INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

DISSERTATION

EFFECTS OF HEAT AND ACID STRESS ON BACTERIAL POPULATIONS OF BEEF, AND ON SUSCEPTIBLE AND MULTI-ANTIMICROBIAL RESISTANT *SALMONELLA* ISOLATED FROM BEEF

Submitted by

Richard Todd Bacon

Department of Animal Sciences

In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2002

COLORADO STATE UNIVERSITY

June 28, 2002

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RICHARD TODD BACON ENTITLED "EFFECTS OF HEAT AND ACID STRESS ON BACTERIAL POPULATIONS OF BEEF, AND ON SUSCEPTIBLE AND MULTI-ANTIMICROBIAL RESISTANT *SALMONELLA* ISOLATED FROM BEEF" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Keith Belk

Gary C. Smith

Norene K. Scott

Adviser

Department Head

ABSTRACT OF DISSERTATION

EFFECTS OF HEAT AND ACID STRESS ON BACTERIAL POPULATIONS OF BEEF, AND ON SUSCEPTIBLE AND MULTI-ANTIMICROBIAL RESISTANT *SALMONELLA* ISOLATED FROM BEEF

Studies were conducted to serve as initial investigations evaluating antimicrobial efficacies of decontamination treatments applied to beef carcass tissue following chilling, prevalence of susceptible and antimicrobial resistant *Salmonella* on beef animal hides and carcasses, and whether antimicrobial resistance provided cross-protection against low-pH or heat stress. Results of studies evaluating methods to improve microbiological quality of chilled beef carcasses, subprimal cuts, and fabrication table surfaces, suggested reduced antimicrobial efficacy ($\leq$ 1 log CFU/100 cm^2 reduction) of steam-vacuuming and lactic acid solution rinsing when applied during beef carcass fabrication. Study results also supported concerns regarding increased antimicrobial resistance among foodborne bacterial populations, as data indicated that 10.7% of beef animal hide samples contained *Salmonella* resistant to at least one of thirteen antimicrobials screened. *Salmonella* isolated from beef animal hides and carcasses were used to evaluate acid and heat tolerance among susceptible and multi-antimicrobial resistant strains. Under the conditions of these studies, results comparing the stress resistance between susceptible and multi-antimicrobial resistant *Salmonella*, following preparation under acid tolerance-inducing and noninducing conditions, suggested that previous induction of a stationary-phase acid tolerance response afforded increased acid and heat resistance. However, the

ability of *Salmonella* to avoid or repair damage associated with low-pH or thermal stress appeared to be independent of antimicrobial susceptibility.

Richard Todd Bacon
Department of Animal Sciences
Colorado State University
Fort Collins, CO 80523
Summer 2002

ACKNOWLEDGMENTS

I start by giving thanks to my committee: Dr. John N. Sofos, for serving as my committee chair; Drs. Gary C. Smith and Keith E. Belk, for playing instrumental roles in my development during my tenure as a graduate student; and, Dr. Doreene R. Hyatt, for serving not only as my outside committee member, but as an invaluable resource. Additional gratitude is owed to Dr. Phillip Chapman, for the willingness to commit his time, and Warren Mirtsching and Dr. Jerome Lawler for making the commercial, in-plant research possible. The intricacies of conducting research more often than not required the commitment of more than one individual. For your time, knowledge and/or technical assistance, I thank Dr. Henry Zerby, Dr. Rob Cannell, Dr. John Scanga, Dr. John Samelis, Lorenzo Ware, Elizabeth Duffy, Justin Ransom, Bill Platter, Derek Vote, Deb Roeber, Aaron Wyle, Jarret Stopforth, Emily McClure, Jennifer Stefanek, Lora Wright, Chris Bullin and Virginia Littlefield. A special thanks to Dr. Henry Zerby, Dr. Rob Cannell, Dr. John Scanga, Elizabeth Duffy, Derek Vote, Bill Platter, Lorenzo Ware and Justin Ransom for the memories and your friendship, and to all of my graduate student colleagues, best of luck in your future endeavors. I also offer a sincere "thank you" to the departmental staff who have provided their assistance during my tenure at Colorado State University: Margo Allmaras, Shelly Castillion, Barb Holst, Cheryl Miller, Krissy Myatt and Jackie Whittemore.

I have been extremely blessed with wonderful friends and family. To my best friend Chad A. Roberts, "we went back"; thank you for being much more than a brother to me. To Melanie and Aimee, thank you both for being such wonderful sisters. I am very proud of both of you and wish for you all of the wonderful things that this life has to offer. To Mom and Dick, thank you for all of the unconditional love and support you have provided Rendi and I over the years. Last, but certainly not least, to the Doll, Cowart, Whately, Murphree and Davis families, thank you for your love and support.

DEDICATION

I dedicate this dissertation to Rendi M. Bacon, my wife, companion, and friend,

with a debt of gratitude for the inspiration, love and support

that has made my accomplishments possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

OBJECTIVES OF DISSERTATION

The objectives of the studies reported in this dissertation were:

Overall Objective:

To increase current knowledge of bacterial behavior, and the factors that influence it, in response to stress associated with the application of decontamination treatments during the animal-to-muscle food conversion process.

Specific Objectives:

(1) To determine whether steam-vacuuming was an effective means of beef carcass tissue decontamination when applied following chilling, thereby allowing an extended period of time for attachment, penetration, and/or biofilm formation of inoculated *Salmonella*.

(2) To determine the efficacy of lactic acid solution rinsing in reducing or controlling bacterial populations on beef carcasses and subprimal cuts, and on table surfaces, during commercial fabrication.

(3) To determine antimicrobial susceptibility of *Salmonella* isolated from beef animal hides and carcass surfaces to a panel of thirteen antimicrobials.

(4) To determine whether an association exists between antibiotic susceptibility and the ability to avoid or repair damage associated with low-pH exposure by evaluating induced and noninduced stationary-phase acid tolerance response between susceptible and multi-antimicrobial resistant strains of *Salmonella*.

(5) To determine if heat resistance differences exist between cultures of susceptible and multi-antimicrobial resistant *Salmonella* strains prepared under acid-tolerance inducing or noninducing conditions.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

In the United States, presence of biological hazards (i.e., bacterial, parasitic and viral) in food results in millions of illnesses and thousands of deaths annually, with an economic impact estimated at approximately 8.4 billion dollars per year (Mead *et al.*, 1999; Todd, 1989). Clearly, the majority of foodborne disease remains unreported and undiagnosed, as unknown agents cause an estimated 62 million illnesses and 3,200 deaths annually in the United States; in contrast, an estimated 14 million illnesses and 1,800 deaths involve identified etiology (Mead *et al.*, 1999). Disease can be classified based on the role that the pathogen plays during the disease-causing process. An infection ensues when a host is invaded directly by a microorganism, which after being established, proliferates in the host. Conversely, intoxication ensues following the introduction of a specific, pre-formed toxin into the body of a host, subsequently inducing disease in the presence or absence of the toxin-producer. A food source may serve as a vehicle of transmission for the biological hazard itself (infection) and/or any metabolic products, including various toxins (intoxication). Typically, the clinical symptoms involved in the majority of foodborne diseases include acute diarrhea, vomiting and/or some other manifestation of the gastrointestinal tract. However, syndromes involving the central

nervous system or various organs, in addition to other chronic sequelae may be the direct or indirect result of foodborne pathogenic microorganisms (Lindsay, 1997).

BEEF CARCASS DECONTAMINATION

Meat is a food product derived from animal tissues, and in general, the internal tissues of healthy animals are sterile at the time of slaughter (Jay, 2000; Judge *et al.*, 1989). This said, contamination of otherwise sterile carcass surfaces, and subsequently fabricated subprimal cuts, is an inherent part of the animal-to-muscle food conversion process; although, minimizing the inevitable transfer or introduction, and proliferation, of microbial contamination on these surfaces is imperative. Jay (2000) suggested that the primary sources and routes of microbial contamination of fresh meats include: (1) the stick knife, as an unsterile knife may introduce bacteria that are then circulated throughout the carcass via the bloodstream; (2) the hide, bacterial populations associated with the hide may be transferred to the underlying carcass surface during the dehiding process either through physical contact or through the production of aerosols; (3) the gastrointestinal tract, punctures or "busted guts" occurring during the evisceration process may result in the transfer of microorganisms from the gastrointestinal tract to the carcass; (4) employees, repeated contact between employees and carcasses or subprimal cuts may result in cross-contamination and/or the introduction of biological hazards (e.g., *Staphylococcus*) not typically associated with muscle foods; (5) the processing environment, in addition to aerosols, physical contact between carcasses or subprimal cuts and slaughter/fabrication equipment (e.g., saws, knifes, tables, etc.) may contribute

to cross-contamination; and, (6) lymph nodes, may serve as a source of contamination if they are cut open or ground during non-intact meat production.

In 1992-1993, an outbreak of *Escherichia coli* O157:H7 transmitted in undercooked ground beef patties served by a fast-food restaurant chain resulted in hundreds of cases of foodborne illness, and set into motion events that changed the way the industry produced beef (Bell *et al.*, 1994; Sofos and Smith, 1993). The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) issued a policy of "zero tolerance" for visible carcass contamination, which was followed by additional mandates requiring implementation of Hazard Analysis and Critical Control Point (HACCP) systems, and compliance with microbiological performance criteria and standards, respectively, for *E. coli* and *Salmonella* (FSIS-USDA, 1996a, 1996b). In order to produce product of improved microbiological quality, and to meet performance criteria, the red meat and poultry industries improved existing, and developed new, antimicrobial technologies. Beef carcass decontamination technologies and good manufacturing practices involving the use of chemicals (e.g., dehairing), knife trimming, steam, ambient temperature to hot water, and sanitizers such as organic acid solution rinses have been extensively researched (Dickson and Anderson, 1992; Dorsa, 1997; Dorsa *et al.*, 1996b; Gorman *et al.*, 1995; Hardin *et al.*, 1995; Kochevar *et al.*, 1997; Nutsch *et al.*, 1997; Phebus *et al.*, 1997; Smulders and Greer, 1998; Sofos and Smith, 1998).

