21, 28, 60 and 90 d of storage at 4°C and -18°C (Table 16). It was observed that microbial populations were reduced as the storage time was increased at both refrigeration and freezing conditions for the three groups of microorganisms. For non acid-adapted organisms stored at 4°C, mean counts were not

TABLE 12. *Mean counts (log CFU/ml) for non acid-adapted fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *strains in phosphate buffered saline at 4°C*

| | Mean count (log CFU/ml) ± SD[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time (days) | | | | | | |
| Microorganism | 0 | 7 | 14 | 21 | 28 | 60 | 90 |
| *E. coli* RFP-1 | 7.2 ± 0.1A[b] | 7.1 ± 0.1AB | 7.0 ± 0.1ABC | 6.9 ± 0.1AB | 6.8 ± 0.0AB | 6.1 ± 0.0A | 5.7 ± 0.4A |
| *E. coli* GFP-3 | 7.2 ± 0.1A | 7.0 ± 0.0AB | 7.0 ± 0.2ABC | 7.0 ± 0.1AB | 6.9 ± 0.0A | 6.0 ± 0.1A | 5.3 ± 0.8ABC |
| *E. coli* YFP-14 | 7.2 ± 0.0A | 6.9 ± 0.1ABC | 6.5 ± 0.2CDE | 5.8 ± 0.1C | 5.6 ± 0.3C | 5.1 ± 1.1ABC | 4.6 ± 0.7ABCD |
| *E. coli* YFP-66 | 7.1 ± 0.1A | 7.1 ± 0.2A | 7.1 ± 0.1A | 7.1 ± 0.0A | 7.0 ± 0.1A | 6.0 ± 0.0AB | 6.0 ± 0.1A |
| *E. coli* O157:H7-2 | 7.1 ± 0.1AB | 7.0 ± 0.2AB | 6.8 ± 0.1ABC | 6.9 ± 0.2AB | 6.3 ± 0.5ABC | 4.7 ± 0.8ABC | 3.8 ± 0.2CD |
| *E. coli* O157:H7-26 | 7.1 ± 0.1AB | 7.0 ± 0.2AB | 6.9 ± 0.1ABC | 6.8 ± 0.2AB | 6.4 ± 0.6ABC | 5.0 ± 0.4ABC | 4.4 ± 0.8ABCD |
| *E. coli* O157:H7-36 | 7.1 ± 0.1AB | 7.0 ± 0.1AB | 6.6 ± 0.3BCDE | 6.8 ± 0.0AB | 6.3 ± 0.7ABC | 4.7 ± 0.5ABC | 3.6 ± 0.5D |
| *E. coli* O157:H7-38 | 7.1 ± 0.0AB | 6.9 ± 0.1AB | 6.9 ± 0.0ABC | 6.8 ± 0.4AB | 5.8 ± 0.3BC | 5.6 ± 0.4ABC | 5.4 ± 0.5ABC |
| *E. coli* O157:H7-43895 | 7.1 ± 0.1AB | 6.6 ± 0.3C | 6.3 ± 0.6DE | 6.3 ± 0.9ABC | 6.2 ± 0.7ABC | 4.5 ± 0.4BC | 3.9 ± 0.4BCD |
| *S.* Agona | 7.1 ± 0.1AB | 6.9 ± 0.0AB | 6.9 ± 0.1ABC | 6.4 ± 0.6ABC | 6.2 ± 1.0ABC | 5.0 ± 1.5ABC | 4.6 ± 2.0ABCD |
| *S.* Anatum | 7.2 ± 0.1A | 7.0 ± 0.0AB | 7.0 ± 0.1AB | 6.5 ± 0.5ABC | 7.0 ± 0.0A | 6.2 ± 0.0A | 6.1 ± 0.1A |
| *S.* Montevideo | 6.9 ± 0.1B | 6.7 ± 0.1BC | 6.2 ± 0.3E | 5.8 ± 0.2C | 5.4 ± 0.2C | 4.3 ± 0.6C | 3.9 ± 0.5CD |
| *S.* Typhimurium | 7.0 ± 0.1AB | 7.0 ± 0.0AB | 6.9 ± 0.1ABC | 6.5 ± 0.5ABC | 6.8 ± 0.1AB | 6.0 ± 0.1A | 5.9 ± 0.2A |
| *S.* Senftenberg | 7.1 ± 0.1AB | 6.8 ± 0.1ABC | 6.7 ± 0.0ABCD | 6.2 ± 0.3BC | 6.3 ± 0.1ABC | 5.7 ± 0.4ABC | 5.6 ± 0.3AB |

[a] Standard deviation

[b] Mean counts in the same column with the same letter (ABC) are not significantly different ($P>0.05$)

TABLE 13. *Mean counts (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *strains in phosphate buffered saline at 4 ℃*

| | Mean count (log CFU/ml) ± SD[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time (days) | | | | | | |
| Microorganism | 0 | 7 | 14 | 21 | 28 | 60 | 90 |
| *E. coli* RFP-1 | 7.1 ± 0.0A[b] | 6.6 ± 0.4AB | 6.1 ± 0.4ABC | 5.9 ± 0.6ABC | 5.6 ± 0.5ACD | 4.4 ± 1.5ABCD | 4.2 ± 1.3A |
| *E. coli* GFP-3 | 7.0 ± 0.1A | 7.0 ± 0.1A | 6.7 ± 0.3AB | 6.9 ± 0.1A | 6.6 ± 0.3A | 5.5 ± 0.2AB | 4.2 ± 1.5A |
| *E. coli* YFP-14 | 7.1 ± 0.1A | 6.8 ± 0.0AB | 6.5 ± 0.3AB | 6.0 ± 0.3ABC | 5.3 ± 0.1CD | 4.0 ± 1.0ABCD | 3.8 ± 0.8A |
| *E. coli* YFP-66 | 7.0 ± 0.1A | 6.8 ± 0.1AB | 6.8 ± 0.2A | 6.5 ± 0.6AB | 6.4 ± 0.4AB | 5.9 ± 0.1A | 4.7 ± 0.4A |
| *E. coli* O157:H7-2 | 7.1 ± 0.1A | 6.9 ± 0.1AB | 6.5 ± 0.6AB | 5.9 ± 0.7ABC | 5.8 ± 0.1BCD | 4.2 ± 1.6ABCD | 4.0 ± 1.8A |
| *E. coli* O157:H7-26 | 7.0 ± 0.1AB | 6.4 ± 0.7ABC | 6.1 ± 0.8ABC | 6.0 ± 1.0ABC | 5.4 ± 0.2CD | 4.0 ± 1.9ABCD | 3.9 ± 0.9A |
| *E. coli* O157:H7-36 | 6.9 ± 0.2A | 6.7 ± 0.1AB | 5.8 ± 0.8ABCD | 5.2 ± 0.5BCD | 4.5 ± 0.2E | 2.5 ± 0.1D | 3.3 ± 0.0A |
| *E. coli* O157:H7-38 | 7.0 ± 0.1AB | 5.8 ± 0.3DE | 5.3 ± 0.5CD | 5.1 ± 0.4CD | 4.4 ± 0.6E | 3.2 ± 0.5ABCD | 3.0 ± 0.4A |
| *E. coli* O157:H7-43895 | 7.0 ± 0.0A | 5.2 ± 0.1E | 4.9 ± 0.3D | 4.4 ± 0.3D | 4.4 ± 0.1E | 2.7 ± 1.2CD | 3.3 ± 0.0A |
| *S.* Agona | 7.0 ± 0.1AB | 6.7 ± 0.1AB | 6.2 ± 0.0C | 6.5 ± 0.8AB | 5.7 ± 0.3BCD | 5.2 ± 0.1ABC | 4.3 ± 0.1A |
| *S.* Anatum | 7.0 ± 0.0A | 6.8 ± 0.1AB | 6.8 ± 0.0A | 6.9 ± 0.3A | 5.9 ± 0.2BC | 3.1 ± 0.4BCD | 3.3 ± 0.1A |
| *S.* Montevideo | 6.7 ± 0.3B | 6.1 ± 0.1CD | 6.0 ± 0.2ABC | 6.1 ± 1.0ABC | 5.0 ± 0.1DE | 2.3 ± 0.4D | 3.3 ± 0.0A |
| *S.* Typhimurium | 6.9 ± 0.0AB | 6.7 ± 0.1AB | 5.7 ± 0.2BCD | 6.4 ± 0.7ABC | 5.8 ± 0.2BCD | 4.3 ± 1.3ABCD | 3.9 ± 0.4A |
| *S.* Senftenberg | 6.9 ± 0.1AB | 6.3 ± 0.2BC | 5.9 ± 0.2ABCD | 6.7 ± 0.5A | 5.6 ± 0.4CD | 3.9 ± 1.9ABCD | 3.8 ± 0.7A |

[a] Standard deviation

[b] Mean counts in the same column with the same letter (ABC) are not significantly different ($P$>0.05)

TABLE 14. *Mean count (log CFU/ml) for non acid-adapted fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *strains in phosphate buffered saline at -18°C*

| | Mean count (log CFU/ml) ± SD[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time (days) | | | | | | |
| Microorganism | 0 | 7 | 14 | 21 | 28 | 60 | 90 |
| *E. coli* RFP-1 | 7.2 ± 0.1A[b] | 6.8 ± 0.2A | 6.8 ± 0.0A | 5.8 ± 1.6AB | 4.8 ± 2.7AB | 4.6 ± 1.9ABC | 5.1 ± 0.5AB |
| *E. coli* GFP-3 | 7.2 ± 0.1A | 6.7 ± 0.0A | 6.7 ± 0.4AB | 6.4 ± 0.6A | 6.1 ± 0.0A | 4.2 ± 1.8ABCD | 4.3 ± 1.4A |
| *E. coli* YFP-14 | 7.2 ± 0.0A | 6.6 ± 0.1AB | 6.3 ± 0.5AB | 6.3 ± 0.7A | 6.1 ± 0.7A | 5.1 ± 1.1AB | 5.3 ± 0.2A |
| *E. coli* YFP-66 | 7.1 ± 0.1A | 6.8 ± 0.0A | 6.8 ± 0.2A | 6.4 ± 0.8A | 6.5 ± 0.4A | 3.9 ± 1.2ABCD | 3.3 ± 1.1BCD |
| *E. coli* O157:H7-2 | 7.1 ± 0.1A | 6.9 ± 0.2A | 6.8 ± 0.2A | 6.3 ± 0.8A | 6.2 ± 0.0A | 5.7 ± 0.1A | 5.3 ± 0.1AB |
| *E. coli* O157:H7-26 | 7.1 ± 0.1A | 6.0 ± 0.1ABCD | 5.8 ± 0.2ABCD | 6.1 ± 0.3A | 5.1 ± 1.3AB | 5.6 ± 0.2A | 5.2 ± 0.1AB |
| *E. coli* O157:H7-36 | 7.1 ± 0.1AB | 6.8 ± 0.1A | 6.6 ± 0.0AB | 6.6 ± 0.4A | 6.1 ± 0.0A | 5.1 ± 0.1AB | 4.2 ± 1.6ABCD |
| *E. coli* O157:H7-38 | 7.1 ± 0.0A | 6.4 ± 0.3ABC | 6.1 ± 1.0ABC | 6.0 ± 0.3A | 4.8 ± 1.6AB | 5.1 ± 0.2AB | 4.7 ± 0.6ABC |
| *E. coli* O157:H7-43895 | 7.1 ± 0.1A | 5.8 ± 0.0CDE | 5.9 ± 0.3ABCD | 6.1 ± 0.8A | 5.1 ± 1.3AB | 3.7 ± 1.9BCDE | 3.7 ± 1.2ABCD |
| *S.* Agona | 7.1 ± 0.1AB | 5.1 ± 1.0E | 3.7 ± 0.8E | 4.4 ± 1.5BC | 3.0 ± 0.4B | 2.6 ± 0.0DEF | 2.7 ± 0.2CD |
| *S.* Anatum | 7.2 ± 0.1A | 5.8 ± 0.4BCDE | 5.7 ± 0.2BCD | 5.8 ± 0.6AB | 5.3 ± 0.9AB | 2.1 ± 0.7EF | 3.7 ± 0.5ABCD |
| *S.* Montevideo | 6.9 ± 0.1AB | 3.6 ± 0.2F | 3.7 ± 0.4E | 3.3 ± 0.3C | 3.3 ± 0.7B | 1.9 ± 0.0F | 2.6 ± 0.0D |
| *S.* Typhimurium | 7.0 ± 0.1AB | 5.5 ± 0.2DE | 5.1 ± 0.1CD | 5.5 ± 0.3AB | 4.8 ± 0.7AB | 3.2 ± 0.8CDEF | 2.3 ± 0.4D |
| *S.* Senftenberg | 7.1 ± 0.1AB | 6.1 ± 0.3ABCD | 5.1 ± 0.0D | 5.7 ± 0.6AB | 4.4 ± 1.9AB | 4.9 ± 0.4ABC | 2.8 ± 1.0CD |

[a] Standard deviation

[b] Mean counts in the same column with the same letter (ABC) are not significantly different ($P$>0.05)

TABLE 15. *Mean counts (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli*,* E. coli *O157:H7 and* Salmonella *strains in phosphate buffered saline at -18°C*

| | Mean count (log CFU/ml) ± SD[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time (days) | | | | | | |
| Microorganism | 0 | 7 | 14 | 21 | 28 | 60 | 90 |
| *E. coli* RFP-1 | 7.1 ± 0.0A[b] | 5.2 ± 0.2BCD | 5.5 ± 0.6AB | 5.0 ± 0.0ABCD | 3.9 ± 1.3ABCD | 3.3 ± 0.1BC | 2.3 ± 0.4A |
| *E. coli* GFP-3 | 7.0 ± 0.1A | 5.7 ± 0.5 AB | 5.7 ± 0.4AB | 6.0 ± 0.2A | 5.6 ± 0.2A | 4.0 ± 1.0AB | 2.6 ± 0.0A |
| *E. coli* YFP-14 | 7.1 ± 0.1A | 6.0 ± 0.2AB | 5.5 ± 0.4AB | 5.5 ± 0.5ABC | 5.0 ± 0.3ABC | 2.9 ± 0.2C | 2.6 ± 0.0A |
| *E. coli* YFP-66 | 7.0 ± 0.1A | 6.3 ± 0.1A | 6.0 ± 0.1A | 5.8 ± 0.0AB | 5.3 ± 0.5AB | 3.0 ± 0.5C | 3.1 ± 0.8A |
| *E. coli* O157:H7-2 | 7.1 ± 0.1A | 5.1 ± 0.6BCD | 5.6 ± 0.5AB | 6.0 ± 0.1A | 5.6 ± 0.2A | 4.5 ± 0.3A | 3.1 ± 0.7A |
| *E. coli* O157:H7-26 | 7.0 ± 0.1AB | 5.2 ± 0.4BCD | 4.4 ± 1.5ABC | 4.3 ± 1.2ABCD | 5.1 ± 0.1ABC | 2.6 ± 0.0C | 2.9 ± 0.1A |
| *E. coli* O157:H7-36 | 6.9 ± 0.2A | 5.5 ± 0.3ABC | 5.6 ± 0.5AB | 4.8 ± 0.5ABCD | 3.7 ± 0.2BCD | 3.0 ± 0.3C | 2.7 ± 0.2A |
| *E. coli* O157:H7-38 | 7.0 ± 0.1AB | 4.8 ± 0.4CD | 4.3 ± 0.3ABC | 3.9 ± 0.3CDE | 3.4 ± 0.4CD | 2.6 ± 0.0C | 2.6 ± 0.0A |
| *E. coli* O157:H7-43895 | 7.0 ± 0.0A | 4.6 ± 0.3D | 2.3 ± 0.5D | 3.3 ± 0.0DE | 3.3 ± 0.0CD | 2.6 ± 0.0C | 2.6 ± 0.0A |
| *S.* Agona | 7.0 ± 0.1AB | 5.2 ± 0.6BCD | 4.7 ± 0.0BCD | 4.1 ± 0.6BCDE | 4.1 ± 1.2ABCD | 1.5 ± 0.0D | 2.6 ± 0.0A |
| *S.* Anatum | 7.0 ± 0.0A | 5.8 ± 0.0AB | 5.6 ± 0.3AB | 4.9 ± 1.5CD | 4.2 ± 1.4ABCD | 1.5 ± 0.0D | 2.6 ± 0.0A |
| *S.* Montevideo | 6.7 ± 0.3AB | 3.2 ± 0.5E | 2.7 ± 0.5CD | 2.5 ± 0.6E | 2.4 ± 1.3D | 1.5 ± 0.0D | 2.6 ± 0.0A |
| *S.* Typhimurium | 6.9 ± 0.0AB | 4.6 ± 0.2D | 4.2 ± 0.0BC | 3.7 ± 0.2DE | 4.0 ± 0.9ABCD | 1.5 ± 0.0D | 2.3 ± 0.4A |
| *S.* Senftenberg | 6.9 ± 0.1AB | 5.9 ± 0.1AB | 3.7 ± 1.5CD | 4.3 ± 1.4CD | 3.5 ± 1.0BCD | 1.3 ± 0.4D | 2.6 ± 0.0A |

[a] Standard deviation

[b] Mean counts in the same column with the same letter (ABC) are not significantly different ($P$>0.05)