Chemical Dehairing

Chemical dehairing was developed as a means for removing the hair and associated contaminants (e.g., dirt, feces and biological hazards) before opening the hide of beef cattle in order to reduce the amount of contamination introduced to the underlying, otherwise sterile, carcass surface (Castillo *et al.*, 1998; Graves Delmore *et al.*, 1997; Schnell *et al.*, 1995). Laboratory studies reported 3.0 to 6.0 log reductions in pathogenic and non-pathogenic bacterial populations following the application of the chemical dehairing process to inoculated hide samples (Castillo *et al.*, 1998; Graves Delmore *et al.*, 1997).

Steam-Vacuuming

In April 1996, steam-vacuuming was approved, as an alternative to knife trimming, to be used as a spot decontaminant (FSIS-USDA, 1996a). The idea behind steam-vacuuming was that microbiological populations associated with the visible contamination would be inactivated by the heat, or at least removed by the subsequent vacuuming of condensate, thereby increasing microbiological quality. Studies evaluating the antimicrobial efficacy of steam-vacuuming have reported reductions in microbial populations ranging from 1.0 to 3.0 logs, and have concluded it is at least as effective as knife trimming (Dorsa *et al.* 1996b; Kochevar *et al.*, 1997). However, some researchers have acknowledged steam-vacuuming as being effective in removing visible contamination, but reported limited reductions in bacterial numbers as a result of its application (Gill and Bryant, 1997). The overall effectivness of steam-vacuuming in reducing microbial populations is dependent upon the method of application, as the

extent of microbial inactivation is directly related to the duration of contact between heat and bacterial cells.

Steam Pasteurization

The process of steam pasteurization involves three phases: (1) removing surface water, in order to eliminate a potential barrier to the steam; (2) pasteurizing, saturating the carcass surfaces with steam in an enclosed chamber; and, (3) surface cooling, to minimize tissue discoloration associated with the heat exposure (Nutsch *et al.*, 1997; Sofos and Smith, 1998). During laboratory studies evaluating the efficacy of the steam pasteurization process in reducing microbiological populations on beef carcass tissue, researchers reported reductions on the order of 3.0 to 4.0 logs (Phebus *et al.*, 1996, 1997); however, researchers evaluating the effectiveness of this technology in reducing microbial populations on commercially slaughtered beef carcasses have reported reductions on the order of 1.0 to 2.0 logs, depending on the anatomical region of the carcass (Nutsch *et al.*, 1997, 1998; Phebus *et al.*, 1996). This difference may be due, at least in part, to higher pre-treatment microbiological populations in inoculated beef tissue (>5.0 log CFU/cm^2) studies, compared to commensal microbial counts (<2.5 log CFU/cm^2) on carcass surfaces (Nutsch *et al.*, 1997, 1998; Phebus *et al.*, 1996, 1997).

Spray-Water Washing

Antimicrobial efficacy of carcass spray-washing has been reviewed extensively, and in general, researchers conducting laboratory and commercial studies have reported reductions in microbial populations on the order of 1.0 to >3.0 logs (Dickson and

Anderson, 1992; Dorsa, 1997; Hardin *et al.*, 1995; Sofos *et al.*, 1999a, b, c; Sofos and Smith, 1998). In addition, the effects of spray-washing water temperature and pressure have been thoroughly explored. Although discoloration has been reported, and although concerns exist regarding increased bacterial penetration into fat/lean tissues of carcasses associated with the high application pressures, efficacy of spray-washing as a means for reducing microbiological contamination is enhanced by increased water temperature (>74°C) and pressure (Cabedo *et al.*, 1996; Gorman *et al.*, 1995; Kochevar *et al.*, 1997; Kotula *et al.*, 1974; Sofos and Smith, 1998).

Organic Acid Solution Rinsing

In the last ten years, numerous carcass sanitizers have been evaluated for their ability to decontaminate carcass surfaces and improve microbiological quality. Traditionally, the industry has used organic acids (e.g., acetic and lactic), for beef carcass decontamination, at concentrations ranging from 1.5 to 2.5% (Dickson *et al.*, 1997). The overall antimicrobial efficacy (1.0 to 3.0 log reductions in bacterial populations) of these acids is greatly influenced by acid temperature, application pressure, tissue type, microbial populations and sensitivity, acid concentration, duration of application, and the presence of organic materials (Dickson, 1992; Hardin *et al.*, 1995). Similar to spray-water washing, as the temperature increases (i.e., from 25 to 70°C), so does its sanitizing effect (Anderson and Marshall, 1989, 1990; Cutter and Siragusa, 1994). Acid concentration has been reported insignificant at higher application temperatures (i.e., 70°C), mainly due to the overriding antimicrobial effect of the heated solution itself and

evaporation/diffusion; however, acid concentration is significant as solution temperature decreases (Anderson and Marshall, 1989, 1990).

Sequential-Intervention Decontamination Systems

Technologies available for beef carcass decontamination have also been evaluated when applied in combination, rather than individually. Researchers have reported increased reductions in microbial populations and improved microbiological quality following sequential decontamination treatment applications, when compared to the antimicrobial efficacy of the treatments applied singly (Dorsa *et al.*, 1996b; Graves Delmore *et al.*, 1998; Hardin *et al.*, 1995; Phebus *et al.*, 1997). Commercial use of a multiple, sequential decontamination system has provided a more effective means for reducing microbial contamination on beef carcass surfaces (Bacon *et al.*, 2000; Dorsa *et al.*, 1996b; Graves Delmore *et al.*, 1998; Phebus *et al.*, 1997).

SALMONELLA

It is estimated that bacterial agents are responsible for approximately 30% of all foodborne illness, resulting in more than four million cases annually in the United States. Despite causing only 30% of all foodborne illness, bacterial agents in foods result in approximately 1,300 deaths annually, accounting for 72% of total deaths attributed to the consumption of contaminated foods (Mead *et al.*, 1999).

In 1880, Eberth discovered *Salmonella* Typhi, the etiologic agent involved in typhoid fever. In 1884, Gaffky isolated the organism and in 1900, Lignieres named the genus after Dr. Salmon who, with help from Smith, isolated *Bacillus cholerae-suis* from

swine suffering from hog cholera (ICMSF, 1996; Le Minor, 1981). The first scheme for *Salmonella* classification, based on antigenic variation, was proposed in 1926 and was expanded in 1941 into the Kauffmann-White scheme (D'Aoust, 1997). Between 1988 and 1992, *Salmonella* was implicated in 69% of the documented bacterial foodborne disease outbreaks in the United States, of which 60% involved *S.* Enteritidis (CDC, 1996). *Salmonella* is currently one of the most common bacteria implicated in foodborne disease outbreaks in the United States, with nontyphoidal strains causing an estimated 1.3 million illnesses annually, with a case fatality rate of 0.0078 (Mead *et al.*, 1999).

S*almonella*, belonging to the family *Enterobacteriaceae*, are gram-negative, facultatively anaerobic, nonspore-forming rods. The two species currently recognized within the genus are *S. enterica*, possessing six subspecies, and *S. bongori* (Popoff and Le Minor, 1997). *Salmonella enterica* subsp. I strains are typically found in warm-blooded animals, while subspecies II, IIIa, IIIb, IV, and VI strains and *S. bongori* are typically found in cold-blooded animals or in the environment. There are approximately 2,463 *Salmonella* serotypes, and in the United States, the two most prevalent are *S.* Typhimurium and *S.* Enteritidis (Brenner *et al.*, 2000; CDC, 1997). *Salmonella*, a zoonotic pathogen, is most commonly isolated from the intestines of mammals, and although not all serotypes demonstrate host specificity, several do (e.g., *S.* Pullorum and *S.* Gallinarum in poultry; *S.* Cholerae-suis and *S.* Typhi-suis in pigs; and, *S.* Dublin and *S.* Typhimurium in cattle; ICMSF, 1996).

Salmonella grow at temperatures ranging from 5.2 to 46.2°C, but prefer a temperature between 35 and 43°C. They can grow in environments ranging from pH 3.8 to 9.5, although they grow best at pH 6.5 to 7.5. Additionally, *Salmonella* require a a_w

above 0.93, and under ideal conditions, can grow in sodium chloride concentrations as high as 4% (Garcia del-Portillo, 2000; ICMSF, 1996).

Foodborne Illness

Typhoidal strains, including *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi C and *S.* Sendai, can cause a serious bloodstream infection known as typhoid fever; although this condition has been virtually eradicated in the United States (approximately 400 cases annually), typhoid fever results in many more deaths in developing countries around the world (ICMSF, 1996; Mermin *et al.*, 1998).

Nontyphoidal *Salmonella* strains typically cause an intestinal infection after an incubation period ranging from 5 h to 5 days, resulting in clinical symptoms including diarrhea, nausea, mild fever, chills, vomiting and abdominal cramping. These manifestations typically last 1 to 2 days, but may persist longer, and affect individuals of all ages with the highest incidence occurring in infants (Bopp *et al.*, 1999; ICMSF, 1996). Infectious doses in foods have been found to be as low as 10^0 to 10^2, depending on the serotype, the specific food, and the individual host (D'Aoust *et al.*, 1985; Fontaine *et al.*, 1978; Kapperud *et al.*, 1990).

Salmonella live in the intestinal tract of the infected host or carrier and cells are shed in the feces. Salmonellosis can result from the ingestion of contaminated food or water, contact with host animals, and even contact with an infected human (Miller *et al.*, 1995; Tauxe, 1996). This organism is widely distributed throughout the environment and is usually associated with human illness transmitted by foods of animal origin, such as beef, pork, poultry, eggs, raw milk and milk products such as dried milk, ice cream and

cheese; however, other foods including fresh fruits, unpasteurized juices, and vegetables have also been implicated as vehicles of transmission (Bryan, 1983; ICMSF, 1996; Jay, 2000; Tauxe, 1991).

Pathogenicity

Salmonella, in general, invade and multiply in the lumen of the small intestine where they colonize and enter intestinal columnar epithelial and M cells overlying Peyer's patches (D'Aoust, 1997, 2000; Galan, 1996; Garcia-del Portillo and Finlay, 1994). Contact between the pathogen and target epithelial cells induce the formation of temporary attachment/adhesion mechanisms (e.g., proteinaceous appendages; D'Aoust, 1997; Galan and Ginocchio, 1994; Ginocchio *et al.*, 1994). During this temporary attachment period, the host epithelial cell undergoes massive structural rearrangement in the vicinity of the adherent pathogen, resulting in the formation of membrane "ruffles" and eventually the energy-dependent internalization of a pathogen-containing pinocytotic vacuole (D'Aoust, 1997; Garcia-del Portillo *et al.*, 1994).

Salmonella enterotoxin is a thermolabile protein released into the cytoplasm of host cells where it activates adenyl cyclase found in the cell membrane, leading to an increase in the cytoplasmic concentration of cyclic AMP. The gene that codes for the enterotoxin is not limited to the chromosome and is transcribed within hours of target cell contact (D'Aoust, 1991, 1997, 2000; Jayasheela *et al.*, 1987). In addition to the enterotoxin, a thermolabile cytotoxic protein is normally produced and released extracellularly in response to a hostile environment. The cytotoxin inhibits protein

synthesis and promotes host cell lysis in an attempt to facilitate pathogen dissemination throughout the host tissue (Ashkenazi *et al.*, 1988; D'Aoust, 1991; Koo *et al.*, 1984).

Siderophore production is another virulence factor that is chromosomally regulated by the *fur* gene, and is transcribed in response to limited availability of Fe^{3+} in host tissue (Crosa, 1989). The ability to retrieve Fe^{3+} is essential for cellular functions such as the electron transport chain and enzyme production.

There are three virulence determinants located within or on the external outer membrane. Capsular polysaccharide Vi antigens, as well as serotypic lipopolysaccharides, protrude from the outer cellular membrane and function in defending the organism from lytic attack by inhibiting the host complement system (D'Aoust, 1991). Additionally, porins are transcribed in response to microenvironmental changes (e.g., low osmolarity, low nutrient availability, low temperature), and function in regulating the influx of small-molecules (D'Aoust, 1997; Csonka, 1989).

ANTIMICROBIAL RESISTANCE

As they have done in the past, microorganisms continue to evolve and their large genetic variability and short generation times increase their potential for survival in environmentally unfavorable conditions (Hall, 1997). The emergence of antibiotic-resistant bacteria as a result of the ubiquity of environmental antimicrobials has received worldwide attention due to potential public health risks (Morse, 1995). In 1997, the United States experienced its first pentadrug-resistant outbreak involving a strain of *S.* Typhimurium phage type 104 (DT 104), previously seen in the United Kingdom, that exhibited resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and

tetracycline (CDC, 1997). In addition to penta-resistant *S.* Typhimurium, there has been a general emergence of quinolone-resistant nontyphoidal *Salmonella* (Herikstad *et al.*, 1997b). Isolation of antimicrobial resistant *Salmonella* has been on the rise since 1979-1980 when 16% were resistant, to 1989-1990 when 29% were resistant, and in 1996, 37% of isolates were resistant to at least one agent (Dabney *et al.*, 1997; Lee *et al.*, 1994a; MacDonald *et al.*, 1987; Riley *et al.*, 1984). In addition to *Salmonella*, other examples of antimicrobial resistant bacteria include, but are not limited to, members of the genera *Shigella* and *Staphylococcus*. *Shigella* have developed resistance to sulfonamides, ampicillin, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol and streptomycin, and in areas of Africa and Asia, *S. dysenteriae* 1 serotypes have been reported as being resistant to all locally available antibiotic agents (Bennish and Salam, 1992; Bopp *et al.*, 1999). Similarly, methicillin-resistant *S. aureus*, emerging during the 1980s and early 1990s, has impacted human health, especially in hospital settings (Kluytmans *et al.*, 1997; Lelievre *et al.*, 1999).

In addition to the emergence of antibiotic resistance, common foodborne pathogenic bacteria have demonstrated resistance and cross-protective capabilities to food preservation stresses, as well as increased virulence (Archer, 1997; Sheridan and McDowell, 1998). In order for a foodborne pathogen to cause disease, it must survive exposure to a wide range of stresses associated with both the vehicle of transmission and host immune defenses. Foodborne infection illustrates a pathogen's ability to adapt and survive exposure to environmental stresses. The induction of bacterial resistance to environmental stresses such as temperature and pH extremes involves the production of "protective" shock proteins, some of which possess cross-protective capabilities, or the

ability to confer protection to more than one type of stress (Sheridan and McDowell, 1998). For example, the ability of *E. coli* O157:H7 to resist the antimicrobial effects of acid is increased following heat shocking (Wang and Doyle, 1998). In addition to increased heat tolerance following shocking, *Listeria monocytogenes* express increased tolerance to ethanol and NaCl. *Listeria monocytogenes* also demonstrate increased tolerance to low pH and H_2O_2, following adaptation to ethanol (Lou and Yousef, 1996, 1997). Exposure to sublethal stress during the food manufacturing process may result in stress-hardened pathogens. These pathogens may more readily survive subsequent antimicrobial treatment applications aimed at improving microbiological food quality, potentially resulting in persistent microbiological populations possessing elevated virulence factor expression (Lou and Yousef, 1997; Rees *et al.*, 1995). The ability of an organism to resist environmental stresses, both individually or in combination, and with or without previous exposure, is an important consideration when developing future systems aimed at improving microbiological quality within a particular food; and, furthermore, deserves consideration during predictive microbiology and modeling efforts. The sections that follow review the antimicrobial properties/mechanisms of action of antibiotic, acid and heat stresses, and include discussion regarding defense mechanisms utilized by resistant bacteria.

Antibiotic Stress

Intensive agricultural production practices have resulted in greater animal-to-animal contact due to less available space, and subsequently, preventive-health measures may be more important than therapeutic ones (NRC, 1999). Currently, the production of

animals intended for harvest and, ultimately, for consumption as food products, heavily depend upon the availability and use of pharmacologically active compounds (i.e., drugs). The majority of drugs used during food-animal production can be classified as one of the following: (1) topical antiseptics, bactericides and fungicides, used to treat or prevent skin surface or hoof infections; (2) ionophores, primarily used to increase "rate-of-gain" or production efficiency by promoting activity of specific microorganisms of the commensal rumen biota that provide more favorable and efficient energy-substrate-byproducts as a result of metabolic activities involved in the conversion of feed; (3) steroid anabolic growth promoters (involving the interaction of estrogen-, progesterone- or testosterone-like compounds with specific hormone receptors in animal cells) or peptide production enhancers (e.g., recombinant bovine somatotropin); (4) antiparasitic drugs; and, (5) antibiotics, used to control disease through therapy or prophylaxis, or to promote growth efficiency (NRC, 1999).

Benefits of drug use during food-animal production largely include the maintenance of acceptable animal health, which in turn reduces the risk of transmission of diseases to humans. However, drug residues in food products derived from treated animals may pose a risk to public health due to allergic responses and/or physiological activity. The use of one class in particular, antibiotics, has been a topic of controversy since being recognized as an allergin and due to recognition, or emergence, of antibiotic resistance among pathogenic and nonpathogenic foodborne bacteria. Therapeutic use of antibiotics during food-animal production generally occurs following the diagnosis of disease and should be performed following label instructions. Subtherapeutic use of antibiotics in the United States—defined as the use of an antibiotic as an additive to feed

at less than 200 g per ton of feed—results in the delivery of therapeutic antibiotics, usually through feed or water, at levels below those required to treat infection (NRC, 1999). In 1987, 60% of consumers viewed the use of antibiotics in poultry and livestock production as a serious health hazard, with an additional one-third having some degree of concern (Scroggins, 1988). Public health concerns have primarily focused on increased human morbidity and mortality resulting from failing antimicrobial treatment regimens, and increased costs associated with health care due to the need for additional research and development of new, efficacious antimicrobial drugs (Cohen, 1997; Morse, 1995; Tollefson *et al.*, 1999). Food-animal industry concerns have primarily focused on the diminishing availability and spectrum of approved, efficacious antibiotics, and the impact that developing resistance may have on the industry's ability to maintain production capacity, economic return and animal well-being, ultimately affecting human health (NRC, 1999).

Generally stated, antibiotics are compounds produced by microorganisms, especially *Streptomyces* spp. and fungi, which at low concentrations, possess bactericidal or bacteriostatic properties against other microorganisms (Prescott and Baggot, 1993). In order to minimize toxicity to the infected host, most antibiotics exploit differences in structure or biochemical function between prokaryotic and eukaryotic cells through site recognition of cellular targets unique to bacteria (e.g., peptidoglycan, bacterial ribosomes, or enzymes involved in folate metabolism or DNA supercoiling; Prescott and Baggot, 1993). "Broad-spectrum" antibiotics are those drugs that kill or inhibit the growth of many types of bacteria. Since different agents can cause the same or similar clinical manifestations, broad-spectrum activity is desirable when the time needed to isolate and

identify the etiologic agent cannot be afforded before initiating treatment. The downside to broad-spectrum activity is that, in addition to reducing populations of disease-causing organisms, commensal flora also are reduced, allowing for normally noninfectious bacteria to thrive in an environment free from competitive growth and in some cases, resulting in an even more serious secondary infection (Salyers and Whitt, 1994). Conversely, "narrow-spectrum" antibiotics are more selective in their bacterial targets, requiring isolation and proper identification before therapy can begin. To effectively treat an infection, adequate amounts of an antibiotic must be able to penetrate or reach the site of its molecular target (i.e., infecting bacteria; Eliopoulos, 1998). Distribution capabilities of an antibiotic within an infected individual deserves careful consideration, as not all antimicrobial agents are capable of being absorbed from the gastrointestinal tract; furthermore, some enter the circulatory system but cannot cross the blood-brain barrier, while others cannot penetrate abscesses well or do not enter phagocytic cells (Salyers and Whitt, 1994). Additionally, an antibiotic must encounter target molecules while escaping intra- and/or extra-cellular enzymatic inactivation in order to initiate its desired antimicrobial effect (Eliopoulos, 1998).