TABLE 16. *Mean counts (log CFU/ml) for fluorescent protein-marked* E. coli *surrogates,* E. coli *O157:H7 and* Salmonella *strains stored at low temperatures*

| | Mean count (log CFU/ml) ± SD[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Time (days) | | | | | | |
| | 0 | 7 | 14 | 21 | 28 | 60 | 90 |
| Microorganism | Non acid-adapted/4°C | | | | | | |
| *E. coli* surrogates[b] | 7.2 ± 0.1A[e]x[f] | 7.0 ± 0.1Ax | 6.9 ± 0.3Ax | 6.7 ± 0.5Ax | 6.6 ± 0.6Ax | 5.8 ± 0.6Ay | 5.4 ± 0.7Ay |
| *E. coli* O157:H7[c] | 7.1 ± 0.1Aw | 6.9 ± 0.2Aw | 6.7 ± 0.3Awx | 6.7 ± 0.4Awx | 6.2 ± 0.5Ax | 4.9 ± 0.6By | 4.2 ± 0.8Bz |
| *Salmonella*[d] | 7.1 ± 0.1Ax | 6.9 ± 0.1Ax | 6.8 ± 0.3Ax | 6.3 ± 0.5Ax | 6.3 ± 0.7Ax | 5.4 ± 0.9ABy | 5.2 ± 1.1Ay |
| | Acid-adapted/4°C | | | | | | |
| *E. coli* surrogates | 7.1 ± 0.1Ax | 6.8 ± 0.2Axy | 6.5 ± 0.4Axy | 6.3 ± 0.5Axy | 6.0 ± 0.6Ay | 4.9 ± 1.1Az | 4.3 ± 0.9Az |
| *E. coli* O157:H7 | 7.0 ± 0.1ABw | 6.2 ± 0.7Bwx | 5.7 ± 0.8Bxy | 5.3 ± 0.7Bxy | 4.9 ± 0.7By | 3.3 ± 1.2Bz | 3.5 ± 0.8Az |
| *Salmonella* | 6.9 ± 0.2Bx | 6.5 ± 0.3ABx | 6.1 ± 0.4ABxy | 6.5 ± 0.6Ax | 5.6 ± 0.4Ay | 3.8 ± 1.3Bz | 3.7 ± 0.5Az |
| | Non acid-adapted/-18°C | | | | | | |
| *E. coli* surrogates | 7.2 ± 0.1Ax | 6.7 ± 0.1Axy | 6.6 ± 0.3Axy | 6.2 ± 0.8Axy | 5.9 ± 1.3Ay | 4.5 ± 1.3Az | 4.5 ± 1.1Az |
| *E. coli* O157:H7 | 7.1 ± 0.1ABx | 6.4 ± 0.5Axy | 6.3 ± 0.5Axy | 6.2 ± 0.5Axy | 5.5 ± 1.0Ayz | 5.0 ± 1.0Az | 4.6 ± 1.0Az |
| *Salmonella* | 7.1 ± 0.1Bx | 5.2 ± 1.0By | 4.7 ± 0.9By | 4.9 ± 1.2By | 4.2 ± 1.2Byz | 2.9 ± 1.2Bz | 2.9 ± 0.6Bz |
| | Acid-adapted/-18°C | | | | | | |
| *E. coli* surrogates | 7.1 ± 0.1Aw | 5.8 ± 0.5Ax | 5.7 ± 0.4Axy | 5.6 ± 0.5Axy | 5.0 ± 0.9Ay | 3.3 ± 0.6Az | 2.7 ± 0.5Az |
| *E. coli* O157:H7 | 7.0 ± 0.1ABx | 5.0 ± 0.5By | 4.5 ± 1.3By | 4.5 ± 1.0ABy | 4.2 ± 1.0By | 3.1 ± 0.8Az | 2.8 ± 0.3Az |
| *Salmonella* | 6.9 ± 0.2Bv | 4.9 ± 1.1Bw | 4.1 ± 1.2Bwx | 3.9 ± 1.1Bwx | 3.7 ± 1.1Bxy | 1.5 ± 0.2Bz | 2.6 ± 0.2Ayz |

[a] Standard deviation
[b] Includes fluorescent protein-marked *E. coli* surrogates RFP-1, GFP-3, YFP14 and YFP-66
[c] Includes *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895
[d] Includes *Salmonella* serotypes Agona, Anatum, Montevideo, Typhimurium and Senftenberg
[e] Mean values in the same column within the same condition and temperature followed by the same letter (ABC) are not different ($P$>0.05)
[f] Mean values in the same row with the same letter (vwx) are not different ($P$>0.05)

significantly different at 28 d ($P>0.05$), but were significantly lower after 60 and 90 d of storage ($P<0.05$). When the organisms were acid-adapted and stored at 4°C, *E. coli* O157:H7 counts were significantly lower at 14 d of storage, while counts for fluorescent protein-marked *E. coli* surrogates and *Salmonella* were lower after 28 d ($P<0.05$). Non acid-adapted *E. coli* surrogates and *E. coli* O157:H7 counts were significantly lower at 28 d, while *Salmonella* counts were significantly reduced after 7 d at -18°C. Mean counts for the three groups of microorganisms were significantly lower at 7 d of storage at -18°C when they were acid-adapted. These results demonstrate that previous exposure of the microorganisms to reduced pH conditions does not confer a cross-protection against cold stress, and contrary to this, it reduces their survival capacity when exposed to low temperatures for prolonged periods of time. Uyttendaele et al. *(245)* reported that survival of *E. coli* O157:H7 strains in beef gravy was prolonged at 7°C and -18°C when the pH of the medium had been reduced from 7.0 to 4.5, suggesting that the acid stress imposed in the microorganism triggered a general stress response system leading to the increased resistance and thus enhanced survival at low temperatures. However, it is possible that the high nutrient content in the medium provided an additional protection effect on the microorganism.

The mean log counts (CFU/ml) for fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* groups were plotted versus time at 4°C (Figures 10 and 11) and at -18°C (Figures 12 and 13). From these charts, it can be noted that in general, the survival ability of the fluorescent protein-marked *E. coli* surrogates was similar or higher to *E. coli* O157:H7 and *Salmonella* at 4°C and -18°C, regardless if the microorganisms

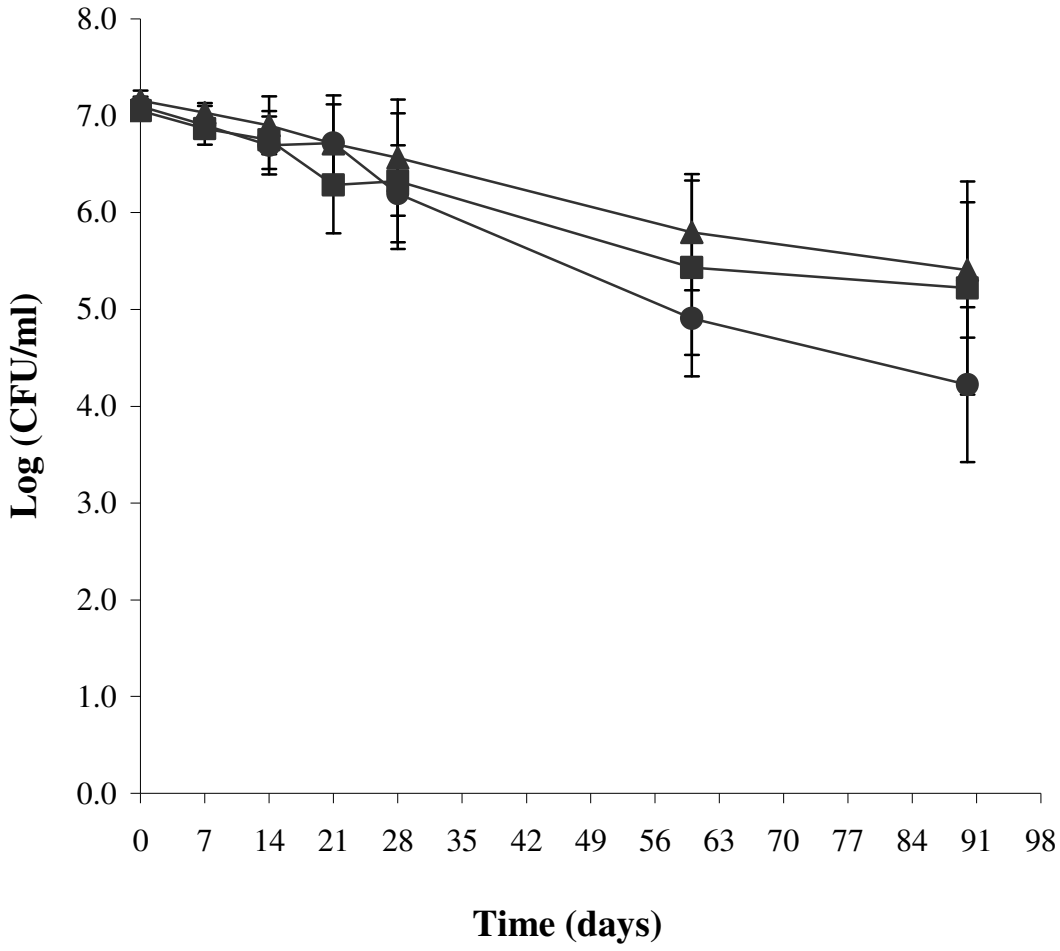


FIGURE 10. *Mean counts (log CFU/ml) for non acid-adapted fluorescent protein-marked* E. coli *surrogates* (▲)*,* E. coli *O157:H7* (●) *and* Salmonella (■) *strains in phosphate buffered saline at 4°C.*

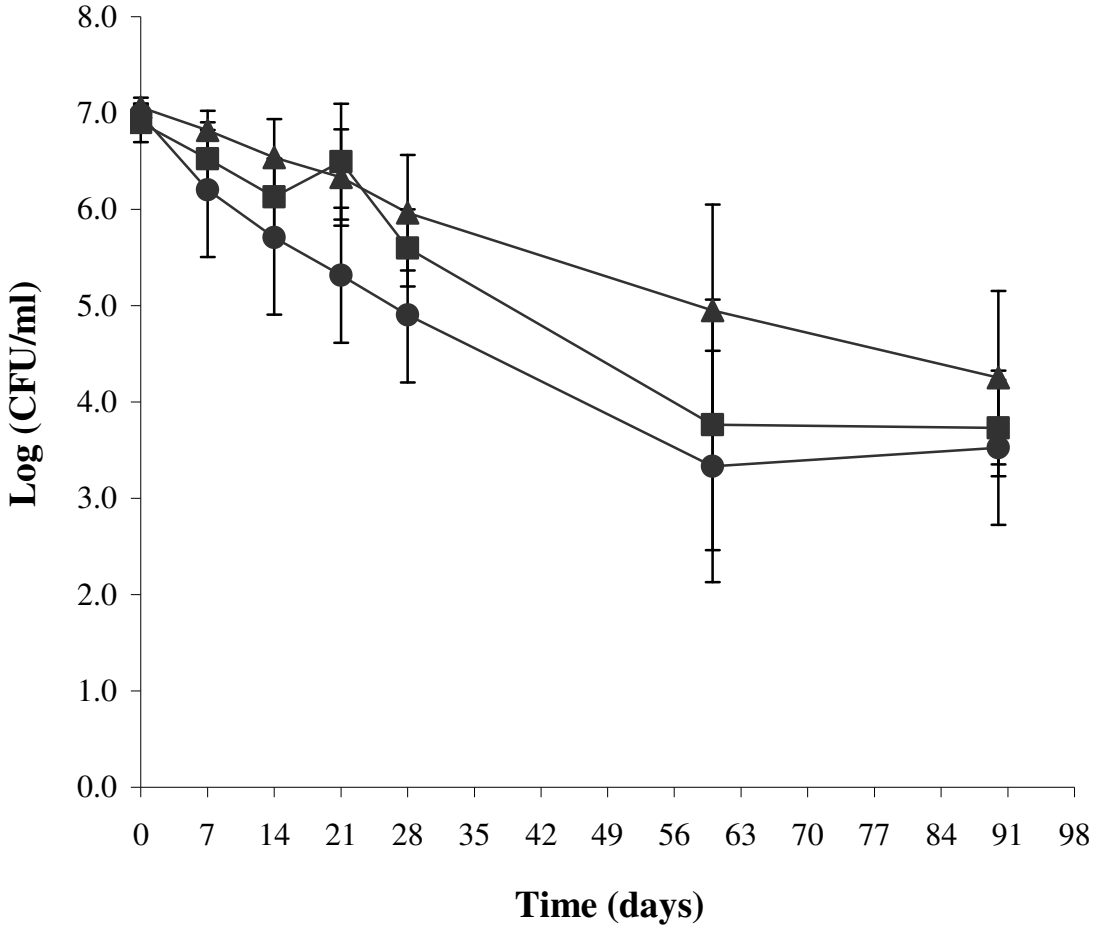


FIGURE 11. *Mean counts (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli *surrogates* (▲)*,* E. coli *O157:H7* (●) *and* Salmonella (■) *strains in phosphate buffered saline at 4°C.*

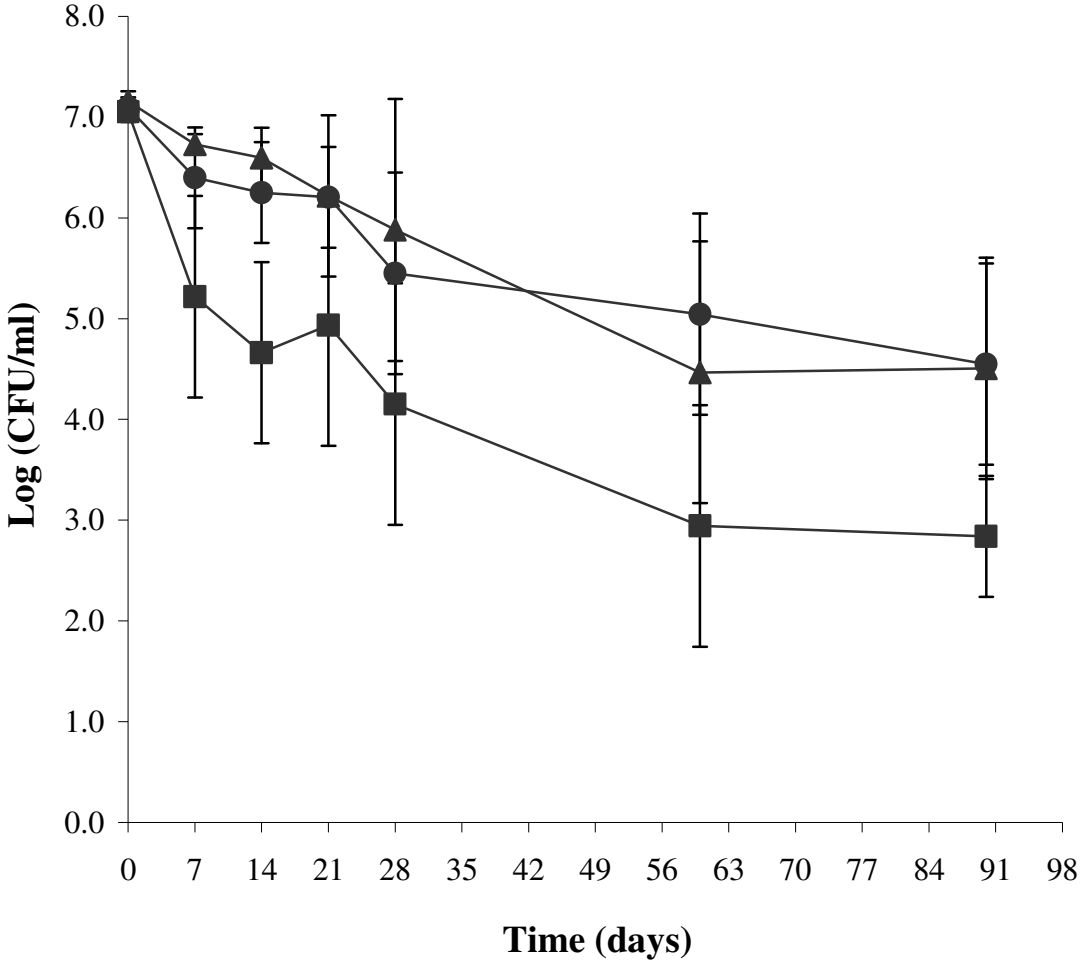


FIGURE 12. *Mean counts (log CFU/ml) for non acid-adapted fluorescent protein-marked* E. coli *surrogates* (▲)*,* E. coli *O157:H7* (●) *and* Salmonella (■) *strains in phosphate buffered saline at -18°C.*