In response to pressures or stresses exerted by antimicrobials, microorganisms can and have mutated to develop or acquire resistance. Resistant bacteria possess one or more mechanisms that: (1) alter permeability or intracellular accumulation of antimicrobials, as any barrier to antibiotic entry or efflux pumping to reduce intracellular accumulation results in reduced antimicrobial activity since the primary targets of most antibiotics lie within the cell and therefore require penetration of the drug; (2) alter the natural molecular targets of antimicrobials by incorporating alternative biochemical

pathways that by-pass the function(s) of the primary target, but do not alter cellular physiologic function(s); or, (3) inactivate or modify antimicrobial targets through intracellular and/or extracellular enzymatic activity (Rice and Bonomo, 1996). In addition to mutation, genetic elements encoding antimicrobial resistance can be shared or acquired between both similar and distinctly different types of bacteria. Bacteriophage transduction (viral transfection) and transformation (uptake and recombination or crossing of homologous DNA from the external environment) occur primarily between similar bacteria belonging to the same species (Alberts *et al.*, 1998; Rice and Bonomo, 1996). While narrow host range transfer of genetic material coding for antimicrobial resistant mechanisms is a fear, a larger concern involves broad host range transfer, or the transfer of genes across genus and species barriers. This less specific type of gene transfer is primarily mediated by conjugation, or the transfer of plasmid or chromosomal (conjugative transposon) DNA through a pore (cytoplasmic bridge) formed between the fused membranes of two bacteria (Alberts *et al.*, 1998; Prescott *et al.*, 1993).

Traditionally, speculation regarding the origins of antimicrobial drug resistance focused on the post-antibiotic era, or the assumption that antimicrobial drug development and widespread use fueled the emergence of resistance-conferring genetic elements (Rice and Bonomo, 1996). Although the environmental ubiquity of these drugs over time has undoubtedly contributed to the increased prevalence of antimicrobial resistance among bacterial populations, the extent of diversity among some resistance genes has suggested their pre-antibiotic evolution. Resistance genes may have developed originally among antibiotic-producing bacteria (e.g., *Streptomyces* spp.) in order to provide protection against their own metabolic products. Antimicrobial genes are often located in the same

gene clusters as those coding for resistance mechanisms, of which the majority have been identified in *Streptomyces* spp. (Salyers and Whitt, 1994). Another theory involves the evolution of antimicrobial resistance genes from housekeeping genes, as inactivation enzymes may have been derived from existent cellular proteases, or efflux proteins or ribosomal protection may have evolved from nutrient transport proteins or from elongation factors, respectively (Salyers and Whitt, 1994).

Regardless of its origin, to understand the increased risk to human health associated with antibiotic resistance and the use of antibiotics during food-animal production, several questions deserve attention: (1) Is the emergence of antibiotic resistance among human-disease-causing etiology a result of antibiotic use during food-animal production? (2) Is a minimum drug level necessary to foster resistance development, and if so, how does that level of stress compare to stress induced at subtherapeutic antibiotic levels? (3) How does antibiotic exposure among nonpathogenic microorganisms, with or without the emergence of resistance, ultimately impact the susceptibility of pathogens? (4) Is the resistant microorganism zoonotic, having the capacity to cause human disease if successfully transferred? (5) What is the potential for human exposure to a particular resistant bacterium, accounting for the animal-to-food conversion process and/or subsequent handling of animal-derived foods during which steps intended to reduce the transmission of zoonotic microorganisms occur? (6) If disease results from transmission, is the etiologic agent more virulent than its less-resistant predecessor? (7) If disease results from transmission, are other antibiotics available to treat the zoonosis? (8) What therapeutic or prophylactic role(s) does the antimicrobial agent play in the treatment of human disease? (9) Is antibiotic development

such that new antimicrobial agents are or will be available to replace those to which resistance has developed (CVM-FDA, 1998; NRC, 1999)?

Antimicrobial drugs can be categorized based upon different agent properties including: (1) group of microorganism(s) targeted (i.e., antibacterial, antiviral, or antifungal); (2) antibacterial activity (i.e., narrow- or broad-spectrum; bactericidal or bacteriostatic); (3) mechanism of action (i.e., inhibition of cell wall synthesis, damage to cell membrane function, inhibition of protein synthesis, or inhibition of nucleic acid synthesis or function); or, (4) antibacterial drug interaction (i.e., synergism, antagonism or indifference; Prescott and Baggot, 1993). The sections that follow identify various classes of antibiotics and include discussion regarding their modes of action, spectrum of activity, and mechanisms by which bacteria avoid or resist their antimicrobial properties.

ß-Lactam Antibiotics

Antimicrobial compounds within this class, including the penicillins (penams), cephalosporins and cephamycins, carbapenems and penems, and monobactams, all possess a ß-lactam ring (Sanders and Thomsom, 1998). ß-lactam antibiotics interfere with the last step in peptidoglycan synthesis, the D-alanyl-D-alanine transpeptidation reaction that cross-links the peptide side chains of the polysaccharide peptidoglycan backbone (Livermore and Williams, 1996). They also bind to and inhibit transpeptidase and other membrane-associated proteins called penicillin-binding proteins (PBPs), which among gram-negative rods, are referred to—in order of their descending molecular weight—as 1a, 1b, 2, 3, 4, 5 and 6 (Livermore and Williams, 1996). The net result of ß-lactam binding to PBPs is the stimulation of endogenous enzymes known as autolysins

that degrade peptidoglycan. Under normal operating conditions, autolysins catalyze the turnover of peptidoglycan that allows the bacteria to grow and divide; however, ß-lactam antibiotics function by removing the controls that keep these enzymes in check and, instead, stimulate their attack on peptidoglycan, resulting in the destruction of the peptidoglycan cell wall and eventually bacterial lysis (Salyers and Whitt, 1994).

ß-lactams are normally bactericidal, and due to their mechanism of action, lyse only cells in which active peptidoglycan synthesis is occurring (i.e., growing cells; Prescott and Baggot, 1993). Their spectrum of activity is drug specific, as some are more effective against gram-negative or gram-positive bacteria, while others are equally effective against both (Salyers and Whitt, 1994).

Mechanisms of resistance to ß-lactams include the modification of normal PBPs, use of alternative peptidoglycan transpeptidases, impermeability, production of ß-lactamases, and most recently, efflux pumping of antibacterial compounds out of the cytoplasm as rapidly as they are taken up (Livermore and Williams, 1996). In gram-positive bacteria, ß-lactam resistance results primarily from the production and extracellular release of ß-lactamases, thereby enzymatically rupturing the ß-lactam ring between a C-N bond and causing the loss of antibacterial activity (Prescott and Baggot, 1993). However, resistance among gram-positive species may also result from modification of the antimicrobial target, or the altered binding specificity of PBPs, rendering the antibiotic unable to bind (Salyers and Whitt, 1994). Many gram-negative bacteria are inherently resistant due to lower membrane permeability, a lack of PBPs and the production and release of ß-lactamases within the periplasmic space (Craig, 1998a; Prescott and Baggot, 1993). Gram-negative bacteria can achieve the same level of

resistance as gram-positive species, while releasing lower levels of ß-lactamases because their ß-lactamases are confined to the periplasm, and porins restrict entry of ß-lactams into this region (Salyers and Whitt, 1994).

Penicillins. In 1928, Alexander Fleming isolated penicillin from a mold (*Penicillium notatum*) after observing the lyses of staphylococci colonies on contaminated plates (Fleming, 1929; Stratton, 1996). Sufficient quantities of the drug were not isolated until 1940, and in 1941, clinical trials began in Great Britain and the United States, leading to the development and availability of the "miracle drug", penicillin G (benzylpenicillin), by the end of the 1940s (Craig, 1998a). Antimicrobial activity of penicillin G (a thiazolidine ring attached to a ß-lactam ring carrying a secondary amino group) included gram-positive and gram-negative cocci, and gram-positive bacilli; however, gram-negative bacilli were only inhibited by high concentrations (Craig, 1998a; Livermore and Williams, 1996). Several characteristics of penicillin G, including the relative low-pH instability of the compound and its susceptibility to ß-lactamases (e.g., staphylococcal ß-lactamases), led to the isolation of the active moiety (6-amino-penicillanic acid (6APA)) and the development of various penicillin derivatives (Prescott and Baggot, 1993). The substitution of selected side chains to the 6APA resulted in staphylococcal ß-lactamase-resistant antibiotics (e.g., dimethoxybenzylpenicillin (methicillin), cloxacillin, oxacillin and flucloxacillin), some of which are still used today (Livermore and Williams, 1996). The downside to these drugs was their lack of effectiveness against non-staphylococcal ß-lactamase-producing bacteria.

The ability to completely synthesize 6APA has allowed for the development of semisynthetic penicillins, including those having activity against gram-negative bacteria. Ampicillin possesses activity against many gram-negative bacteria, including *Haemophilus influenzae*, *E. coli*, *Proteus mirabilis*, *Salmonella* spp. and *Shigella* spp., although it is hydrolyzed by staphylococcal ß-lactamases and most of the ß-lactamases produced by gram-negative species (Livermore and Williams, 1996).

The ureidopenicillins, azlocillin, mezlocillin and piperacillin, are ampicillin derivatives with improved activity against gram-negative bacilli due to an increased affinity for PBP-3, and the decreased likelihood of their presence resulting in the production of ß-lactamases (Craig, 1998a). Unlike ampicillin, they are active against *Enterobacter*, *Citrobacter freundii*, *Serratia* spp., indole-positive *Proteus* and *Pseudomonas aeruginosa*, and display activity against klebsiellae at lower inocula levels (Livermore and Williams, 1996).

Much like the ureidopenicillins, the carboxypenicillins, carbenicillin and ticarcillin, show significant activity against *P. aeruginosa*, and moderate activity against indole-positive *Proteus* and *Enterobacter* strains (Craig, 1998a; Prescott and Baggot, 1993). When compared to ampicillin, these drugs generally express enhanced activity against gram-negative bacteria, including ampicillin-resistant strains; however, they possess decreased activity against gram-positive bacteria, especially *Enterococcus faecalis* (Craig, 1998a; Livermore and Williams, 1996). Temocillin, a drug similar to ticarcillin both in structure and activity against susceptible microorganisms, is the only penicillin completely resistant to transferable ß-lactamase and chromosomal AmpC

enzyme hydrolysis by gram-negative bacteria; however, temocillin is not effective against gram-positive bacteria (Prescott and Baggot, 1993).