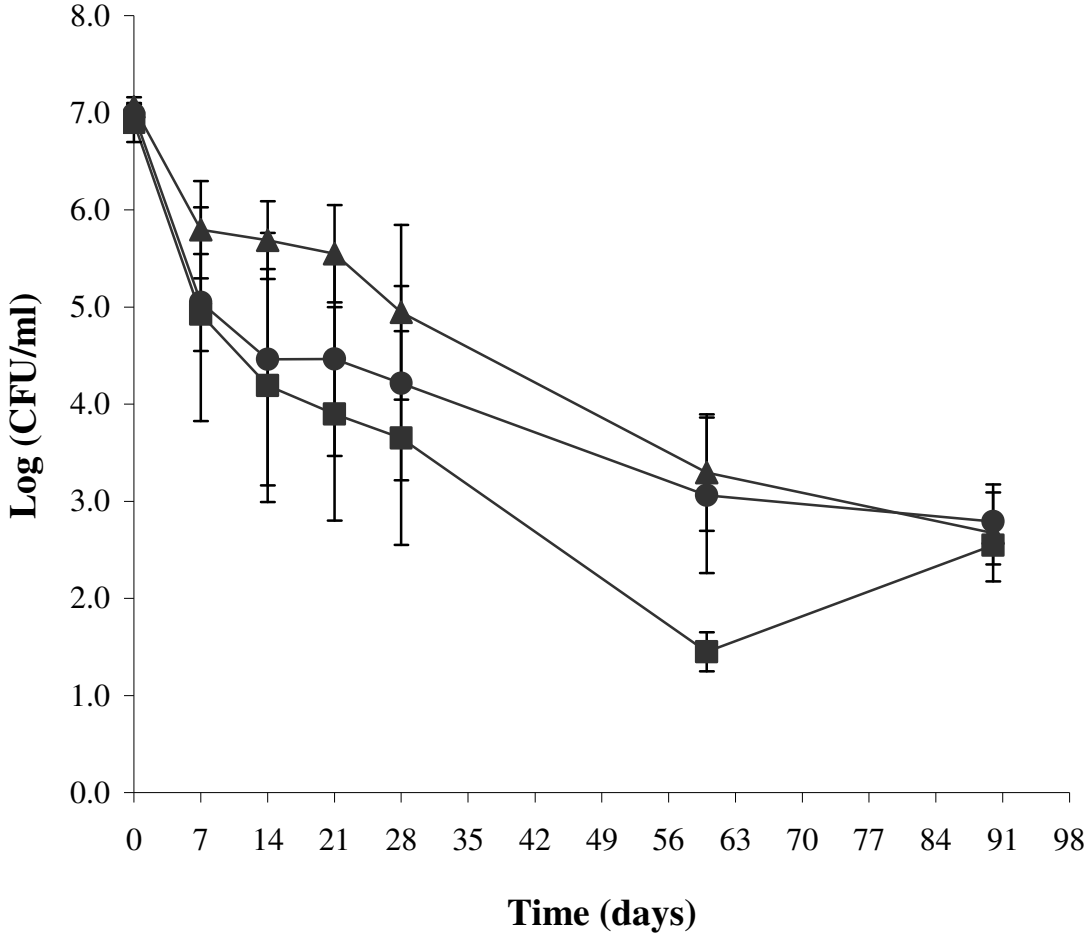


FIGURE 13. *Mean counts (log CFU/ml) for acid-adapted fluorescent protein-marked* E. coli *surrogates* (▲)*,* E. coli *O157:H7* (●) *and* Salmonella (■) *strains in phosphate buffered saline at -18°C.*

had been previously acid-adapted or not. According to the results obtained, the fluorescent protein-marked *E. coli* strains may be appropriate surrogates for *E. coli* O157:H7 and *Salmonella* in validation studies for food products stored at low temperatures; however, evaluation studies in specific food products are required.

**Cell surface hydrophobicity and attachment properties of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains to beef carcass surfaces.** The ability of enteric pathogenic bacteria to adhere to the surface of beef carcasses represents a major food safety problem for the meat industry. Bacterial attachment to beef tissues is a complex phenomenon. Factors involved in the attachment process include the physicochemical properties of the surfaces such as charge and hydrophobicity, as well as specific molecular interactions between the surface of the bacterial cell and the tissue. Bacterial adhesion progresses in two stages: an initial approach governed by electrostatic repulsion and van der Waals attraction, and a stabilization phase where secondary interactions are established through fimbriae (pili) in gram negative organisms, or through carbohydrate polymers in gram positive organisms *(256)*. The hydrophobic nature of the outer surface of bacterial cells has been related to their ability to attach to surfaces. Cell-hydrophobicity represents an attractive force that is strongly correlated with microbial adhesion to surfaces. When bacterial and support surfaces are both hydrophobic, microbial adhesion is highly facilitated, and an increased cell surface hydrophobicity favors the adhesion of cells to hydrophilic and hydrophobic surfaces *(155)*.

A number of methods to determine the hydrophobic interactions of bacterial cells have been developed. Among them, the bacterial adhesion to hydrocarbons (BATH) method proposed by Rosenberg et al. *(209)* is a simple quantitative technique for measuring the cell surface hydrophobicity based on the degree of adherence of cells to hydrocarbons following a brief period of mixing. The hydrophobicity of the fluorescent protein-marked *E. coli, E. coli* O157:H7, and *Salmonella* strains was determined by the BATH method. Each microorganism was previously cultured in TSB and TSB+G, in order to determine if acid adaptation could have an effect on the cell surface hydrophobicity. Previous reports indicate that acid-adapted cells of *S.* Typhimurium showed significantly higher hydrophobicity that non acid-adapted cells *(149).* Increased hydrophobicity may favor the attachment of microorganisms to support surfaces.

Hydrophobicity (expressed as %BATH) for non acid-adapted fluorescent protein-marked *E. coli* strains ranged from 24.9 ± 7.8 to 45.6 ± 3.8%; from 28.2 ± 7.2 to 54.3 ± 3.7% for *E. coli* O157:H7 and from 26.8 ± 7.0 to 43.3 ± 10.6% for *Salmonella* strains (Table 17). *E. coli* O157:H7 #43895 which is a foodborne outbreak isolate, showed significantly higher hydrophobicity than *E. coli* O157:H7 strains #2, 36 and 36 isolated from bovine feces. There was a large variability among microorganisms and also between duplicates for some strains. Due to this phenotypic variation, we found that no single fluorescent-marked *E. coli* strain showed a hydrophobicity pattern that represented all pathogenic strains. The hydrophobicity observed for the fluorescent protein-marked *E. coli* strains covered a wide range of values that were not significantly different ($P>0.05$) to the hydrophobicity values showed by different target pathogens.

TABLE 17. *Bacterial adhesion to hydrocarbons (BATH) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *strains*

| | BATH[a] (%) ± SD[b] | |
|---|---|---|
| Microorganism | Non acid-adapted | Acid-adapted |
| *E. coli* RFP-1 | 29.6 ± 4.6 CDE[c] *x*[d] | 27.2 ± 2.8 CD *x* |
| *E. coli* GFP-3 | 24.9 ± 7.8 E *x* | 25.3 ± 3.8 CD *x* |
| *E. coli* YFP-14 | 45.6 ± 3.8 ABC *x* | 50.1 ± 2.3 A *x* |
| *E. coli* YFP-66 | 23.6 ± 5.1 E *x* | 28.4 ± 4.7 CD *x* |
| *E. coli* O157:H7-2 | 30.1 ± 6.5 CDE *x* | 33.6 ± 10.4 BCD *x* |
| *E. coli* O157:H7-26 | 28.2 ± 7.2 DE *x* | 25.7 ± 3.9 CD *x* |
| *E. coli* O157:H7-36 | 36.3 ± 8.5 BCDE *x* | 29.9 ± 7.7 BCD *x* |
| *E. coli* O157:H7-38 | 47.7 ± 2.8 AB *x* | 37.4 ± 7.1 ABC *x* |
| *E. coli* O157:H7-43895 | 54.3 ± 3.7 A *x* | 52.5 ± 8.2 A *x* |
| *S.* Agona | 26.8 ± 7.0 DE *x* | 19.4 ± 4.6 D *x* |
| *S.* Anatum | 28.9 ± 12.6 CDE *x* | 29.6 ± 11.2 BCD *x* |
| *S.* Montevideo | 43.3 ± 10.6 ABCD *x* | 32.1 ± 13.4 BCD *x* |
| *S.* Typhimurium | 40.5 ± 1.9 ABCDE *x* | 44.9 ± 2.4 AB *x* |
| *S.* Senftenberg | 39.2 ± 21.5 ABCDE *x* | 41.2 ± 5.7 ABC *x* |

[a] Bacterial adhesion to hydrocarbons values represent the mean of six replicates
[b] Standard deviation
[c] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)
[d] Mean values in the same row with the same letter (*xyz*) are not different ($P$>0.05)

Previous studies have also reported differences in the hydrophobicity among pathogenic bacteria from similar or different species. Dickson and Koohmaraie *(79)* reported large differences in the cell surface hydrophobicity of gram negative and gram positive bacteria as determined by different methods such as hydrophobic interaction chromatography (HIC), contact angle and BATH. Ukuku and Fett *(232)* found a substantial variation in the bacterial surface hydrophobicity (using the HIC method) among strains of *Salmonella* but not among *E. coli* O157:H7 and *L. monocytogenes* strains. The highest cell surface hydrophobicity was exhibited by *Salmonella* followed by *L. monocytogenes* and *E. coli* O157:H7. Li and McLandsborough *(150)* compared the cell surface properties among 22 *E. coli* O157 and non-O157 strains by using the BATH method and large differences were observed in the hydrophobicity within strains. Values ranged from 0.4 to 74.1% using the BATH method. Rivas et al. *(205)* found significant differences in the hydrophobicity of 20 STEC strains. BATH values ranged from 21 to 58% for planktonic cultures and from 0 to 36% for sessile cultures. It is difficult to make comparisons among studies when different methods have been used and when the cultures and the testing conditions are not similar, but it is evident that strain to strain variation in cell surface characteristics is present among enteric pathogens.

The hydrophobicity for acid-adapted fluorescent protein-marked *E. coli* strains ranged from 25.3 to 50.1%; from 25.7 to 52.5% for *E. coli* O157:H7 and from 19.4 to 44.9% for *Salmonella* strains (Table 17). No significant differences were observed in hydrophobicity properties between non acid-adapted and acid-adapted organisms ($P>0.05$). Leyer and Jonhson *(149)* had previously reported that acid adaptation

increased the cell surface hydrophobicity in *S.* Typhimurium. The authors related the changes in hydrophobicity to changes in the synthesis of outer membrane proteins and consequently, to the modification of the structure on the bacterial outer membrane. Results from this study and the one reported by Leyer and Johnson *(149)* are difficult to compare, since they used hexadecane instead of xylene for the BATH analysis. Hydrophobicity values observed for *S.* Typhimurium in this study were 40.5% for non acid-adapted and 44.9% for acid-adapted cells; while values reported by those authors ranged from 0.108 to 11.76% in non acid-adapted and acid-adapted cells, respectively.

The hydrophobicity values (%BATH) were averaged by groups of microorganisms and compared. The fluorescent protein-marked *E. coli* surrogates showed no different hydrophobicity than *E. coli* O157:H7 and *Salmonella* groups ($P>0.05$) regardless of acid adaptation (Table 18). The inability to find significant differences among groups may be due to the large variability observed among strains and among some duplicates of the same organism.

The strength of attachment ($S_R$) to beef carcass surfaces was determined and compared among surrogates and target pathogens. The number of loosely and strongly attached cells on the surface of hot-boned outside rounds, briskets and clods was determined for the fluorescent protein-marked *E. coli, E. coli* O157 and *Salmonella* strains and used to calculate their $S_R$ value. Bacteria can be freely located at the water film on the surface of tissues ("loosely" attached) or can be physically associated to the surface ("strongly" attached). Attachment strength increases if the target surface is not

TABLE 18. *Bacterial adhesion to hydrocarbons (BATH) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *groups*

| | BATH[a] (%) ± SD[b] | |
|---|---|---|
| Microorganism | Non acid-adapted | Acid-adapted |
| *E. coli* surrogates | 31.0 ± 10.3 A[c] *x*[d] | 32.7 ± 10.9 A *x* |
| *E. coli* O157:H7 | 39.3 ± 11.7 A *x* | 35.8 ± 11.7 A *x* |
| *Salmonella* | 35.7 ± 13.1 A *x* | 33.4 ± 11.9 A *x* |

[a] Bacterial adhesion to hydrocarbon values represent the mean of six replicates
[b] Standard deviation
[c] Mean values in the same column with the same letter (ABC) are not different ($P>0.05$)
[d] Mean values in the same row with the same letter (*xyz*) are not different ($P>0.05$)

uniformly smooth or flat. A rough surface, like the one found on beef carcasses, reduces near-surface shear forces that negatively affect the initial phases of bacterial adhesion. At the same time, rough surfaces allows bacterial cells to settle into molecular grooves or canyons, increasing the number of possible cell-to-surface contact sites *(256)*.

The mean counts (log CFU/cm$^2$) obtained for the loosely attached cells were not significantly different among the fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* strains regardless of the carcass region ($P>0.05$) (Table 19). Significantly different counts of strongly attached cells were observed only for *Salmonella* on the outside round which showed higher counts than fluorescent protein-marked *E. coli* surrogates and *E. coli* O157:H7 on the clod ($P<0.05$). This may be due to the higher amount of fat tissue present on the outside round which may favor hydrophobic interactions.

$S_R$ values were calculated for each group of microorganisms. The $S_R$ is a measure of the relative strength of bacterial attachment and indicates the proportion of the total population of bacteria which is physically attached to the contact surface *(79)*. The higher the $S_R$ value, the higher the number of bacteria that are physically attached. No significant differences were observed among the $S_R$ values for the fluorescent protein-marked *E. coli*, *E. coli* O157:H7 and *Salmonella* strains, regardless of the carcass region ($P>0.05$) (Table 19). $S_R$ values observed for the fluorescent protein-marked *E. coli* surrogates ranged from 0.162 to 0.224, from 0.099 to 0.236 for the *E. coli* O157:H7 cocktail and from 0.149 to 0.296 for the *Salmonella* cocktail. This $S_R$ values are not very different from those reported by Dickson and Koohmaraie *(79)* for enteric pathogens on

TABLE 19. *Mean counts (log CFU/cm$^2$) of loosely and strongly attached cells and strength of attachment ($S_R$) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *strains on beef carcass surfaces*

| | | Mean count[a] (log CFU/cm$^2$) ± SD[b] | | |
|---|---|---|---|---|
| Carcass region | Microorganism | Loosely attached | Strongly attached | $S_R$[c] |
| Outside round | *E. coli* surrogates[d] | 6.8 ± 0.2 A[g] | 6.1 ± 0.4 AB | 0.224 A |
| | *E. coli* O157:H7[e] | 7.0 ± 0.4 A | 6.3 ± 0.3 AB | 0.236 A |
| | *Salmonella*[f] | 7.0 ± 0.4 A | 6.6 ± 0.3 B | 0.296 A |
| Brisket | *E. coli* surrogates | 6.6 ± 0.5 A | 6.2 ± 0.4 AB | 0.337 A |
| | *E. coli* O157:H7 | 7.0 ± 0.2 A | 6.2 ± 0.2 AB | 0.160 A |
| | *Salmonella* | 7.2 ± 0.2 A | 6.5 ± 0.1 AB | 0.177 A |
| Clod | *E. coli* surrogates | 6.8 ± 0.3 A | 5.9 ± 0.2 A | 0.162 A |
| | *E. coli* O157:H7 | 6.9 ± 0.3 A | 6.0 ± 0.2 A | 0.099 A |
| | *Salmonella* | 7.1 ± 0.3 A | 6.2 ± 0.2 AB | 0.149 A |

[a] Log counts (CFU/cm$^2$) represent the mean of six replicates
[b] Standard deviation
[c] $S_R$ = (strongly attached cells)/(strongly attached cells + loosely attached cells)
[d] Includes fluorescent protein-marked *E. coli* strains RFP-1, GFP-3, YFP-14 and YFP-66
[e] Includes *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895
[f] Includes *Salmonella* serotypes Agona, Anatum, Montevideo, Typhimurium and Senftenberg
[g] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)

meat tissues. According to their report, $S_R$ values for *E. coli* O157:H7 on lean and fat tissues were 0.118 and 0.183, while for *S.* Typhimurium were 0.170 and 0.139, respectively. It is possible that the slightly higher $S_R$ values observed in this study are related to the use of fecal slurries as vehicles for inoculation of the carcass surfaces, instead of bacterial suspensions using buffer solutions. It has been previously demonstrated by Delazari et al. *(75)* that removal of *E. coli* O157:H7 is more difficult if the meat surface has been inoculated with bovine feces, than if the inoculation has been made with a bacterial suspension.

The correlation between bacterial cell surface hydrophobicity and strength of attachment has been established on inert surfaces and also in different food commodities. Dickson and Koohmaraie *(79)* reported a correlation between hydrophobicity and strength of attachment on fat meat tissue. Ukuku and Fett *(232)* found a correlation between bacterial cell surface hydrophobicity and the strength of attachment for *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* to cantaloupe rinds. No correlation was found between %BATH and $S_R$ for the fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella strains* included in this study ($P$>0.05). This may be explained by the large variability observed among strains and among some duplicates due to the complexity of the testing system by using hot-boned carcass surfaces and bovine feces as the inoculation vehicle. Several studies have also failed to find correlation among hydrophobicity and attachment. Gilbert et al. *(111)* reported that surface hydrophobicity did not correlate with the adhesion of *E. coli* to the surface of glass slides. Dickson and Koohmaraie *(79)* did not find a correlation between hydrophobicity and attachment of

several gram positive and gram negative bacteria to lean beef tissue. Bouttier et al. *(23)* did not find any evidence of a role of hydrophobicity interactions in attachment of *S.* Cholerasuis to either fat or lean beef. Rivas et al. *(204)* found no correlation between hydrophobicity of STEC strains and their adhesion to beef tissues. This lack of correlation has been attributed to the complexity and heterogeneous nature of the meat surface tissues.

An important characteristic of organisms intended for use as surrogates for pathogens in validation studies, is that they must demonstrate similar surface characteristics and attachment properties *(30).* According to the results of this study, the cell surface hydrophobicity and the attachment properties to beef tissues of the fluorescent protein-marked *E. coli* strains are not different from those of *E. coli* O157:H7 and *Salmonella,* and when used as a cocktail of strains, they may constitute appropriate surrogates for these target pathogens.

**Response of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains to hot water washes on beef carcasses.** Surrogate organisms intended for use in validation studies must demonstrate similar reductions to target pathogens after the application of those interventions to be validated. Application of hot water washes on beef carcasses pre- or post-evisceration have been proposed as an effective intervention. USDA-FSIS regulations indicate that water applied to the surface of beef carcasses at a temperature greater than 165°F (>74°C) for more than 10 s can be an effective antimicrobial intervention *(241)*. Efficacy of hot water washes reported by

several studies differs and the variation observed may be a consequence of differences in treatment parameters such as water temperature, pressure, contact time, as well as distance between nozzles and carcass surface. The response of the fluorescent protein-marked *E. coli* strains proposed as surrogates was compared to that of *E. coli* O157:H7 and *S.* Typhimurium after application of hot water washes at three different temperatures on the surface of beef carcasses. Log reductions ($CFU/cm^2$) achieved on surrogates and target pathogens at different temperatures are presented in Table 20. According to these data, in general, as the temperature on the carcass surface and the contact time were increased, the log reductions ($CFU/cm^2$) were also increased.

Mean log reductions ($CFU/cm^2$) for all the microorganisms at different temperatures were calculated and plotted versus time. The effect of hot water washes on surrogates and pathogens at increasing temperature and contact time is shown in Figure 14. It is difficult to compare studies reported in the literature due to differences in the application parameters of the hot water washes, type of microbial populations evaluated and reporting units. Dorsa et al. *(86)* obtained reductions of 2.0 and 2.7 log $CFU/cm^2$ on APC and coliforms, respectively, after application of hot water at 82.2°C for 10 s which increased the carcass surface temperature to 72°C. Barkate et al. *(11)* obtained reductions of 1.3 log $CFU/cm^2$ on APC after application of water at 95°C, reaching a carcass surface temperature of 82°C for 10 s. Bosilevac et al. *(22)* reported reductions of 2.7 log $CFU/100cm^2$ on APC and coliforms when the hot water was applied at 74°C and the carcass surface temperature was increased to 70°C for 5.5 s.

TABLE 20. *Mean reductions (log CFU/cm$^2$) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *Typhimurium on beef carcass surfaces by application of hot water washes*

| Temperature (°C)[c] | Time (s) | Mean reduction[a] (log CFU/cm$^2$) ± SD[b] | | |
|---|---|---|---|---|
| | | *E. coli* surrogates[d] | *E. coli* O157:H7 | *S.* Typhimurium |
| 70.9 | 2 | 0.7 ± 0.5 | 0.8 ± 0.8 | 0.8 ± 0.8 |
| | 4 | 1.3 ± 0.4 | 1.4 ± 0.3 | 1.3 ± 0.4 |
| | 6 | 1.8 ± 0.9 | 1.8 ± 0.9 | 1.9 ± 1.2 |
| | 8 | 2.5 ± 1.1 | 2.7 ± 1.3 | 2.3 ± 1.0 |
| | 10 | 2.2 ± 0.6 | 1.9 ± 0.5 | 3.0 ± 1.4 |
| 78.5 | 2 | 1.7 ± 0.8 | 1.7 ± 0.7 | 1.7 ± 0.6 |
| | 3 | 2.7 ± 1.0 | 2.7 ± 1.0 | 2.6 ± 0.9 |
| | 5 | 2.9 ± 0.8 | 3.5 ± 0.7 | 3.1 ± 0.8 |
| | 7 | 3.5 ± 0.9 | 3.7 ± 0.9 | 3.4 ± 0.8 |
| | 9 | 4.1 ± 1.1 | 4.1 ± 0.7 | 4.1 ± 0.6 |
| 84.6 | 1 | 0.9 ± 0.5 | 1.2 ± 0.5 | 1.2 ± 0.5 |
| | 2 | 1.8 ± 1.3 | 2.1 ± 1.4 | 2.0 ± 1.0 |
| | 3 | 3.4 ± 1.3 | 3.5 ± 1.1 | 3.3 ± 0.8 |
| | 4 | 2.6 ± 1.2 | 2.8 ± 1.1 | 2.6 ± 1.0 |
| | 5 | 2.8 ± 1.1 | 2.9 ± 0.8 | 2.9 ± 0.8 |

[a] Log reductions (CFU/cm$^2$) represent the mean of six replicates
[b] Standard deviation
[c] Temperature of the carcass surface during application of hot water washes
[d] Includes fluorescent protein-marked *E. coli* strains RFP-1, GFP-3, YFP-14 and YFP-66

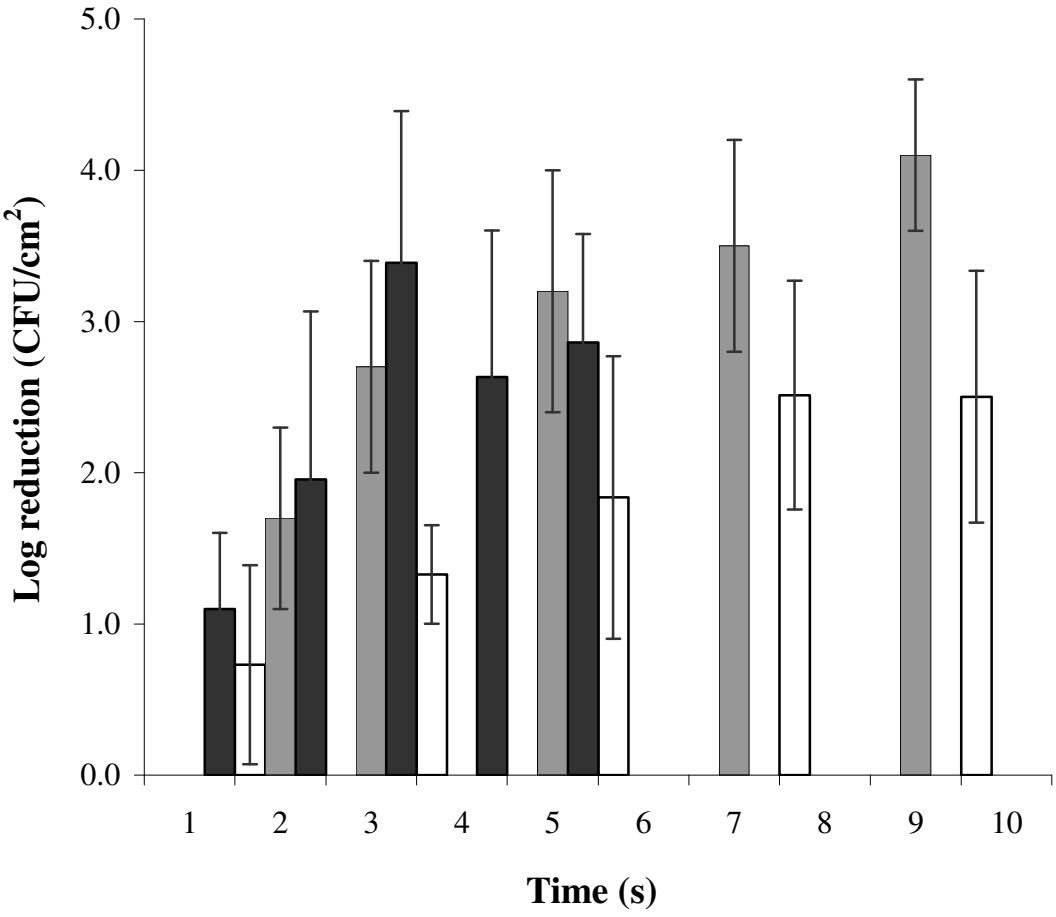


FIGURE 14. *Mean log reductions (CFU/cm$^2$) for fluorescent protein-marked* E. coli *surrogates,* E. coli *O157:H7 and* Salmonella *Typhimurium on beef carcasses by application of hot water washes when the carcass surface temperature was increased to 70.9°C* (□)*, 78.5°C* (■) *and 86.4°C* (■)

Reductions reported by Castillo et al. *(44)* were 1.6, 2.2, 1.6 and 1.9 log CFU/cm$^2$ for APC, coliforms, *E. coli* O157:H7 and *S.* Typhimurium when hot water applied at 95°C increased the carcass surface temperature to 82°C for 5 s. In another study using the same treatment parameters, Castillo et al. *(45)* reported reductions from 0.7 to 2.2 log CFU/cm$^2$ on *E. coli* O157:H7 and *S.* Typhimurium when different carcass surface regions were treated.

In order to compare the thermal inactivation kinetics among surrogates and pathogens, heat inactivation curves were constructed by plotting the survivor data versus contact time after application of the hot water washes at three different temperatures (Figures 15 to 17). After fitting the data by linear regression, the obtained slopes were used to calculate decimal reduction times (D-values) for fluorescent protein-marked *E. coli* surrogates*, E. coli* O157:H7 and *S.* Typhimurium (Table 21). No significant differences were observed in D-values among surrogates and pathogens at each temperature ($P$>0.05). D-values for the fluorescent protein-marked *E. coli* strains either individually enumerated or enumerated as a cocktail were not significantly different to those D-values observed for *E. coli* O157:H7 and *S.* Typhimurium ($P$>0.05). In general, D-values were significantly reduced as the temperature of the carcass surface was increased during the application of hot water washes ($P$<0.05). A large variability was observed in survivor counts after application of hot water among some duplicate samples. This variability is consequently reflected in the standard deviation of the calculated D-values and may be attributed to the uneven topography of the carcass surface. The roughness of beef carcass surfaces may allow bacterial cells to settle into

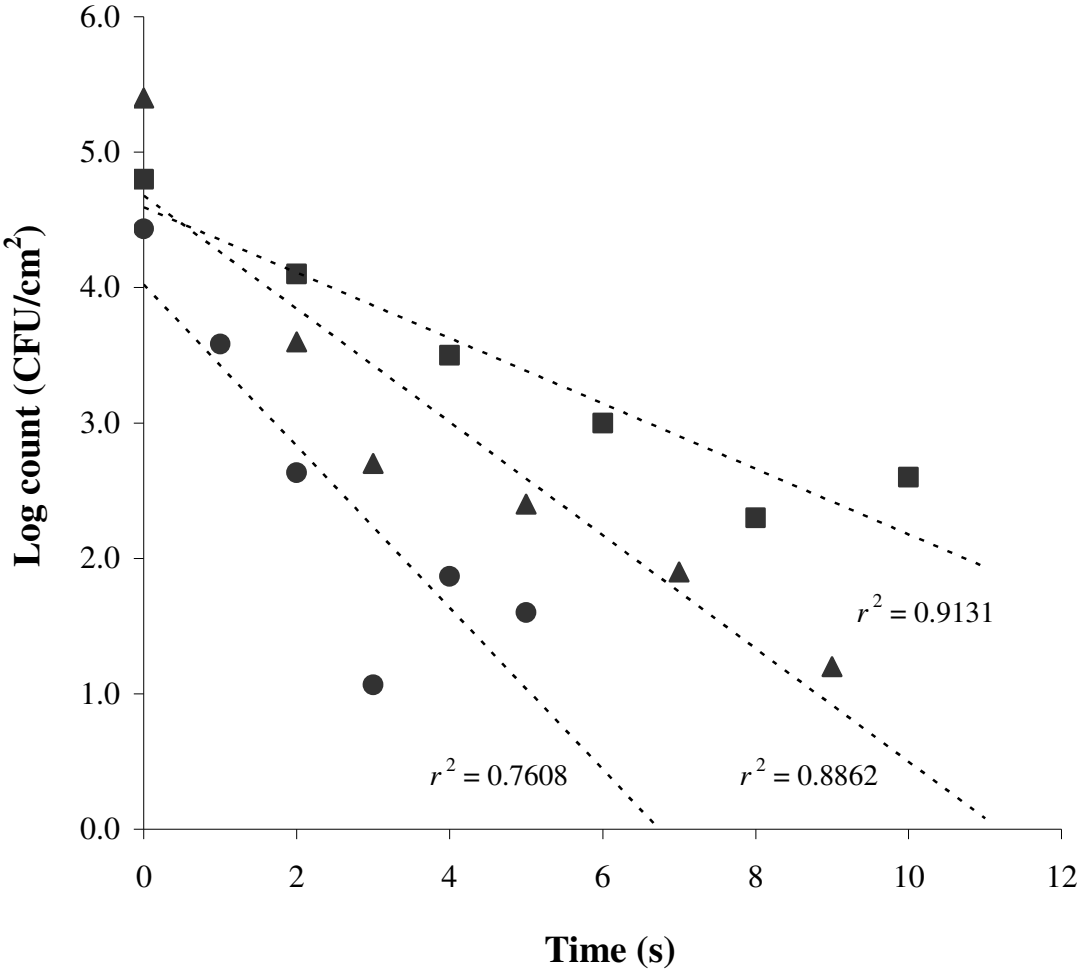


FIGURE 15. *Heat inactivation curves of fluorescent protein-marked* E. coli *surrogates on beef carcasses by application of hot water washes. Each data point represents the mean of six replicates. The dotted lines represent the linear regression of the survival curves at different temperatures achieved on the carcass surface: 70.9°C* (■)*, 78.5°C* (▲) *and 86.4°C* (●).

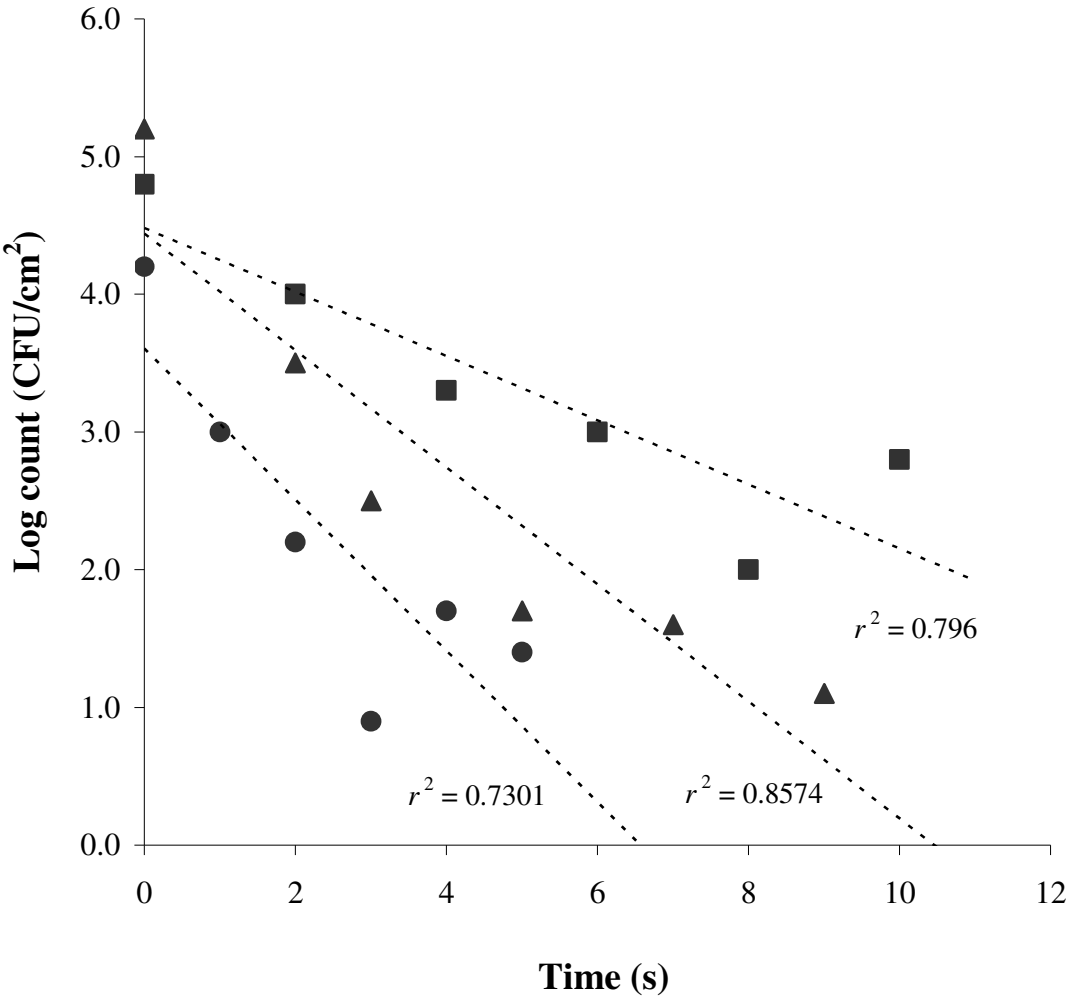


FIGURE 16. *Heat inactivation curves of* E. coli *O157:H7 on beef carcasses by application of hot water washes. Each data point represents the mean of six replicates. The dotted lines represent the linear regression of the survival curves at different temperatures achieved on the carcass surface: 70.9°C* (■)*, 78.5°C* (▲) *and 86.4°C* (●).