The only available amidinopenicillins include amdinocillin, formerly referred to as mecillinam, and its oral ester pivmecillinam. Although their activity against gram-positive bacteria is low, almost all fermentative, gram-negative rods are susceptible, including *E. coli*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Shigella* and *Proteus*; pseudomonads, *Bacteroides fragilis* and *H. influenzae* are resistant to amidinopenicillins (Livermore and Williams, 1996). These drugs are unique in that they bind to PBP-2, rather than PBP-1 or PBP-3 (like other penicillins), therefore synergistic activity against gram-negative bacteria is observed when present in combination with ampicillin or other ß-lactams (Craig, 1998a).

Cephalosporins. Development of the initial cephalosporins, and selection of the first derivatives (e.g., cephalothin and cephaloridine), resulted directly from the high levels of ß-lactamase-producing staphylococci present during the 1950s, and the subsequent inability to treat infections (Moellering and Sentochnik, 1998). During the 1960s and 1970s, increased gram-negative nosocomial infections stimulated the development of ß-lactamase-resistant cephalosporins possessing activity against gram-negative bacteria. Eventually, the production of cephamycins and oxacephems provided activity against anaerobes as well. In general, cephalosporins are broad-spectrum drugs that provide activity against both gram-negative and gram-positive bacteria (Moellering and Sentochnik, 1998). However, differences in spectra exist between various cephalosporins, as a desired increase in activity against one bacterial group often times results in decreased activity against another. Therefore, cephalosporins are subdivided

into one of four groups, or generations, based upon their antimicrobial properties (Livermore and Williams, 1996; Moellering and Sentochnik, 1998).

Group I (first-generation) cephalosporins primarily provide activity against gram-positive bacteria (e.g., staphylococci), and include cephalothin (the first clinically available cephalosporin) which proved metabolically unstable and susceptible to the activity of esterases, and cephaloridine and cefazolin, which were developed later and provided resistance against esterases (Klein *et al.*, 1964; Livermore and Williams, 1996). For good activity against most members of the family *Enterobacteriacea*, first-generation cephalosporins should either be administered in combination with an aminoglycoside, or avoided all together (Moellering and Sentochnik, 1998).

Group II (second-generation) cephalosporins provide activity against members of the family *Enterobacteriaceae*, although degree of activity and susceptibility to ß-lactamases varies between compounds. The first clinically available group II cephalosporins that were stable in the presence of ß-lactamases were cefamandole and cefuroxime. These were later followed by cefotaxime, ceftriaxone and ceftizoxime, all of which exhibited 10- to 100-fold greater activity against gram-negative bacteria (Williams and Moosdeen, 1987). Development of antienterobacterial cephalosporins that could be administered orally began in 1969 with the development of cephalexin. Cephalexin was followed by cephradine and cefaclor in the 1970s, and most recently by cefdinir, cefixime, cefpodoxime, ceftibuten and the carbacephem loracarbef, which have 10- to 100-fold greater activity against enterobacteria, *H. influenzae* and *Moraxella catarrhalis* (Bauernfeind and Jungwirth, 1991; Wise, 1992). Second-generation cephalosporins are particularly useful as single-agent therapy of mixed aerobic and anaerobic infections, but

due to antimicrobial resistance, they are not recommended for use in treating nosocomial infections or when multidrug-resistant gram-negative infections are suspected (Moellering and Sentochnik, 1998).

Group III (third-generation) cephalosporins are clinically important due to their broad range of activity, especially against gram-negative nosocomial infections, and their ability to penetrate into cerebrospinal fluid (Moellering and Sentochnik, 1998). Ceftazidime, cefepime, cefpirome, cefoperazone and cefsulodin provide activity against pseudomonads, and with the exception of cefsulodin, which possesses activity against *P. aeruginosa* only, these antipseudomonal drugs provide good antienterobacterial activity as well (Livermore and Williams, 1996). Cefpirome and cefoperazone are also active against gram-positive species including enterococci (Schafer *et al.*, 1992).

Group IV (fourth-generation) cephalosporins are composed of cephamycins that are resistant to a wide range of ß-lactamases, most notably those produced by *Bacteroides* spp. The first clinically available group IV cephalosporin was cefoxitin, which is naturally produced by *Streptomyces lactamdurans*; however, more recently, semisynthetic cephamycins have been produced, including cefotetan, cefmetazole and moxalactam (1-oxacephamycin), which are more active than any other cephalosporin against strains of *B. fragilis* (Livermore and Williams, 1996).

Carbapenems. Carbapenems resemble penicillins and are based on the *Streptomyces cattleya* product thienamycin (Sanders and Thomson, 1998). Two thienamycin-based derivatives, imipenem and meropenem, have been developed, and exhibit a general ß-lactamase resistance (Edwards *et al.*, 1989; Kropp *et al.*, 1980). Meropenem is four- to eight-fold more active against gram-negative species, but less

active against gram-positive species when compared to imipenem. In general, these carbapenems possess the broadest spectrum of activity of any clinically available ß-lactam, and are more rapidly bactericidal than most penicillins and cephalosporins due to their ability to bind to PBP-1 and PBP-2, rather than PBP-3 (Livermore and Williams, 1996; Prescott and Baggot, 1993).

Monobactams. While all of these drugs possess a single, ß-lactam ring structure, the only clinically available monobactam is aztreonam (Sykes and Bonner, 1985). Their spectrum of activity is specifically against aerobic, gram-negative species of bacteria where they bind to PBP-3; however, they lack activity against gram-positive cocci and anerobes (Prescott and Baggot, 1993).

ß-lactamase Inhibitors. ß-lactamase production is a major factor in constitutive and acquired ß-lactam resistance (Prescott and Baggot, 1993). There are two major classes of clinically important ß-lactamase inhibitors. These include the penicillanic acid sulfones sulbactam and tazobactam, which have been mainly combined with ampicillin and piperacillin, respectively, and clavulanic acid or clavulanate, which has been combined with amoxicillin and ticarcillin (Craig, 1998a; Prescott and Baggot, 1993). ß-lactamase inhibitors generally have little, if any, antibacterial activity, and their activity when administered in combination with a ß-lactam depends upon several factors including: (1) whether the ß-lactamase is able to be inhibited; (2) the amount of ß-lactamase produced, it is easier to protect the drug in the presence of lower enzyme levels; (3) the drug to be protected, because piperacillin is easier to protect than ampicillin, amoxicillin or ticarcillin; (4) the susceptibility of the organism to the inhibitor itself; and, (5) the pH, as sulfones inhibit less than clavulanate at a lower pH (Livermore,

1993; Livermore and Corkill, 1992). ß-lactamase inhibitors function by binding ß-lactamases, thereby allowing the ß-lactams to exert their antibacterial properties (Salyers and Whitt, 1994). In general, both classes of inhibitors provide similar protection against class A ß-lactamases, including staphylococcal penicillinase, the most common plasmid-mediated ß-lactamases produced by gram-negative rods, and those chromosomal ß-lactamases produced by *B. fragilis*, *Proteus vulgaris* and *Citrobacter diversus*. However, in vitro, sulbactam and tazobactam inhibit class C ß-lactamases better than clavulanate (Livermore and Williams, 1996).

Glycopeptides

The glycopeptide vancomycin was isolated among by-products produced during fermentative growth by a strain of *Amycolatopsis orientalis*, formerly referred to as *Streptomyces orientalis*, found in a soil sample taken from Borneo in 1956 (Griffith, 1981). Two years later, vancomycin became clinically available and was used to treat infections caused by penicillin-resistant gram-positive bacteria, especially *Staphylococcus aureus*. Following the production of bactericidal antistaphylococcal penicillins and cephalosporins, and partially due to the perception that it was more toxic, vancomycin became more of an alternative therapy for those individuals allergic to ß-lactam antibiotics (Glew and Keroack, 1998). During the 1980s, vancomycin use increased dramatically, primarily due to the emergence of oxacillin-resistant *S. aureus* (Ena *et al.*, 1993). However, the 1990s saw the epidemic emergence and spread of vancomycin-resistant microorganisms, especially among enterococci (VRE; Glew and Keroack, 1998). Glycopeptide antibiotics include actinoidin, ristocetin and teichoplanin,

in addition to vancomycin, and are much larger than any of the penicillins, cephalosporins, aminoglycosides, macrolides or tetracyclines (Glew and Keroack, 1998). Similar to the ß-lactams, glycopeptides disrupt peptidoglycan synthesis by inhibiting both transglycosylation and transpeptidation by binding to the D-alanyl-D-alanine portion of the UDP-muramyl-pentapeptide, and sterically inhibiting the binding of the peptide to the peptidoglycan synthetase enzyme after being transferred out of the cell cytoplasm (Salyers and Whitt, 1994). Glycopeptides are narrow-spectrum compounds that are active only against gram-positive bacteria, as their molecular size prevents them from crossing the outer cell membrane in gram-negative species. Virtually all gram-positive cocci and bacilli, including methicillin-resistant and –susceptible *S. aureus* are susceptible to vancomycin (Glew and Keroack, 1998). Resistance to vancomycin involves two genes (i.e., *van*A and *van*H) encoding proteins that act to incorporate D-alanyl-D-hydroxybutyrate instead of D-alanyl-D-alanine into the UDP-muramyl-pentapeptide. The D-hydroxybutyrate form does not bind vancomycin, and is still recognized by the transglycosylating and transpeptidation bacterial enzymes (Salyers and Whitt, 1994). Therefore, peptidoglycan synthesis continues in the presence of the glycopeptide.