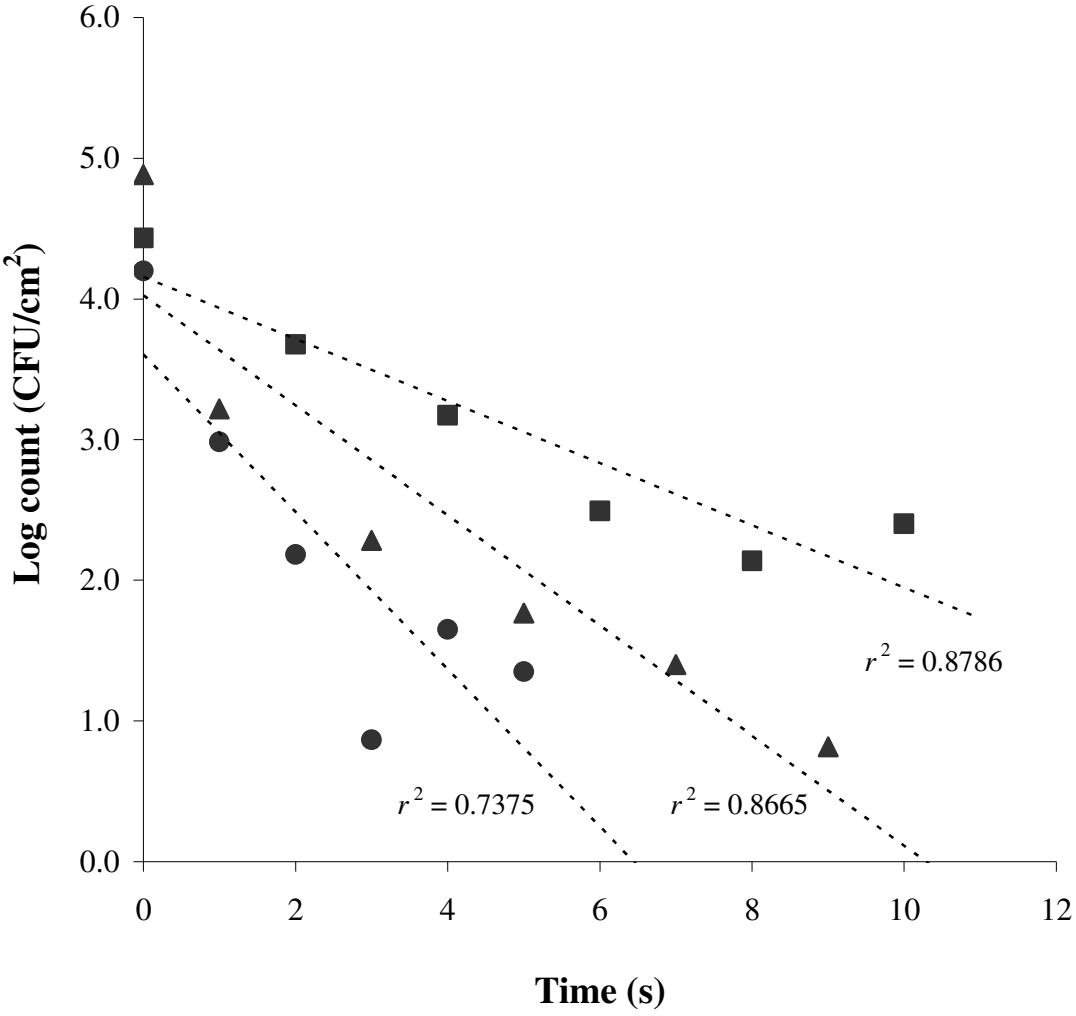


FIGURE 17. *Heat inactivation curves of* S. *Typhimurium on beef carcasses by application of hot water washes. Each data point represents the mean of six replicates. The dotted lines represent the linear regression of the survival curves at different temperatures achieved on the carcass surface: 70.9°C* (■)*, 78.5°C* (▲) *and 86.4°C* (●).

TABLE 21. *Decimal reduction time (D-value) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* S. *Typhimurium strains on beef carcasses treated with hot water washes*[a]

| | D-value[b] (min) ± SD[c] | | |
|---|---|---|---|
| | Temperature of carcass surface (°C) | | |
| Microorganism | 70.9 | 78.5 | 86.4 |
| *E. coli* RFP-1 | 0.07 ± 0.02 A[e]*x*[f] | 0.05 ± 0.01 A*xy* | 0.03 ± 0.01 A*y* |
| *E. coli* GFP-3 | 0.06 ± 0.04 A*x* | 0.04 ± 0.01 A*x* | 0.04 ± 0.02 A*x* |
| *E. coli* YFP-14 and YFP-66 | 0.07 ± 0.04 A*x* | 0.04 ± 0.01 A*y* | 0.03 ± 0.01 A*y* |
| *E. coli* surrogates[d] | 0.07 ± 0.03 A*x* | 0.04 ± 0.01 A*y* | 0.03 ± 0.01 A*y* |
| *E. coli* O157:H7 | 0.07 ± 0.03 A*x* | 0.04 ± 0.00 A*y* | 0.03 ± 0.01 A*y* |
| *S.* Typhimurium | 0.08 ± 0.03 A*x* | 0.04 ± 0.01 A*y* | 0.03 ± 0.01 A*y* |

[a] Hot water was applied at 30 lb/in$^2$ using a flat spray nozzle from a distance of 10 cm
[b] D-values represent the mean of six replicates
[c] Standard deviation
[d] Represent the mean D-value for *E. coli* RFP-1, GFP-3, YFP-14 and YFP-66 enumerated as a cocktail
[e] Means in the same column with the same letter (ABC) are not different ($P$>0.05)
[f] Means in the same row with the same letter (*xyz*) are not different ($P$>0.05)

molecular grooves or crevices, making difficult for the hot water to reach them. Log D-values were plotted as a function of temperature and from the fitted curves (Figure 18) theoretical D-values were calculated for fluorescent protein-marked *E. coli* surrogates, *E. coli* O157:H7 and *S.* Typhimurium at different temperatures. The theoretical D-values were used to estimate the temperature on the carcass surface and the contact time required to achieve a 5-log reduction on surrogates and target pathogens (Table 22). According to these results for example, if a 5-log reduction is targeted, a hot water treatment that is able to increase the carcass surface temperature to 70°C must be applied for approximately 21 s. If the hot water treatment is able to increase the carcass temperature to 80°C, the contact time should be approximately 12.7 s to achieve the same target reduction. Using the equations obtained from the fitted curves presented in Figure 18, theoretical D-values at different temperatures can be calculated and then used to estimate the required contact time to achieve different target reductions. Thermal inactivation studies to validate the process lethality of heat treatments in meat and meat products are common in the literature *(129, 133-135, 173, 174, 188, 189)*. This study constitutes a first attempt to use the same approach for the validation of hot water treatments to reduce enteric pathogens on beef carcasses. More research is required to have more precise estimations of the thermal death kinetics of enteric pathogens by application of hot water treatments on beef carcasses.

The heat loss during the spraying of hot water must be considered to ensure that the target temperature at the carcass surface has been achieved. Heat loss observed

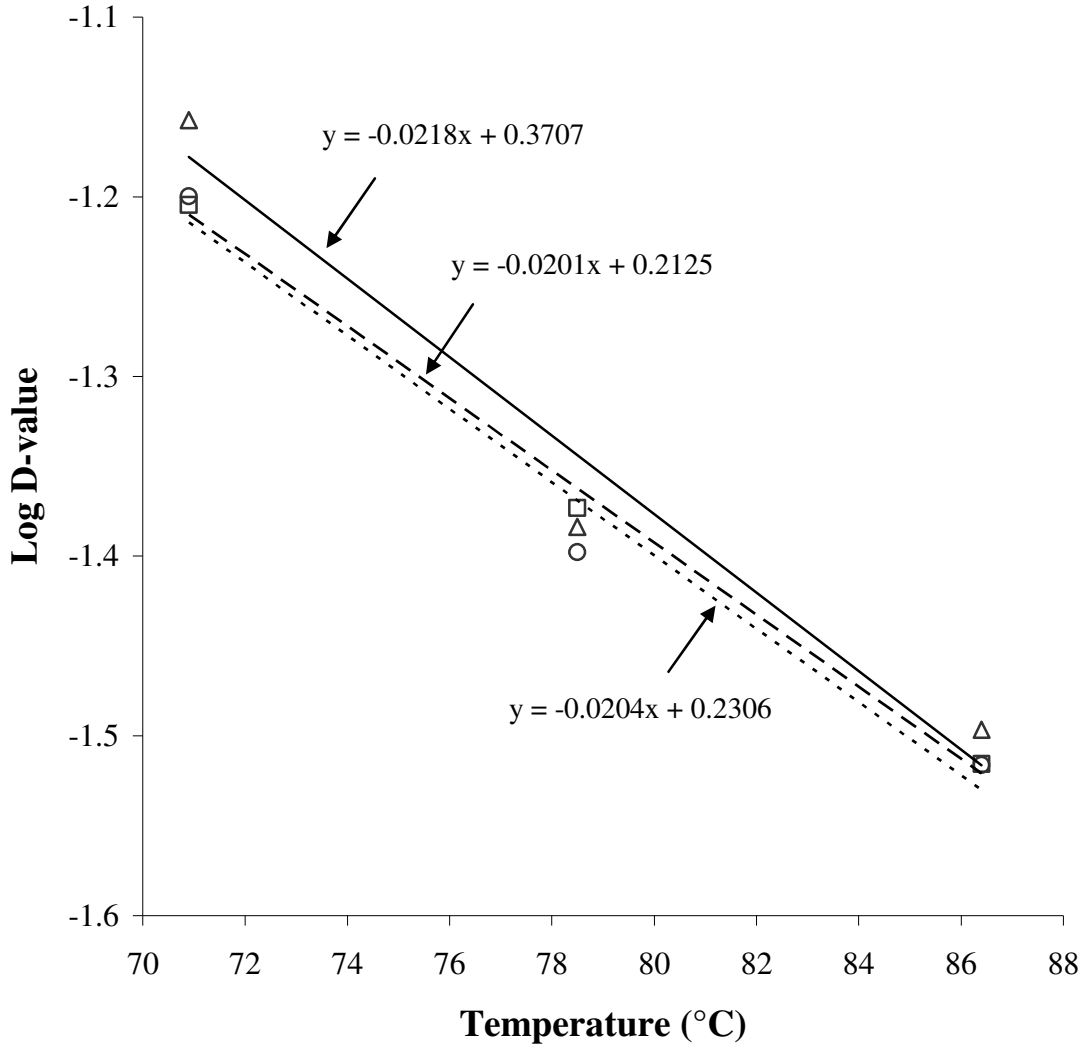


FIGURE 18. *Logarithmic plot of D-values versus temperature for fluorescent protein-marked* E. coli (□), E. coli *O157:H7* (○) *and* S. *Typhimurium* (△) *on beef carcasses*. *The dashed line represents the fitted curve for the fluorescent protein-marked* E. coli; *the dotted line represents the fitted curve for* E. coli *O157:H7 and the solid line represent the fitted curve for* S. *Typhimurium*.

TABLE 22. *Estimated parameters to achieve a 5-log reduction in fluorescent protein-marked* E. coli *surrogates,* E. coli *O157:H7 and* S. *Typhimurium by application of hot water washes on beef carcasses*[a]

| Temperature[b] (ºC) | Contact time (s) | | |
|---|---|---|---|
| | *E. coli* surrogates[c] | *E. coli* O157:H7 | *S.* Typhimurium |
| 70 | 19.2 | 19.0 | 21.0 |
| 72 | 17.5 | 17.3 | 19.0 |
| 74 | 15.9 | 15.8 | 17.2 |
| 76 | 14.5 | 14.4 | 15.5 |
| 78 | 13.2 | 13.1 | 14.0 |
| 80 | 12.1 | 11.9 | 12.7 |
| 82 | 11.0 | 10.8 | 11.5 |
| 84 | 10.0 | 9.9 | 10.4 |
| 86 | 9.1 | 9.0 | 9.4 |
| 88 | 8.3 | 8.2 | 8.5 |
| 90 | 7.6 | 7.4 | 7.7 |

[a] Hot water wash applied at 30 lb/in$^2$ using a flat spray nozzle from a 10-cm distance

[b] Temperature on the carcass surface

[c] Includes fluorescent protein-marked *E. coli* RFP-1, GFP-3, YFP-14 and YFP-66

between the water source or the nozzle and the carcass surface in this study is presented in Table 23. By delivering water at 75.2 ± 1.2°C from a distance of approximately 10 cm, the temperature of the carcass surface was increased to 70.9 ± 2.6°C. This represents a heat loss of 4.7 ± 2.5°C during the spraying of the hot water. As the temperature of the water was increased at the source, the heat loss was higher. Spraying water at 96.6 ± 1.1°C from a 10-cm distance produced a temperature on the carcass surface of 86.4 ± 2.1°C, which represents a heat loss of 10.3 ± 2.3°C. The equipment used in the meat industry for application of hot water washes varies in design and specifications, such as nozzle type, delivery pressure, droplet size, distance between nozzles and carcass surface and, therefore, in the heat loss during the water spraying. It is important to conduct validation studies under commercial operation conditions to determine the parameters that result in maximal reductions for enteric pathogens on beef carcasses.

The fluorescent protein-marked *E. coli* strains evaluated in this study may constitute appropriate surrogates to conduct validation studies for hot water interventions because they showed similar inactivation to enteric pathogens by application of hot water washes on the surface of beef carcasses. The use of surrogates also provides an opportunity of having a high initial concentration as well as a controlled inoculum consisting of marker organisms which facilitates the tracking of microbial reductions.

**Response of fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains to lactic acid sprays on beef carcasses.** Lactic acid is the organic acid most commonly used as an antimicrobial intervention on beef carcasses. It has been

TABLE 23. *Temperature of water and beef carcass surfaces during application of hot water washes*[a]

| Mean temperature (ºC) ± SD[b] | | | | |
|---|---|---|---|---|
| Water at source | Water at nozzle | Carcass surface | Heat loss | |
| | | | Nozzle-carcass[c] | Source-carcass[d] |
| 76.3 ± 1.1 | 75.2 ± 1.2 | 70.9 ± 2.6 | 4.7 ± 2.5 | 5.5 ± 3.4 |
| 86.0 ± 0.2 | 85.2 ± 1.3 | 78.5 ± 2.6 | 7.6 ± 2.7 | 7.5 ± 2.7 |
| 97.1 ± 0.9 | 96.6 ± 1.1 | 86.4 ± 2.1 | 10.3 ± 2.3 | 10.7 ± 1.9 |

[a] Hot water was applied at 30 lb/in$^2$ using a flat spray nozzle from a 10-cm distance
[b] Standard deviation
[c] Temperature difference between water out of the nozzle and the carcass surface
[d] Temperature difference between water at the source and the carcass surface

adopted for pre-evisceration, post-evisceration and post-chilling treatments in commercial facilities, based on the substantial number of studies that support its efficacy. The application of 2.5-5% lactic acid solution on beef carcasses prior to fabrication (either as a pre-chill or post-chill intervention) has been approved by FSIS-USDA regulations *(237).* Surrogate organisms intended to be used for the validation of lactic acid interventions on beef carcasses must demonstrate similar reductions to their target pathogens after the application of lactic acid treatments. For this reason, the response of the fluorescent protein-marked *E. coli* strains proposed as surrogates in this study was compared to that of *E. coli* O157:H7 and *Salmonella* after application of different volumes of 2% L-lactic acid at two different temperatures on the surface of beef carcasses. Mean counts (log CFU/cm$^2$) observed for fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* before and after the application of different volumes of 2% L-lactic acid at 25°C and 55°C are presented in Table 24. The initial concentration of the three groups of microorganisms on the surface of hot-boned outside rounds was approximately 4.7-4.8 log CFU/cm$^2$. According to these data, the mean counts of surrogates and pathogens on the meat surface were significantly lower ($P$<0.05) after application of 2% L-lactic acid regardless of the temperature and the volume of the sprayed solution. The pH of the carcass surface before the application of acid solutions was 8.0 ± 0.4 and it was significantly reduced ($P$<0.05) to 2.6-3.0 units after the spraying 2% L-lactic acid at 25°C and to 3.0-3.1 after applying 2% L-lactic acid at 55°C (Table 24). The mean reductions (log CFU/cm$^2$) calculated for fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* after the application of different

TABLE 24. *Mean counts (log CFU/cm$^2$) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *on beef carcasses by application of 2% L-lactic acid at two temperatures*

| Temperature (°C) | Spraying[a] time (s) | Volume (ml) | Carcass surface pH | Mean reduction[b] ± SD[c] | | |
|---|---|---|---|---|---|---|
| | | | | *E. coli* surrogates[d] | *E. coli* O157:H7[e] | *Salmonella*[f] |
| 22.8 ± 0.1 | 0 | 0 | 8.0 ± 0.4 A[g] | 4.7 ± 0.4 A | 4.7 ± 0.4 A | 4.7 ± 0.3 A |
| | 2 | 47 ± 1.0 | 2.6 ± 0.2 B | 3.2 ± 1.2 B | 3.2 ± 1.3 B | 3.1 ± 1.3 B |
| | 4 | 86 ± 1.6 | 3.0 ± 0.4 B | 2.6 ± 0.6 BC | 2.6 ± 0.6 BC | 2.4 ± 0.5 BC |
| | 6 | 126 ± 1.9 | 2.9 ± 0.1 B | 2.1 ± 1.0 C | 2.2 ± 1.0 BC | 1.8 ± 1.1 C |
| | 8 | 154 ± 4.9 | 3.0 ± 0.3 B | 1.9 ± 1.0 C | 1.8 ± 0.9 C | 1.7 ± 1.1 C |
| 54.4 ± 0.8 | 0 | 0 | 7.9 ± 0.7 A | 4.8 ± 0.3 A | 4.7 ± 0.2 A | 4.8 ± 0.1 A |
| | 2 | 47 ± 1.0 | 3.2 ± 0.2 B | 2.5 ± 0.3 BC | 2.5 ± 0.3 BC | 2.3 ± 0.4 BC |
| | 4 | 86 ± 1.6 | 3.0 ± 0.2 B | 2.6 ± 0.4 BC | 2.6 ± 0.7 BC | 1.9 ± 1.2 C |
| | 6 | 126 ± 1.9 | 3.1 ± 0.3 B | 2.7 ± 0.4 BC | 2.9 ± 0.5 B | 2.5 ± 0.6 BC |
| | 8 | 154 ± 4.9 | 3.1 ± 0.3 B | 2.7 ± 0.6 BC | 2.9 ± 0.7 B | 2.6 ± 0.7 BC |

[a] L-lactic acid was sprayed on carcass surfaces of ~300 cm$^2$ at 10 lb/in$^2$ using a hand sprayer from a 15-cm distance
[b] Log counts (CFU/cm$^2$) represent the mean of six replicates
[c] Standard deviation
[d] Includes fluorescent protein-marked *E. coli* strains RFP-1, GFP-3, YFP-14 and YFP-66
[e] Includes *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895
[f] Includes *Salmonella* serotypes Agona, Anatum, Montevideo, Typhimurium and Senftenberg
[g] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)

volumes of 2% L-lactic acid at 25°C and 55°C are presented in Figures 19 and 20. These reduction data and the correspondent results of the statistical analysis are presented in Table 25. The application of 47 ± 1.0 ml of 2% L-lactic acid at 25°C (spraying during 2 s) produced initial reductions of 1.6 log CFU/cm$^2$ on the fluorescent protein-marked *E. coli* surrogates, *E. coli* O157:H7 and *Salmonella*. When the volume was increased to ≥126 ± 1.9 ml (spraying for ≥ 6 s) the reductions were significantly increased to 2.5-2.7 log CFU/cm$^2$ for the three groups of microorganisms ($P$<0.05). In comparison, the application of an initial volume of 47 ± 1.0 ml of 2% L-lactic acid at 55°C (spraying for 2 s) produced reductions of 2.2-2.5 log CFU/cm$^2$ among surrogates and pathogens and subsequent increase in the sprayed volume did not produced significantly larger reductions on the microbial populations ($P$>0.05).