Aminoglycosides

The first aminoglycoside antibiotic, streptomycin, was isolated in the early 1940s from *Streptomyces griseus* following initiation of the first systematic examination of soil microorganisms for antibacterial activity (Lerner *et al.*, 1998). The emergence of antimicrobial resistance quickly compromised the activity of streptomycin against

aerobic, gram-negative bacteria; however, the use of streptomycin in combination drug therapy proved effective for the treatment of tuberculosis, and resulted in minimal resistance among *Mycobacterium tuberculosis* (Lerner *et al.*, 1998). The spectrum of activity against aerobic, gram-negative bacteria was increased with the isolation and identification of kanamycin from a strain of *Streptomyces kanamyceticus* during the 1950s (Lerner *et al.*, 1998). Still, a broader spectrum accompanied the discovery of gentamicin from a strain of *Micromonospora purpurea*, which provided activity against *P. aeruginosa* and many kanamycin-resistant members of the family *Enterobacteriaceae.* The subsequent development of other aminoglycosides, including tobramycin, amikacin and netilmicin, focused on expanding the drugs' activity against gram-negative bacteria, while reducing the degree of nephro- and oto-toxicity (Lerner *et al.*, 1998). In general, aminoglycosides possess bactericidal activity against aerobic, gram-negative bacteria (Stratton, 1996). They are inactive against strict anaerobes and facultative anerobes under anaerobic conditions, and possess limited activity against gram-positive bacteria (Prescott and Baggot, 1993).

Aminoglycosides are comprised of a central, six-membered aminocyclitol ring linked to two or more amino sugar residues by glycosidic linkages (Stratton, 1996). Streptomycin has been the aminoglycoside studied most, and functions by targeting the bacterial ribosome—following part active transport and part passive diffusion—where it binds to the 30S subunit at its interface with the 50S subunit, and inhibits active ribosome formation, peptide chain elongation, and ultimately, protein synthesis (Prescott and Baggot, 1993; Salyers and Whitt, 1994; Stratton, 1996). Streptomycin also causes incorrect codon-anticodon interaction in the ribosome, resulting in the formation of

missense proteins due to a misreading of the genetic code (Prescott and Baggot, 1993). In addition to protein synthesis inhibition, other cellular effects include the impairment of cellular respiration (i.e., electron transport system), inhibition of RNA and DNA synthesis, breakdown of RNA, and damage to the plasma membrane (Lerner *et al.*, 1998; Prescott and Baggot, 1993). Their bactericidal activity probably results from the incorporation of false proteins into the cell membrane, creating abnormal channels and disrupting normal permeability (Stratton, 1996).

There are three mechanisms utilized by bacteria to resist the activity of aminoglycosides: (1) production of aminoglycoside-modifying enzymes; (2) mutational alterations of the ribosomes; and, (3) mutations interfering with aminoglycoside uptake (Prescott and Baggot, 1993). The main mechanism of aminoglycoside resistance is through enzymatic inactivation. In contrast to ß-lactamases that cleave between a C-N bond in the compound, aminoglycoside-modifying enzymes inactivate by adding groups (phosphoryl, adenyl or acetyl) to the antimicrobial compound (Salyers and Whitt, 1994). Among gram-negative bacteria, the modifying enzymes are located on the outside of the cytoplasmic membrane, resulting in reduced transportation of the modified aminoglycoside into the cell and a reduced ability to interfere with protein synthesis (Salyers and Whitt, 1994). Prolonged use of aminoglycosides can lead to hearing loss and impaired kidney function (Prescott and Baggot, 1993; Salyers and Whitt, 1994).

Tetracyclines

Tetracyclines, the first broad-spectrum antibiotics used widely to combat bacterial infections in humans, were discovered in the late 1940s when chlortetracycline was

isolated from *Streptomyces aureofaciens* (Stratton, 1996). However, due to the emergence of resistance and the development of more effective chemotherapeutic agents, their use has decreased significantly (Williams, 1998). Tetracyclines can be placed into three categories, based upon their pharmacological characteristics: (1) short-acting, including chlortetracycline, oxytetracycline and tetracycline; (2) intermediate, including demeclocycline and methacycline; and, (3) long-acting, including doxycycline and minocycline (Williams, 1998). Tetracyclines are comprised of four fused six-membered benzene rings, and like aminoglycosides, target the bacterial ribosome where they bind to the 30S subunit (Salyers and Whitt, 1994). After active carrier-mediated transport across the inner cytoplasmic membrane, tetracyclines distort the A site on the 30S ribosomal subunit, preventing the alignment of aminoacylated tRNA with the appropriate codon on mRNA (Prescott and Baggot, 1993). In addition to disrupting protein synthesis, some atypical, more recently identified tetracyclines (e.g., chelocardin) act by disrupting the bacterial membrane; however, they are too toxic for human use (Salyers and Whitt, 1994). Tetracyclines are generally bacteriostatic and are known to be the least toxic type of antimicrobial drug ever produced (Prescott and Baggot, 1993; Williams, 1998). Tetracycline resistance is primarily accomplished through interference with active transport into the cell, and efflux pumping of the antimicrobial compounds out of the cytoplasm (Prescott and Baggot, 1993). The efflux mechanism identified involves a cytoplasmic membrane-bound protein involved in the energy-dependent transport of tetracycline out of a bacterium (Salyers and Whitt, 1994). Tetracycline efflux pumping genes have been found in both gram-negative (*tet*A-*tet*G) and gram-positive (*tet*K and *tet*L) bacteria. Another type of tetracycline resistance involves ribosomal protection

through the actions of a cytoplasmic binding protein. This type of resistance is widespread among gram-positive, and occurs among some gram-negative pathogenic bacteria, including members of the genera *Neisseria*, *Haemophilus* and *Bacteroides* (Salyers and Whitt, 1994). The combination of low toxicity and the ability to administer tetracyclines orally has led to their overuse in clinical practice. Tetracyclines have been used to treat acne and as a feed additive to promote growth during livestock production. Although widespread resistance to tetracyclines has developed, they still play some important antibacterial roles, including the treatment of Lyme disease (Salyers and Whitt, 1994).

Macrolides and Lincosamides

Like the tetracyclines, macrolides are another class of antibacterial compounds that are not very toxic to humans. Macrolides (e.g., erythromycin, tylosin, spiramycin, tilmicosin and roxithromycin) target the 50S ribosomal subunit where they bind and inhibit chain elongation of the peptide by peptidyltransferase and/or prevent translocation of the ribosome (Prescott and Baggot, 1993). These protein synthesis inhibitors are primarily bacteriostatic, although for some gram-positive species, the inhibition of critical proteins results in a bactericidal effect (Stratton, 1996). Macrolides also have been used to treat livestock, primarily to prevent shipping fever, and have generated concern regarding resistance as a result of their subtherapeutic applications (Salyers and Whitt, 1994).

Lincosamides (e.g., clindamycin) are considerably different than macrolides in their structure, although both classes of drugs have the same antibacterial mechanism of

action, and even bind the ribosome (50S subunit) at or near the same site (Stratton, 1996). The ribosomal binding site for both macrolides and lincosamides contains a portion of 23S rRNA. One mechanism of resistance to both antimicrobials involves the methylation of an adenine residue on the 23S rRNA by rRNA methylase, resulting in the inability of the drug to bind to the target site (Prescott and Baggot, 1993). This type of resistance, involving rRNA methylase, has been found mainly among gram-positive cocci and members of the genus *Bacteroides* (Salyers and Whitt, 1994).

Quinolones

Quinolones are broad-spectrum antimicrobials that inhibit bacterial DNA replication by binding to the ß-subunit of DNA gyrase—an enzyme (topoisomerase) that is essential to DNA replication, allowing supercoils to be relaxed and reformed—and inhibiting the enzymes' activity (Andriole, 1998; Prescott and Baggot, 1993; Stratton, 1996). Nalidixic acid, introduced in 1962, was used to inhibit bacterial DNA replication in the laboratory, but was never considered a clinically important antibacterial agent (Andriole, 1998). More recently, the emergence of fluoroquinolones, a new class of quinolones, has generated great enthusiasm due to their high antibacterial activity against gram-negative aerobes (1000-fold higher than nalidixic acid), including *Enterobacteriacea*, and their good pharmacological properties (Andriole, 1998; Prescott and Baggot, 1993). Quinolones exert a bactericidal effect, and are less likely than other antibiotics to disrupt commensal microflora, as they have poor activity against the streptococci and anaerobes that comprise the majority of resident flora found in the mouth, colon and vaginal tract (Prescott and Baggot, 1993; Stratton, 1996). Quinolones

also have the ability to penetrate polymorphonuclear leukocytes (PMNs; i.e., basophils, eosinophils and neutrophils) and macrophages better than most other antibiotics and are therefore useful in treating infections caused by bacteria capable of surviving in phagocytes (Salyers and Whitt, 1994). Resistance to quinolones does not impact bacterial growth and results from either point mutations that alter outer membrane porins or the affinity of the DNA gyrase ß-subunit for the antibiotic, or through the acquisition of genetic material encoding the resistant form of the ß-subunit (Andriole, 1998; Salyers and Whitt, 1994). The major drawback associated with quinolone use is that a single mutation in the DNA gyrase results in resistance to this class of drugs, which has already been seen among several groups of bacteria (Prescott and Baggot, 1993).

Rifamycins

Rifamycin antibiotics were first isolated as products of *Streptomyces mediterranei* fermentation in 1957 (Sensi, 1983). The first clinically available semisynthetic compounds were rifamycin SV and rifamide, but were both poorly absorbed when administered orally (Craig, 1998b). Rifampin, also referred to as rifampicin, was discovered in 1965 and found to be the most active rifamycin derivative following oral administration (Maggi *et al.*, 1966). More recently, other derivatives have been developed, including rifabutin (Ansamycin), rifapentine, rifaximin and the benzoxazinorifamycins, that exhibit increased activity against mycobacteria, (Arioli *et al.*, 1981; Della Bruna *et al.*, 1983; Saito *et al.*, 1991). The rifamycins are comprised of a naphthalenic ring that is spanned by an activity-dependent, long aliphatic loop (Craig, 1998b). Rifamycins inhibit bacterial DNA-dependent RNA polymerase by binding to its

ß-subunit (Salyers and Whitt, 1994). This results in the interference with protein synthesis by preventing chain initiation, not elongation (Craig, 1998b). Inhibition of enzymes within mammalian cells requires a 1000-fold increase in concentration (Buss *et al.*, 1978). Rifampin is active against a wide range of aerobic and anaerobic bacteria. Among gram-positive bacteria, rifampin is most active against staphylococci (methicillin-resistant or –susceptible), while among gram-negative species, it provides excellent antibacterial activity against members of the genera *Brucella*, *Neisseria*, *Moraxella* and *Haemophilus* (Craig, 1998b). Furthermore, it is one of the most active drugs against all species of *Legionella* (Thornsberry *et al.*, 1983). Resistance to rifamycins results from the insertion, deletion or point mutations in the gene (*rpo*B) encoding the ß-subunit of RNA polymerase, and results in reduced affinity of the subunit for the antibiotic, while still allowing RNA polymerase to function (Craig, 1998b; Salyers and Whitt, 1994). Rifamycins are one of a small number of drugs available for the treatment of tuberculosis, especially since the emergence of isoniazid-resistant *M. tuberculosis* (Salyers and Whitt, 1994). However, their effectiveness is heavily dependent upon the mycobacterial strain, and with the exception of prophylaxis, rifamycins should never be administered solely, due to the emergence of resistant bacteria (Craig, 1998b). For example, rifampin-resistant *M. tuberculosis* occurs in approximately 3.5% of newly diagnosed patients in the United States (Bloch *et al.*, 1994).