In general, observed reductions for fluorescent protein-marked *E. coli* surrogates, *E. coli* O157:H7 and *Salmonella* ranged from 1.6 to 3.0 log CFU/cm$^2$ when applying 2% L-lactic acid at 25°C and from 1.8 to 2.9 log CFU/cm$^2$ when the acid solution was applied at 55°C. These finding are in agreement with previous studies. A large number of reports on the efficacy of lactic acid washes to reduce microbial populations on beef carcasses have been published in the past 20 years. Some of these studies are summarized in Table 26. Maximum reductions of 3.0, 2.7, 2.6, 2.6 and 2.6 log CFU/cm$^2$ have been reported on APC, coliforms, *E. coli*, *E. coli* O157:H7 and *S.* Typhimurium, respectively. The large variation observed in microbial reductions among reports may be due to differences in treatment parameters, microorganisms evaluated and sampling procedures.

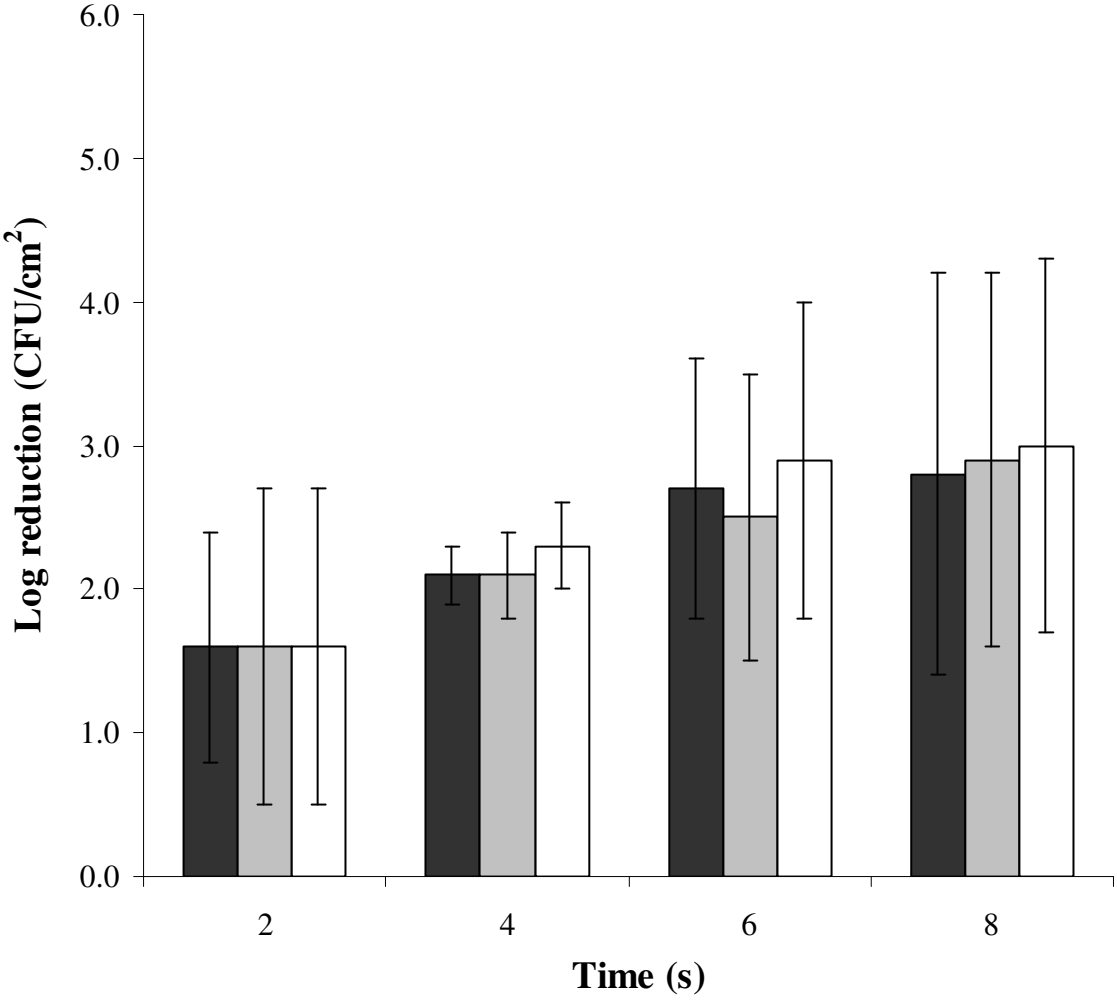


FIGURE 19. *Mean reductions (log CFU/ml) for fluorescent protein-marked* E. coli (■)*,* E. coli *O157:H7* (■) *and* Salmonella (□) *strains after application of 2% L-lactic acid at 25°C at different spraying times on beef carcass surfaces.*

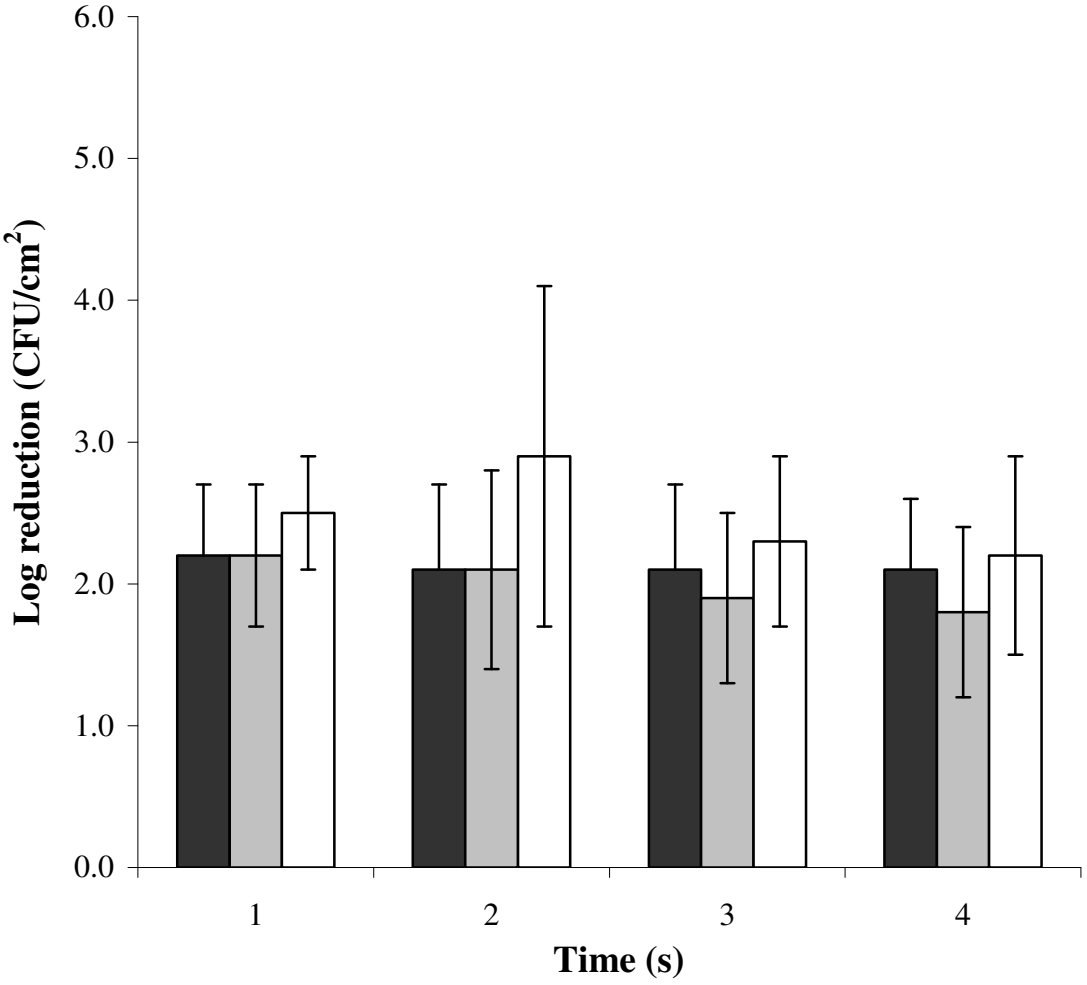


FIGURE 20. *Mean reductions (log CFU/ml) for fluorescent protein-marked* E. coli (■)*,* E. coli *O157:H7* (■) *and* Salmonella (□) *strains after application of 2% L-lactic acid at 55°C at different spraying times on beef carcass surfaces.*

TABLE 25. *Mean reductions (log CFU/cm$^2$) for fluorescent protein-marked* E. coli, E. coli *O157:H7 and* Salmonella *on beef carcasses by application of 2% L-lactic acid at two temperatures*

| Temperature (°C) | Spraying[a] time (s) | Volume (ml) | Carcass surface pH | Mean reduction[b] ± SD[c] | | |
|---|---|---|---|---|---|---|
| | | | | *E. coli* surrogates[d] | *E. coli* O157:H7[e] | *Salmonella*[f] |
| 22.8 ± 0.1 | 2 | 47 ± 1.0 | 2.6 ± 0.2 B[g] | 1.6 ± 0.8 A*x*[h] | 1.6 ± 1.1 A*x* | 1.6 ± 1.1 A*x* |
| | 4 | 86 ± 1.6 | 3.0 ± 0.4 B | 2.1 ± 0.2 AB*x* | 2.1 ± 0.3 ABC*x* | 2.3 ± 0.3 AB*x* |
| | 6 | 126 ± 1.9 | 2.9 ± 0.1 B | 2.7 ± 0.9 B*x* | 2.5 ± 1.0 BC*x* | 2.9 ± 1.1 B*x* |
| | 8 | 154 ± 4.9 | 3.0 ± 0.3 B | 2.8 ± 1.4 B*x* | 2.9 ± 1.3 C*x* | 3.0 ± 1.3 B*x* |
| 54.4 ± 0.8 | 2 | 47 ± 1.0 | 3.2 ± 0.2 B | 2.2 ± 0.5 AB*x* | 2.2 ± 0.5 ABC*x* | 2.5 ± 0.4 AB*x* |
| | 4 | 86 ± 1.6 | 3.0 ± 0.2 B | 2.1 ± 0.6 AB*x* | 2.1 ± 0.7 ABC*x* | 2.9 ± 1.2 B*x* |
| | 6 | 126 ± 1.9 | 3.1 ± 0.3 B | 2.1 ± 0.6 AB*x* | 1.9 ± 0.6 AB*x* | 2.3 ± 0.6 AB*x* |
| | 8 | 154 ± 4.9 | 3.1 ± 0.3 B | 2.1 ± 0.5 AB*x* | 1.8 ± 0.6 AB*x* | 2.2 ± 0.7 AB*x* |

[a] L-lactic acid was applied on carcass surfaces of ~300 cm$^2$ at 10 lb/in$^2$ using a hand sprayer from a 15-cm distance
[b] Log reductions (CFU/cm$^2$) represent the mean of six replicates
[c] Standard deviation
[d] Includes fluorescent protein-marked *E. coli* strains RFP-1, GFP-3, YFP-14 and YFP-66
[e] Includes *E. coli* O157:H7 strains #2, 26, 36, 38 and 43895
[f] Includes *Salmonella* serotypes Agona, Anatum, Montevideo, Typhimurium and Senftenberg
[g] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)
[h] Mean values in the same row with the same letter (*xyz*) are not different ($P$>0.05)

TABLE 26. Reduction of microbial populations by application of lactic acid solutions (LA) on the surface of beef carcasses

| Treatment | Microbial population | Log reduction and reporting units | | Reference |
|---|---|---|---|---|
| 1% LA/room temperature | APC | 1.8 | $CFU/cm^2$ | Snidjers et al. |
| | *Enterobacteriaceae* | ≥ 0.7 | | *(222)* |
| 1% LA/24°C | *E. coli* O157:H7 | 1.0 | $CFU/cm^2$ | Cutter and |
| 3% LA/24°C | *E. coli* O157:H7 | 1.8 | | Siragusa *(68)* |
| 5% LA/24°C | *E. coli* O157:H7 | 2.6 | | |
| 2% LA/55°C/200ml | *E. coli* O157:H7 | 1.2 | $CFU/cm^2$ | Hardin et al. |
| | *S.* Typhimurium | 2.6 | | *(125)* |
| 2% LA/55°C/11s | APC | 3.0 | $CFU/cm^2$ | Castillo et al. |
| | *Enterobacteriaceae* | 2.6 | | *(44)* |
| | Coliforms | 2.7 | | |
| | *E. coli* | 2.6 | | |
| | *E. coli* O157:H7 | 2.2 | | |
| | *S.* Typhimurium | >2.6 | | |
| 2% LA | APC | 0.9 | $CFU/cm^2$ | Dormedy et al. |
| | Coliforms | 0.8 | | *(85)* |
| | *E. coli* | 1.1 | | |
| 2% LA/55°C/30s post-chilling | *E. coli* | >1.8 | $CFU/cm^2$ | Castillo et al. |
| 2% LA/65°C/30s post-chilling | *E. coli* | >1.5 | | *(48)* |
| 4% LA/55°C/30s post-chilling | *E. coli* | >1.9 | | |
| 4% LA/65°C/30s post-chilling | *E. coli* | >1.7 | | |
| 4% LA/55°C/30s post-chilling | *E. coli* O157:H7 | 1.9 | | |
| 4% LA/55°C/30s post-chilling | *S.* Typhimurium | 2.4 | | |
| 4% LA/55°C/500ml/post-chilling | APC | 3.3 | $CFU/100cm^2$ | Castillo et al. |
| | Coliforms | >1.6 | | *(47)* |
| | *E. coli* | >0.2 | | |
| 2% LA/38-46°C/125ml | *E. coli* O157:H7 | 2.3 | $CFU/cm^2$ | Calicioglu et al. *(35)* |
| 2% LA/pre-evisceration | APC | <1.0 | $CFU/cm^2$ | Gill and Landers *(114)* |
| 4% LA/7°C/50ml post-chilling | APC | >2.0 | $CFU/cm^2$ | Gill and Badoni |
| | Coilforms | >1.5 | | *(113)* |
| 2% lactic acid/42°C | APC | 1.6 | $CFU/100cm^2$ | Bosivelac et al. |
| | *Enterobacteriaceae* | 1.0 | | *(22)* |

It was expected that larger reductions in microbial populations would be found as the temperature and the volume of lactic acid applied to the carcass surface were increased. However, no significant differences in mean log reductions for fluorescent protein-marked *E. coli* surrogates, *E. coli* O157:H7 and *Salmonella* were observed regardless of the volume applied of 2% L-lactic acid and the temperature of the solution ($P>0.05$). It was also observed that the carcass surface pH was not significantly different ($P>0.05$), regardless of the volume of 2% L-lactic acid applied and the temperature of the solution. Possibly this is the reason why the microbial counts were not significantly reduced as the volume and temperature of the solutions were increased. The limited efficacy of lactic acid sprays to reduce microbial populations reported in some studies maybe a consequence of a poor distribution of the acid solution on the carcass surface.

The main purpose of this study was to determine if the proposed surrogates responded in a similar way to the target pathogens. According to the obtained results, the reduction in counts produced by 2% L-lactic acid sprays on beef carcasses was not significantly different ($P>0.05$) among fluorescent protein-marked *E. coli* surrogates, *E. coli* O157:H7 and *Salmonella*, regardless of the temperature of the acid solution and the volume applied (Table 25).

**Validation of antimicrobial interventions in commercial beef slaughter establishments using fluorescent protein-marked *E. coli* strains as surrogates for enteric pathogens.** As previously described, the process of identifying surrogate organisms for validation studies requires the evaluation of their growth, attachment and

resistance properties to determine if their behavior is similar to that of selected target pathogens. The evaluation procedures must be performed first under laboratory controlled conditions. Once potential surrogates have demonstrated similar behavior to the target pathogens, it is then necessary to evaluate their response under commercial operating conditions. It is very difficult to extrapolate the results obtained in the laboratory to the actual situations that occur in a commercial processing environment. After it was determined that the fluorescent protein-marked *E. coli* strains had similar growth, attachment characteristics and resistance properties to selected *E. coli* O157:H7 and *Salmonella* strains under laboratory controlled conditions, they were used in commercial beef slaughter establishments to validate the efficacy of antimicrobial interventions. The potential surrogates were inoculated in the neck area of beef carcasses and enumerated before and after the application of water washes and lactic acid sprays at each establishment. Simultaneously, traditional indicator groups such as APC, coliforms and *E. coli* were enumerated for comparison with the proposed surrogates.

Five small beef slaughter establishments located in the state of Texas were included in this study. Each establishment utilized lactic acid sprays as an antimicrobial intervention within their HACCP plan. Each establishment included either a water wash at room temperature or a hot water wash before the application of the lactic acid intervention (Table 27). The water washes were primarily for improving quality and even when they were not considered as CCPs, their effect on the fluorescent protein-marked *E. coli* and traditional indicators populations was evaluated for comparative purposes and to collect more data about the performance of the potential surrogates.

Application parameters for water washes and lactic acid sprays were different at each establishment as shown in Table 27. The temperature of the water washes ranged from 25 to 64°C and the washing time per carcass side ranged from 0.9 to 5.9 min. The lactic acid solutions were applied at a concentration of 2.0-2.1% and at temperatures from 22 to 57°C. Different types of manual sprayers were used at each establishment. According to the flow rate of each sprayer and the spraying time, the volume of lactic acid delivered on the carcass surface was calculated and found to range between 90 ± 31 to 1,266 ± 394 ml per carcass side. The amount of lactic acid applied on the carcasses and the resulting surface pH were different among establishments. The surface pH achieved by the lactic acid sprayed on the beef carcasses was plotted with respect to the establishment (Figure 21) to graphically represent the variation observed. The carcass surface pH was also plotted against the volume of lactic acid sprayed on carcass sides at each establishment (Figure 22). A significant correlation ($P<0.05$) was found between the volume sprayed and the carcass surface pH. This correlation may be a consequence of the poor coverage of the acid on the surface of the carcass when small amounts of the solution were applied.

A large variation in the application parameters for the lactic acid sprays were observed at each establishment and the monitoring activities conducted by the plant personnel were limited to measuring the concentration of the lactic acid solution. Other parameters such as spraying time, volume delivered or carcass surface pH were not measured since they are not considered as critical limits; however, these parameters may provide useful information to monitor and detect variations in the intervention.

TABLE 27. *Parameters of water washes and lactic acid sprays applied on beef carcasses at five small slaughter establishments*

| Establishment | Water wash | | Lactic acid spray | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Mean ± SD[c] | | |
| | Temperature (°C) | Time (min/side) | Concentration (%) | Temperature (°C)[a] | Flow rate (ml/s)[b] | Time (s/side) | Volume (ml/side) | Surface pH[d] |
| A | 69 | 2.5 | 2.1 | 57 | 23.5 | 54 ± 17 | 1,266 ± 398 | 3.5 ± 0.2 |
| B | 25 | 2.0 | 2.0 | 24 | 18.0 | 53 ± 12 | 944 ± 209 | 3.6 ± 0.3 |
| C | 64 | 2.3 | 2.1 | 22 | 16.0 | 28 ± 8 | 453 ± 61 | 4.6 ± 0.8 |
| D | 25 | 0.9 | 2.0 | 25 | 10.0 | 12 ± 1 | 121 ± 8 | 5.2 ± 0.7 |
| E | 29 | 5.1 | 2.0 | 29 | 3.3 | 27 ± 9 | 90 ± 31 | 4.8 ± 1.4 |

[a] Temperature of the lactic acid solution out of the sprayer nozzle
[b] Volume of lactic acid delivered by the sprayer per second
[c] Standard deviation
[d] pH on the carcass surface immediately after application of the lactic acid spray

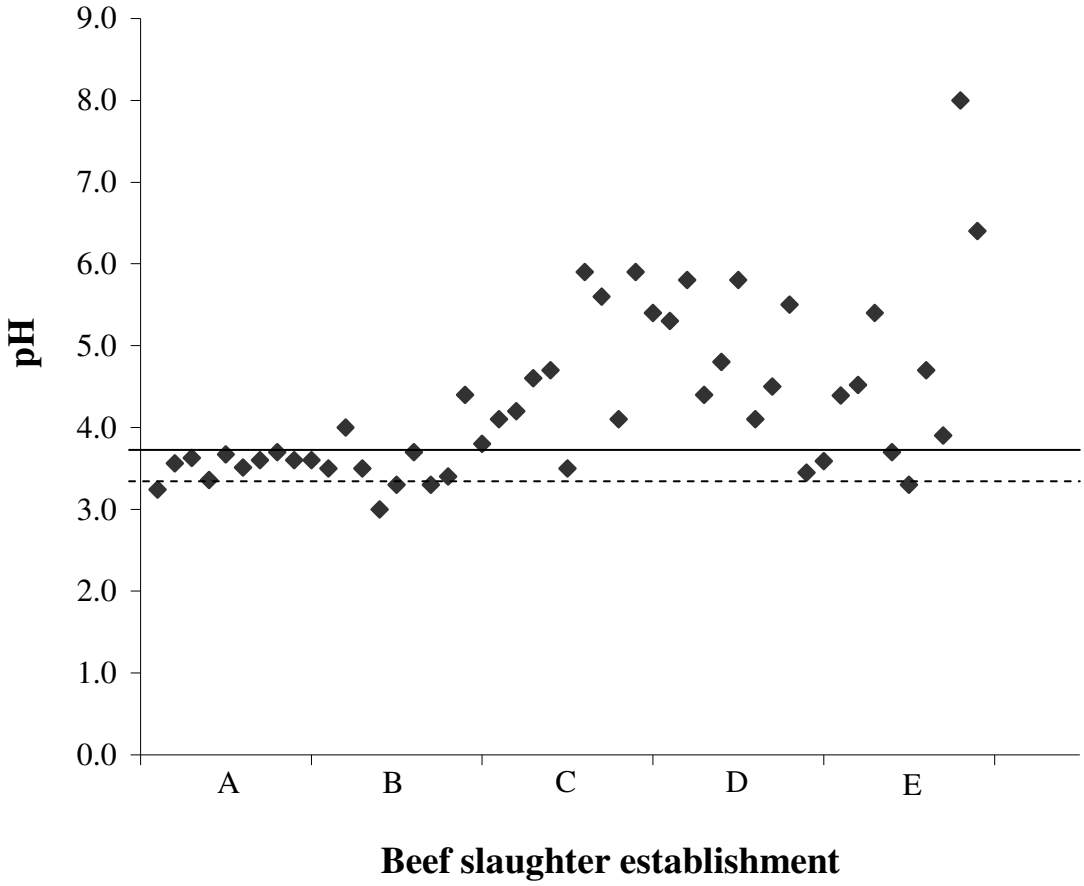


FIGURE 21. *Carcass surface pH after application of lactic acid sprays at five small beef slaughter establishments (A-E). The dashed line represents the mode and the solid line represents the mean of the data.*

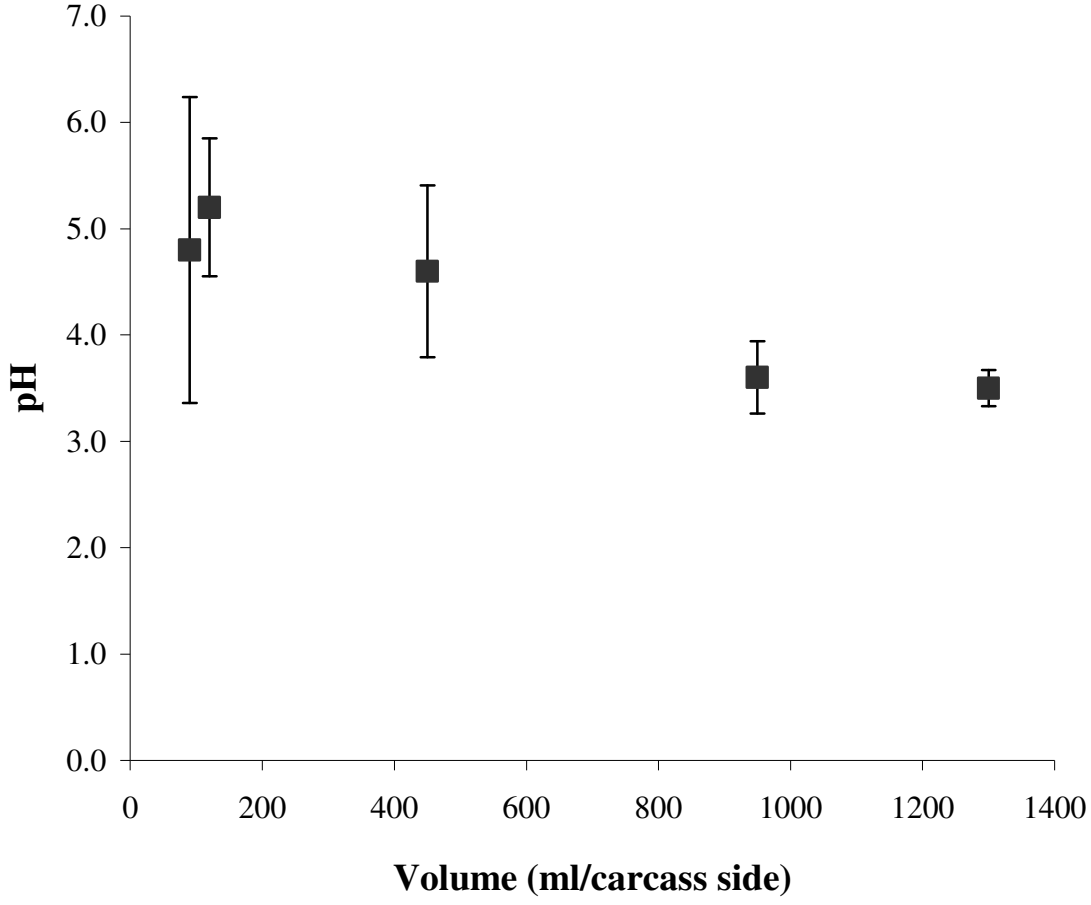


FIGURE 22. *Carcass surface pH and volume of lactic acid sprayed on beef carcasses at five small beef slaughter establishment.*

Mean reductions (log CFU/cm$^2$) observed for fluorescent protein-marked *E. coli* surrogates, APC, coliforms and *E. coli* after the application of water washes on inoculated beef carcasses in commercial processing establishments are shown in Table 28. Reductions ranged from 0.1 to 1.2 log CFU/cm$^2$ for all the microbial populations. These results are in agreement with other reports. Anderson et al. *(3)* reported an average reduction of 1.0 log CFU/200 cm$^2$ on APC by application of a hand-water wash at 14.4°C on beef carcasses. Cutter and Siragusa *(68)* used an automated carcass washer and found that *E. coli* O157:H7 was reduced 1 to 1.9 log CFU/cm$^2$ from the surface of beef by washing with water at 24°C. Prasai et al. *(195)* reported reductions of 0.3 log CFU/cm$^2$ on APC by water washing of beef carcasses. Gorman et al. *(119)* reported reductions on APC of 1.7 CFU/cm$^2$ after application of water washes at low pressure (40 psi). No significant differences in log reductions were observed among fluorescent protein-marked *E. coli* surrogates and traditional indicators, regardless of the temperature of the water wash and the washing time ($P$>0.05). However, the variation observed in log reductions for the indicators was larger than the variation observed for the surrogates, as denoted by the standard deviation of the mean reductions. As noted previously, the water washes were not considered an intervention and were applied to remove contamination such as blood, tissue fragments, hair, or bone particles. However, it was important to determine if the fluorescent protein-marked *E. coli* surrogates were affected in the same way as the traditional indicators. Mean log counts remaining on the inoculated surfaces after the water washes were 5.7 ± 0.9, 4.4 ± 1.3, 3.6 ± 1.3 and 3.4 ± 1.1 log CFU/cm$^2$ for the surrogates, APC, coliforms and *E. coli*, respectively.

TABLE 28. *Mean reductions (log CFU/cm2) of fluorescent protein-marked* E. coli *surrogates, aerobic plate count (APC), coliforms and* E. coli *after application of water washes on beef carcasses in five small slaughter establishments*

| Establishment | Temperature (°C) | Time (min/side) | Mean[a] ± SD[b] | | | |
|---|---|---|---|---|---|---|
| | | | *E. coli* surrogates[c] | APC | Coliforms | *E. coli* |
| A | 69 | 2.5 | 0.4 ± 0.7 A[d]*x*[e] | 0.7 ± 0.9 A*x* | 0.8 ± 1.0 A*x* | 0.6 ± 0.8 A*x* |
| B | 25 | 2.0 | 0.9 ± 0.4 A*x* | 0.3 ± 0.7 A*x* | 0.3 ± 0.7 A*x* | 0.3 ± 0.7 A*x* |
| C | 64 | 2.3 | 0.2 ± 0.8 A*x* | 1.2 ± 2.1 A*x* | 0.9 ± 2.2 A*x* | 0.9 ± 1.8 A*x* |
| D | 25 | 0.9 | 0.5 ± 0.6 A*x* | 0.1 ± 0.5 A*x* | 0.1 ± 0.6 A*x* | 0.1 ± 0.7 A*x* |
| E | 29 | 5.1 | 1.2 ± 1.1 A*x* | 0.8 ± 1.1 A*x* | 0.8 ± 1.2 A*x* | 0.8 ± 1.1 A*x* |

[a] Mean log reduction = log count before water wash – log count after water wash. Log reductions represent the mean of six duplicates

[b] Standard deviation

[c] Include fluorescent protein-marked *E. coli* strains RFP-1, GFP3, YFP-14 and YFP-66

[d] Mean values in the same column with the same letter (ABC) are not different ($P>0.05$)

[e] Mean values in the same row with the same letter (*xyz*) are not different ($P>0.05$)

The mean reductions (log CFU/cm$^2$) calculated for fluorescent protein-marked *E. coli* surrogates, APC, coliforms and *E. coli* after the application of lactic acid sprays on the inoculated beef carcasses are shown in Table 29. Mean reductions ranged from 0.1 to 1.6 log CFU/cm$^2$ for the fluorescent protein marked *E. coli* surrogates, from -0.2 to 1.5 log CFU/cm$^2$ for APC, from -0.3 to 0.8 log CFU/cm$^2$ for coliforms and from –0.3 to 1.0 log CFU/cm$^2$ for *E. coli*. Significantly larger reductions were observed for the surrogates in establishment "A" when compared to establishment "B" and "D" and larger reductions for APC were also observed in establishment "A" when compared to establishment "C" and "D". These larger reductions may be related to the larger volume of lactic acid applied to the carcasses in establishment "A" (Figure 23). However, that was not the case for coliforms and *E. coli.* It is possible that when the amount of lactic acid is increased, the probability of having a good coverage on the carcass surface and the opportunity for the acid to contact the microorganisms is also increased. The mean log reductions observed for microbial populations after application of 2% lactic acid sprays were in some cases similar to those reductions reported in the literature, but in most cases were observed to be lower. Dormedy et al. *(85)* reported reductions from 0.8 to 1.1 log CFU/cm$^2$ on APC, coliforms and *E. coli* after application of 2% lactic acid. Gill and Landers *(114)* observed reductions of <1.0 log CFU/cm$^2$ on APC when 2% lactic acid was applied as a pre-eviscertion intervention. Bosilevac et al. *(22)* reported reductions of 1.0 and 1.6 log CFU/100 cm$^2$ for *Enterobacteriaceae* and APC, respectively, after application of 2% lactic acid at 42°C. Castillo et al. *(47)* reported reductions on APC of 3.0 to 3.3 log CFU/100 cm$^2$ on chilled beef carcasses while coliforms and *E. coli* were