Trimethoprim and Sulfonamides

Trimethoprim and sulfonamides are inhibiters of enzymes required for the production of tetrahydrofolic acid in bacteria (Salyers and Whitt, 1994). Tetrahydrofolic

acid is an essential cofactor for 1-carbon transfer reactions that occur during synthesis of nucleic acids and formyl methionine (fMet). Mammalian cells require preformed folic acid as they are unable to synthesize their own tetrahydrofolate, and therefore, are not affected by pathway inhibitors (Salyers and Whitt, 1994). Trimethoprim is structurally similar to dihydrofolic acid and competitively inhibits dihydrofolate reductase, the enzyme that catalyzes the last step in the pathway. Sulfonamides also act through competitive inhibition, as they are structurally similar to *p*-aminobenzoic acid (PABA), the substrate for the first enzyme in the pathway (Prescott and Baggot, 1993). Resistance to trimethoprim and sulfonamides results from chromosomal point mutations that either: (1) impair drug penetration, (2) alter the affinity of the enzymes inhibited for the antimicrobial, or (3) hyperproduce PABA (Prescott and Baggot, 1993). Although mutations resulting in resistance to one of the antimicrobials occur rather frequently, mutations conferring resistance to both are rare. Therefore, trimethoprim and sulfonamides are often administered in combination.

Chloramphenicol

Following the discovery of penicillin, organisms derived from the soil were routinely screened for their ability to produce antimicrobial compounds. Chloramphenicol, originally released in 1949 under the name Chloromycetin, was isolated from a strain of *Streptomyces venezuelae* discovered in Caracas, Venezuela (Calia and Oldach, 1998). The drug proved to be active against gram-positive and gram-negative bacteria, including anaerobes and ricksettsiae, and for a long period of time, was the only consistently active drug against *Salmonella* species including *S.* Typhi (Kucers,

1980). The recognition of life-threatening side effects (e.g., gray syndrome in premature and newborn infants and aplastic anemia) has resulted in its restricted use, and subsequently, less toxic antibiotic alternatives (e.g., cephalosporins and quinolones) have been substituted in many cases (Calia and Oldach, 1998).

Chloramphenicol is a relatively simple structure comprised of a *p*-nitrobenzene ring attached to a propanediol moiety with a dichloracetamide side chain, and can be readily produced by chemical synthesis (Calia and Oldach, 1998; Malik, 1972). Its mechanism of antibacterial action involves protein synthesis inhibition without disturbing the production of nucleic acids. High-affinity, reversible binding occurs on the 50S subunit of the 70S ribosome, resulting in the blockage of new amino acids to the growing protein chain by inhibiting peptide bond formation. More specifically, the amino acid-containing end of transfer RNA is not allowed to bind to its 50S subunit binding site, rendering the amino acid substrate unavailable to peptidyl transferase and unable to form the peptide bond (Pestka, 1971). Chloramphenicol does not inhibit protein synthesis in host cells, since synthesis occurs on 80S ribosomes in mammals. Chloramphenicol exerts a general bacteriostatic effect, although it is bactericidal for certain organisms including *Streptococcus pneumoniae*, *H. influenzae* and *Neisseria meningitidis* (Calia and Oldach, 1998; Rahal and Simberkoff, 1979). It is a broad-spectrum drug that exhibits activity against bacteria, ricksettsiae, ehrlichiae, chlamydiae and spirochetes (Dajani and Kauffman, 1981; Feder *et al.*, 1981). Most aerobic and anaerobic gram-positive bacteria, except for *Nocardia* spp., group D streptococci and methicillin-resistant *S. aureus*, are susceptible to chloramphenicol, (MRSA; Finland, 1972). Most *Enterobacteriaceae*, especially *E. coli* and *P. mirabilis*, and other gram-negative species, including members

of the genera *Neisseria*, *Haemophilus*, *Vibrio*, *Brucella*, *Bordetella*, and *Salmonella* are usually quite sensitive to chloramphenicol; however, with the exception of *Pseudomonas pseudomallei*, *Pseudomonas* species and *Acinetobacter* species are almost always resistant (Calia and Oldach, 1998).

Several mechanisms of chloramphenicol resistance have been identified and include: (1) chloramphenicol inactivation through acetylation or nitroreduction (e.g., *Bacteroides* and *Clostridium* strains); (2) alteration of bacterial ribosomal binding proteins (e.g., *Bacillus subtilis*); (3) alteration of chloramphenicol transport across the bacterial cell wall (e.g., *E. coli*, *P. aeruginosa*, *Serratia* and *H. influenzae*); and (4) active efflux pumping (e.g., *P. aeruginosa*; Calia and Oldach, 1998). The most significant of the resistance mechanisms is the plasmid-mediated production of acetyltransferases, which may confer resistance to other antibiotics in addition to chloramphenicol, and have been implicated in outbreaks of chloramphenicol-resistant typhoid fever and shigella dysentery (Benveniste and Davies, 1973; Goldstein *et al.*, 1986; Murray, 1986; Smith *et al.*, 1984). In California, an outbreak involving chloramphenicol-resistant *Salmonella* Newport was linked to contaminated hamburgers prepared from the meat derived from dairy cattle raised on a farm where chloramphenicol was commonly used (Spika *et al.*, 1987). Although chloramphenicol use during food-animal production has never been legal in the United States, this case demonstrated a direct link between antibiotic use, selection of resistant phenotypes, and subsequent human disease (Calia and Oldach, 1998).

Acid Stress

The ability of a bacterium to sense potentially lethal environmental changes and initiate appropriate cellular activities in response to those changes is a characteristic critical to survival. Among many encountered stresses (e.g., starvation, oxidation, osmolarity, extreme temperatures, heavy metals and nucleic acid damaging agents), bacteria may face transient or prolonged exposures to potentially lethal or sublethal pH conditions in both natural and host environments (Cirillo *et al.*, 1998; Foster and Spector, 1995; Rathman *et al.*, 1996; Renberg *et al.*, 1993). Acid-induced injury or inactivation is the direct result of H^+ ions and the presence of weak acids. Generally speaking, the bacterial outer membrane is not very permeable to protons; however, under conditions in which the extracellular environment (pH_o) is extremely acidic, protons will permeate the membrane, subsequently reducing intracellular pH (pH_i), and ultimately disrupting crucial biochemical reactions and macromolecular structures (Hill *et al.*, 1995). Bacterial stress associated with moderate-to-low pH_o is amplified by the presence of weak acids (e.g., organic or short-chain fatty acids), including volatile fatty acids such as butyrate, propionate or acetate. The lower the pH_o, the more protonated or undissociated the weak acid (depending on the respective pK_a), which can permeate across the cell membrane and dissociate, thereby lowering pH_i (Foster, 2000). If the pH gradient is 2 units or more (e.g., pH_i of 7.0 and pH_o of 5.0), the internal weak acid concentration can exceed 100-fold that of the external environment (Hill *et al.*, 1995). Survival of a microorganism under these acid-stressing conditions ultimately depends on the presence of adaptive mechanisms and the ability of the organism to mount an appropriate molecular response in order to avoid or repair damage associated with low-pH exposure (Foster, 2000;

Neidhardt, 1987). While acidophiles have evolved over time to claim the low-pH environment as their niche, neutralophiles (e.g., *E. coli* and *S.* Typhimurium) have developed inducible low-pH survival systems that allow for survival over a 1,000,000-fold range of H^+ ion concentration (Hall *et al.*, 1995).

Among both gram-negative and gram-positive bacteria, there exist pH homeostatic systems that function to regulate pH_i following mild shifts in pH_o. Enteric gram-negative organisms work to maintain a consistent pH_i (pH 7.6 to 7.8) by moving protons into the cell at alkaline pH_o, and out of the cell at acidic pH_i (Hall *et al.*, 1995; Hill *et al.*, 1995). The two systems considered the primary pH gradient generators are potassium-proton antiporters, which alkalinize pH_i, and sodium-proton antiporters, which acidify pH_i (Brey *et al.*, 1979; Dibrov, 1991; Kroll and Booth, 1983; Zilberstein *et al.*, 1984). In contrast to gram-negative bacteria that attempt to maintain a constant pH_i, gram-positive bacteria allow pH_i to decrease as pH_o decreases, attempting to maintain a difference between pH_i and pH_o (ΔpH) of 0.5 to 1.0 units (Foster, 2000). Regulation of ΔpH primarily depends on the activity of pH-dependent F_oF_1 proton translocating ATPase, rather than antiporters, for the removal of protons (Foster, 2000). In addition to these "housekeeping" homeostatic antiporters and ATPase, gram-negative and gram-positive bacteria possess adaptive acid stress response systems that include, among other inducible pathways, emergency pH homeostasis systems to prevent or repair damage associated with extreme acid stress (Foster, 2000).