TABLE 29. *Mean reductions (log CFU/cm$^2$) of fluorescent protein-marked* E. coli *surrogates, aerobic plate count (APC), coliforms and* E. coli *on beef carcasses after application of lactic acid sprays at five small slaughter establishments*

| | | | Mean[a] ± SD[b] | | | |
|---|---|---|---|---|---|---|
| Establishment | Volume (ml/side) | Carcass surface pH | *E. coli* surrogates[c] | APC | Coliforms | *E. coli* |
| A | 1266 ± 398 | 3.5 ± 0.2 | 1.6 ± 1.0 A[d]*x*[e] | 1.5 ± 1.0 A*x* | 0.8 ± 0.8 A*x* | 1.0 ± 0.6 A*x* |
| B | 944 ± 209 | 3.6 ± 0.3 | 0.2 ± 0.9 B*x* | 1.2 ± 1.6 AB*x* | 0.3 ± 1.3 A*x* | 0.3 ± 1.2 A*x* |
| C | 453 ± 61 | 4.6 ± 0.8 | 0.6 ± 1.0 AB*x* | 0.0 ± 0.8 BC*x* | 1.0 ± 0.6 A*x* | 0.6 ± 0.6 A*x* |
| D | 121 ± 8 | 5.2 ± 0.7 | 0.1 ± 0.5 B*x* | -0.2 ± 0.7 C*x* | -0.3 ± 0.7 A*x* | -0.3 ± 0.7 A*x* |
| E | 90 ± 31 | 4.8 ± 1.4 | 0.5 ± 1.6 AB*x* | 0.4 ± 1.4 AC*x* | 0.4 ± 1.4 A*x* | 0.5 ± 1.3 A*x* |

[a] Mean log reduction = (log count before lactic acid – log count after lactic acid). Log reductions represent the mean of six duplicates

[b] Standard deviation

[c] Include fluorescent protein-marked *E. coli* strains RFP-1, GFP3, YFP-14 and YFP-66

[d] Mean values in the same column with the same letter (ABC) are not different ($P$>0.05)

[e] Mean values in the same row with the same letter (*xyz*) are not different ($P$>0.05)

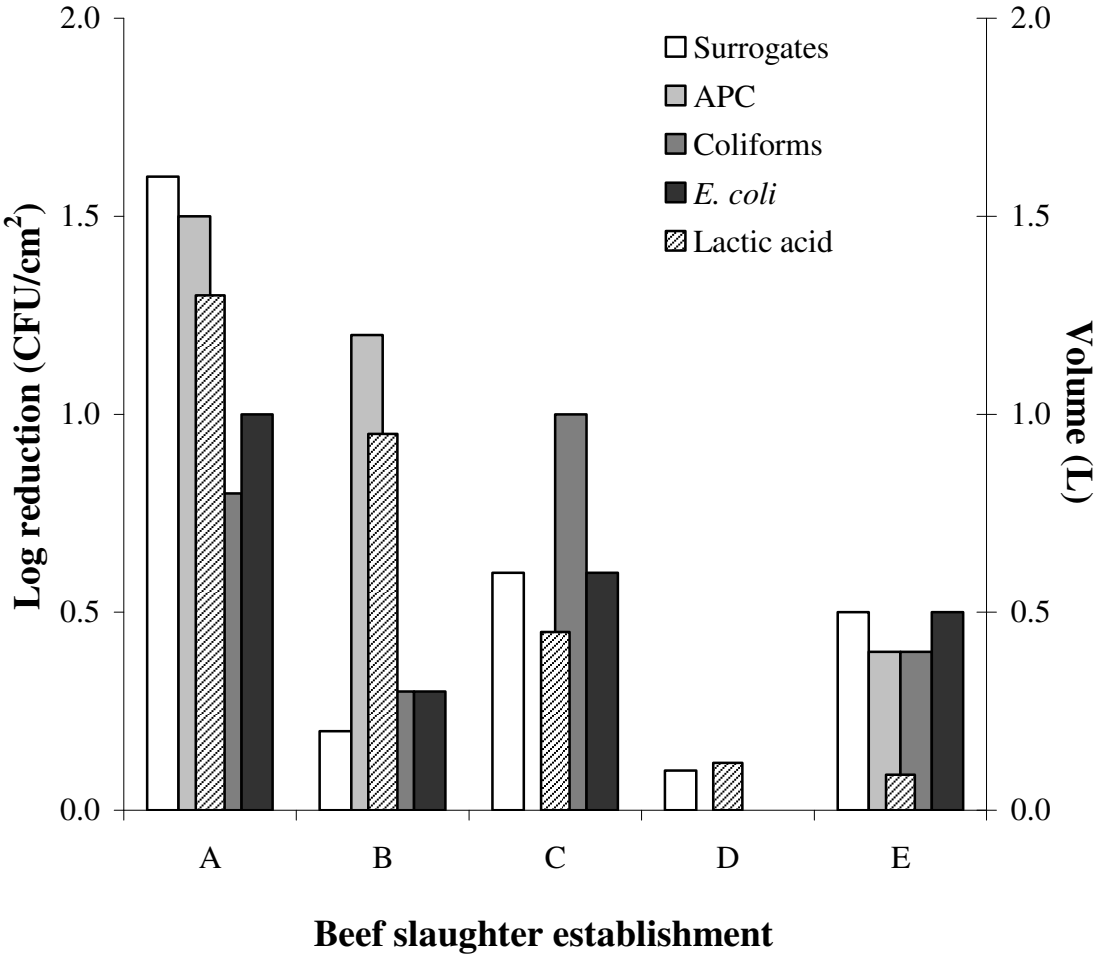


FIGURE 23. *Mean reduction of fluorescent protein-marked* E. coli *surrogates, aerobic plate count (APC), coliforms and* E. coli *on beef carcasses after application of 2% L-lactic acid sprays in small slaughter establishments.*

consistently reduced to undetectable levels after spraying 4% lactic acid as a post-chilling intervention. The lower reductions observed in this study may be due to differences in the methods used to spray the lactic acid. In small slaughter plants the acid solutions are applied with manual hand-held sprayers, while the studies reported by other authors were conducted in large slaughter plants where automated spraying systems are used. Another factor may be the uneven distribution of the contamination on the carcass surface. Other studies evaluate the natural contamination present on the carcass which is not even, while in this study a controlled inoculum was spread on the carcass surface providing larger microbial populations evenly distributed.

Even when no differences were observed in log reductions among surrogates, APC, coliforms and *E. coli* at each establishment ($P>0.05$), the variation found in the reductions of the traditional indicators was larger (Figure 24). The use of surrogates for the validation of lactic acid interventions in commercial slaughter operations offers additional advantages: they represent *E. coli* O157:H7 and *Salmonella* reductions more accurately because they have shown similar resistance properties against lactic acid, and they can be inoculated at high levels, providing a larger and more consistent initial population (Figure 25) which is not dependent on the fecal slurry applied. For all this reasons, track of surrogate reductions can be easier and the results of the validation studies can be more consistent.

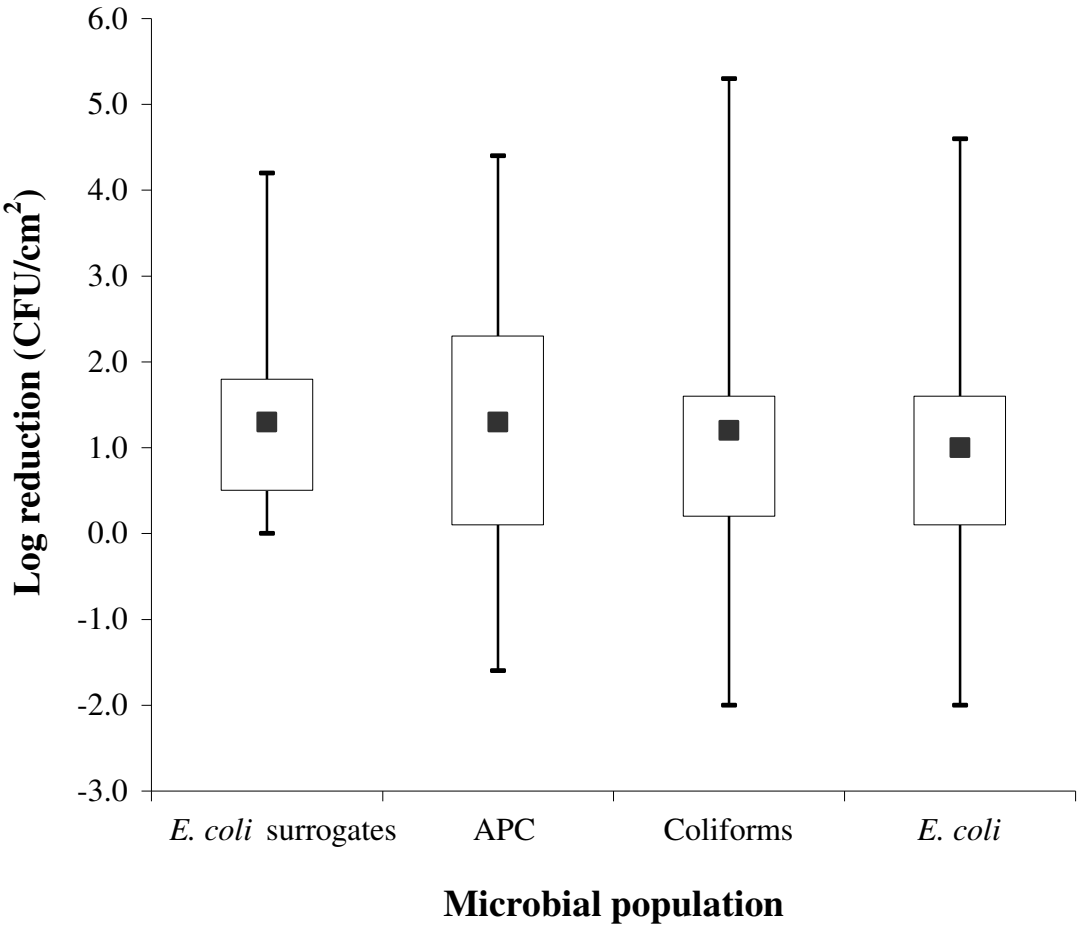


FIGURE 24. *Reduction of microbial populations on beef carcasses after the application of water washes and lactic acid sprays at five small beef slaughter establishments. Each data point represents the mean log reduction obtained from 30 carcass sides.*

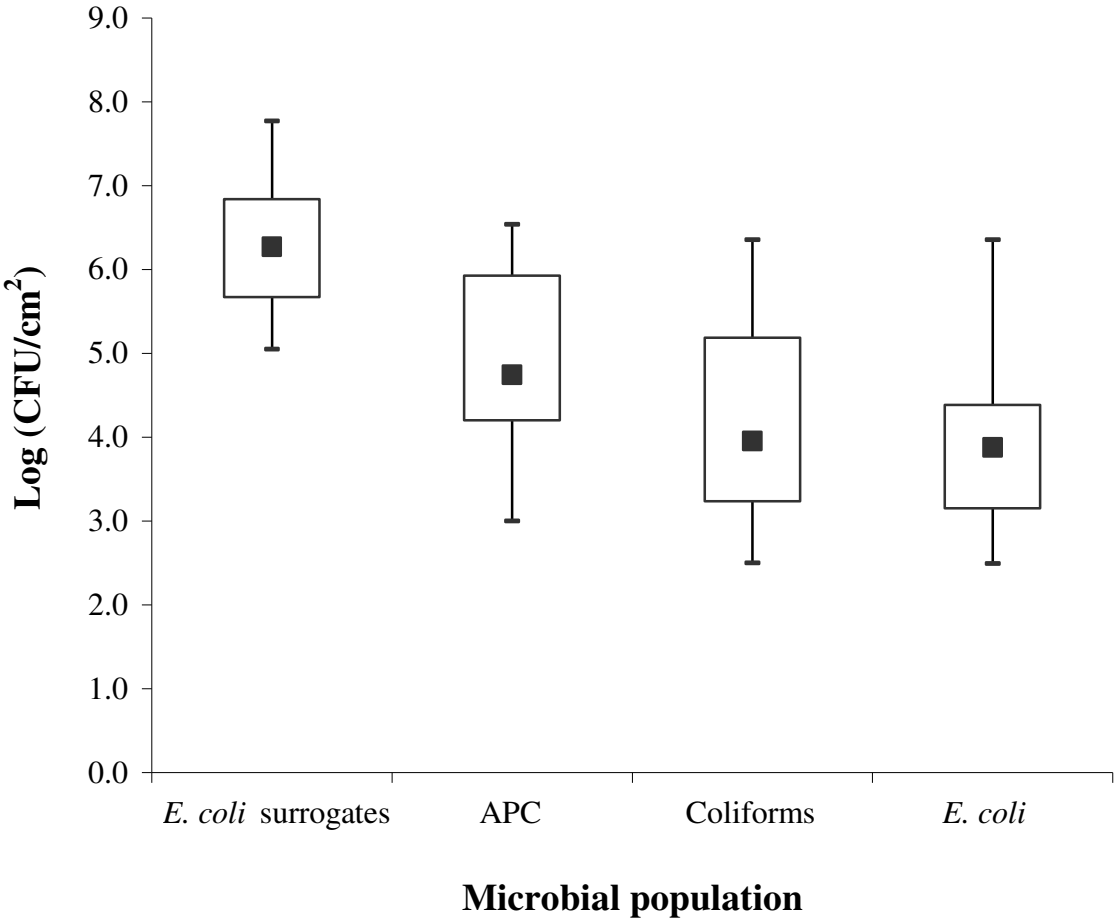


FIGURE 25. *Initial population of fluorescent protein-marked* E. coli *surrogates, aerobic plate count (APC), coliforms and* E. coli *on inoculated beef carcasses. Each data point represents the mean log count obtained from 30 carcass sides.*

# CONCLUSIONS

The identification of appropriate surrogates for the validation of antimicrobial interventions on beef carcasses was addressed through a stepwise process. Potential surrogates were first isolated from similar environments to those of target pathogens in previous collaborative research. The current study included the following steps: i) the transformation of the potential surrogates to obtain marker organisms; ii) the comparison of growth, attachment, and resistance properties between the potential surrogates and target pathogens; iii) the selection of particular antimicrobial interventions to be validated followed by the selection of inoculation and microbial enumeration methods, and iv) the comparison in the response of surrogates and target pathogens to the selected interventions under laboratory controlled conditions as well as during commercial processing operations.

This study demonstrated that the evaluated fluorescent protein-marked *E. coli* strains are appropriate surrogates for *E. coli* O157:H7 and *Salmonella* for use in the validation of hot water washes and lactic acid sprays on beef carcasses. The evaluated surrogates were determined to be nonpathogenic after testing for virulence attributes. Therefore, they can be utilized in commercial processing environments where pathogens cannot be introduced due to safety concerns. The transformation of the surrogates using high-copy plasmids for expression of fluorescent proteins and ampicillin resistance did not alter growth characteristics or biochemical profiles. The expression of different fluorescent proteins provides an effective marker for the individual enumeration of surrogates to evaluate strain to strain variation while the ampicillin resistance conferred

by the plasmids makes the isolation procedure easier by inhibition of the natural background microbiota present on beef carcasses.

In general, growth parameters such as final bacterial cell density, maximum specific growth rate, t-lag and doubling time were not different among the fluorescent protein-marked *E. coli, E. coli* O157:H7 and *Salmonella* strains. Heat resistance of fluorescent protein-marked *E. coli* surrogates was not different or was higher than that of *E. coli* O157:H7 and *Salmonella* as measured by D-values at 55, 60 and 65°C in phosphate buffered saline. Acid resistance at pH 2.5, 3.0 and 3.5 in phosphate buffered saline acidified with lactic acid was not different among the fluorescent protein-marked *E. coli* surrogates and *E. coli* O157:H7, but the surrogates were more acid resistant than some *Salmonella* serotypes. The survival ability of the fluorescent protein-marked *E. coli* surrogates at 4°C and -18°C was not different or was higher than the survival ability demonstrated by *E. coli* O157:H7 and *Salmonella*. Attachment characteristics among the surrogates and the target pathogens were not different as demonstrated by their cell surface hydrophobicity and by the enumeration of loosely and strongly attached cells on the surface of different carcass regions.

The response of fluorescent protein-marked *E. coli* surrogates was not different than the response of *E. coli* O157:H7 and *Salmonella* when exposed to hot water washes and 2% L-lactic acid sprays on the surface of beef carcass tissues under laboratory controlled conditions. D-values observed after application of hot water washes that elevated the temperature of the carcass surface to 70.9, 78.5 and 86.4°C were not different among surrogates and pathogens. Log D-values were used to calculate the

temperature and time required to achieve 5-log cycle reductions by application of hot water washes on beef carcasses. Reductions obtained after application of 2% L-lactic acid sprays at 25 and 55°C were not different among surrogates and target pathogens, regardless of the temperature of the acid solution and the volume applied on the surface of beef carcass tissues. When used for the validation of antimicrobial interventions in commercial slaughter facilities, the response of the fluorescent protein-marked *E. coli* surrogates was not different to the response observed in traditional indicator groups such as APC, coliforms and *E. coli* after application of water washes and lactic acid sprays on beef carcasses.

The fluorescent protein-marked *E. coli* surrogates evaluated in this study offer several advantages for validation of interventions that rely on heat and lactic acid for the reduction of pathogens on beef carcasses. The surrogates may represent *E. coli* O157:H7 and *Salmonella* reductions more accurately than traditional indicators since they have demonstrated similar resistance properties under laboratory controlled studies. Surrogates can be inoculated at high initial levels facilitating the tracing of reductions after sequential interventions, and they demonstrate less variation and more consistent results than traditional indicators under in-plant operation conditions. A cocktail of three different surrogate strains can be used and since each strain can be individually enumerated, strain to strain variation can be evaluated.

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