Salmonella

Although protective mechanisms allowing for an adaptive response to low-pH exposure have been reported among other *Salmonella* serotypes, *S.* Typhimurium has been the primary subject of acid survival investigation due to its well-defined genetics and its ability to survive a wide-range of acid stresses in both intra- and extra-host environments (Foster, 1995; Leyer and Johnson, 1992). *Salmonella* Typhimurium possesses two distinct low-pH inducible systems referred to as acid tolerance responses (ATR), which are distinguished based on the growth phase during which induction occurs (Hall *et al.*, 1995). Induction of the log-phase ATR occurs when exponentially growing cells are suddenly exposed to low pH, resulting in the production of 50 acid shock proteins (ASP; Foster, 2000; Foster and Hall, 1990; Hall *et al.*, 1995). The production of some ASP is induced by changes in pH_i, while others respond to decreased pH_o (Foster, 2000). Log-phase ATR has been demonstrated when cells grown in pH 7.7 minimal media were either subsequently grown in pH 5.8 minimal media, or shocked for 2 h at pH 4.5, thereby providing protection against subsequent acid-challenge at pH 3.0 (Foster, 1991, 1993; Foster and Hall, 1990, 1991). The *rpoS* and *fur* genes, encoding the alternative sigma factor, σ^S, and the iron-regulatory protein Fur, respectively, in addition to the two-component signal transduction system PhoPQ, all play a regulatory role in log-phase acid tolerance (Foster, 2000; Hall and Foster, 1996; Lee *et al.*, 1995).

In the natural and host environments, salmonellae are not always engaged in exponential growth when they encounter acid stress. A second, pH-dependent system known as the stationary-phase ATR is induced when stationary-phase cells are exposed to low pH (Lee *et al.*, 1994b). This system is distinct from the pH-independent general

stress response system that is induced by entry into stationary phase, as the general stress response system requires stationary-phase induction of the alternative sigma factor, σ^S, while the acid-induced stationary-phase ATR does not (Hall *et al.*, 1995). The stationary-phase ATR requires the regulatory gene *ompR* and results in the production of 15 ASP (most of which are distinct from log-phase ASP) that afford a higher level of acid resistance than the log-phase ATR (Bang *et al.*, 2000; Lee *et al.*, 1994b).

In addition to withstanding low pH exposure, acid-adapted *S.* Typhimurium may exhibit cross-protection against heat, osmotic and oxidative stresses (Foster, 1999; Lee *et al.*, 1995; Leyer and Johnson, 1993; Wilde *et al.*, 2000). A strong correlation between the ability to mount an ATR and enhanced virulence also has been suggested (Idziak and Suvanmongkol, 1971; Wilmes-Riesenberg *et al.*, 1996). Stress cross-protection associated with the ATR may prove important during pathogenesis, as cells undergoing acid-adaptation prior to ingestion by the host or by acid shock in the stomach may be better prepared to endure environmental stresses subsequently confronted in the stomach or intestinal and intracellular environments, respectively (Idziak and Suvanmongkol, 1971; Wilmes-Riesenberg *et al.*, 1996). In fact, the induction of numerous genes has been found to coincide with phagosome entry and phagolysosomal formation (Alpuche-Aranda *et al.*, 1992; Cirillo *et al.*, 1998).

Escherichia coli

In *E. coli*, three acid survival systems have been identified: acid tolerance response (ATR), acid habituation, and acid resistance (Hall *et al.*, 1995). In a direct comparison of log-phase ATR between *E. coli* and *S.* Typhimurium, *E. coli* were found to

display similar acid resistance when grown and challenged in non-supplemented minimal media at pH 3.0 for 1 to 2 h (Lin *et al.*, 1995). In contrast to the ATR, acid habituation—another log-phase survival system—is evaluated in complex, nutrient rich media, involving short exposures (<10 min) to pH 3.0 (Goodson and Rowbury, 1989; Raja *et al.*, 1991). This acid survival system involves mechanisms that are only peripherally related to those involved with the ATR (Foster, 2000). Induction of acid habituation, even at neutral pH_o, has been found to be affected by various compounds including, among others, glucose, glutamate, aspartate, KCl, phosphate and cAMP (Foster, 2000; Rowbury and Goodson, 1993, 1998). Clearly, the greatest resistance to the antimicrobial effects of low-pH is seen in stationary-phase *E. coli* cells. These acid resistance (AR) systems protect cells for several hours at pH 2, unlike log-phase ATR or acid habituation (Small *et al.*, 1994).

Castanie-Cornet *et al.* (1999) identified three distinct, stationary-phase AR systems (AR system 1, 2 and 3) in *E. coli.* AR system 1 is RpoS-dependent, glucose repressed, and requires glutamate activation at pH 2.5 (Castanie-Cornet *et al.*, 1999). AR system 2 is RpoS-independent, and requires extracellular glutamate and glutamate decarboxylase during acid exposure at pH 2.0 (Lin *et al.*, 1995, 1996). Protons are used during intracellular amino acid decarboxylation reactions (e.g., glutamate to γ–amino butyric acid), and GadC antiporters function to transport and provide extracellular substrate and remove decarboxylation products (Castanie-Cornet *et al.*, 1999). AR system 3 requires arginine and arginine decarboxylase (*adi*A) during acid-challenge at pH 2.0, and functions similarly to AR system 2 (Lin *et al.*, 1995). Optimum expression of *adi*A occurs at low-pH following anaerobic growth in complex media, at which time

the arginine decarboxylase use protons during the conversion of intracellular arginine to agmatine (Foster, 2000). Lastly, a particular virulent serotype of *E. coli* (i.e., *E. coli* O157:H7) has been shown to possess an AR system 3 that provides increased acid resistance when compared to commensal *E. coli* strains (Lin *et al.*, 1996). Induction of any of these individual, low-pH survival systems depends on many factors including growth phase, substrate medium, and type of acid stress (i.e., organic or inorganic; Foster, 1999). Since the identification of low-pH adaptive systems in *E. coli* and *Salmonella*, inducible acid tolerance has been found among many microorganisms, including members of the genera *Aeromonas*, *Helicobacter*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Mycobacterium*, *Shigella* and *Streptococcus*, and has been suggested as a possible universal phenomenon (Foster, 2000; Hamilton and Buckley, 1991; Hartke *et al.*, 1996; Karem *et al.*, 1994; Karita and Blaser, 1998; Kroll and Patchett, 1992; Lorca *et al.*, 1998; McDonald *et al.*, 1990; Nojoumi *et al.*, 1995; O'Brien *et al.*, 1996; O'Driscoll *et al.*, 1996; Small, 1998). While little is known mechanistically about low-pH inducible acid tolerance systems, their importance to bacterial survival and pathogenesis should not be underestimated.

Heat Stress

The majority of food preservation techniques operate by slowing down or inhibiting the proliferation of microorganisms. The goal of thermal processing, however, is to inactivate vegetative cells and endospores, when applicable, in order to reduce the opportunity for survival of an organism that is capable of growth in a specific food (Gould, 1989). Derivation of determined "acceptable levels", and the practical

application(s) of heat during thermal processing, originated primarily from earlier inactivation studies evaluating the kinetics of *Clostridium botulinum* type A spores, and has since been developed over many years (Esty and Meyer, 1922). Standards for sterilization requiring reductions in *C. botulinum* spore populations by 10^{12} cells have been reported as necessary to ensure the satisfactory safety of low-acid canned foods (Hicks, 1961). Development and recognition of this widely referred "12-D concept" has served as the basis for several analogous concepts, including the "7D", which refers to the 7 log reduction of *Salmonella* required by a thermal process to ensure the satisfactory safety of cooked meat (Angelotti, 1978).

Inactivation kinetics describe the behavior of microorganisms when exposed to heat stress. In general, viable vegetative cells and spores in a uniformly heated, pure population decrease exponentially with time over many orders of magnitude, and are reported in terms of the survival curve slope as decimal reduction time (*D*-value; Gould, 1989; Pflug and Holcomb, 1991). Furthermore, rate of inactivation increases (*D*-value decreases) exponentially with increased temperature, and is expressed in terms of the slope derived from plotting *D*-value vs. challenge temperature as the temperature coefficient of inactivation (z_D-value) in degrees celsius (Gould, 1989). Departures from the traditional log-linear relationship have been described, especially when large inactivations are achieved. In other words, a "tailing" phenomenon is commonly reported when survival populations are followed over extended periods of time, yielding very low populations (Gould, 1989). Similarly, "shouldering", or a rise in microbial populations, has been reported following initial periods of heating (Gould, 1989). Deviations from log-linear inactivation kinetics have been explored, and several

explanations have been provided (Pflug and Holcomb, 1977; Roberts and Hitchins, 1969). These include the assumption that the deviations are artefactual, resulting from methodology involving the enumeration of colony-forming units (CFU) rather than actual surviving cell populations, thereby not providing an accurate estimate of the true inactivation kinetics. This explanation encompasses, among others, the activation of spores or cell clumping (Pflug and Holcomb, 1977; Roberts and Hitchins, 1969). Additionally, deviations from log-linear kinetics can be attributed to heterogeneity of thermal resistance, or phenotypic variation in susceptibility among members of a population, and/or the change in resistance over time of heating (Allwood and Russell, 1970). In other words, if heat is assumed to affect all cells within a given population in a similar, uniform manner, then the shape of the resulting survivor curve is a function of the non-uniform distribution of heat resistance between members of the population (Gould, 1989). While the aforementioned factors may individually contribute to "tailing" or "shouldering", their combination contributes to survival curves that are sigmoidal in shape (Gould, 1989).

Problems with quantifiying lethality, in terms of an expressed *D*-value, using nonlinear survival curves have led to the development of numerous kinetic models of higher orders (Kormendy and Kormendy, 1997; Pflug and Holcomb, 1991; van Gerwen and Zwietering, 1998; Whiting, 1995), and to the idea that, in such cases, the resulting survival curve reflects the presence of two different populations with varying susceptibility/resistance, each of which follows a first order (log-linear) kinetic (Stumbo, 1973). Additionally, it also has been suggested that the idea of reaction kinetics is false, lending to the theory that a microbial survival curve is a cumulative reflection of a

distribution of lethal events (Anderson *et al.*, 1996; Augustin *et al.*, 1998; Casolari, 1988; Peleg, 1999; Peleg and Cole, 1998). Therefore, the varieties of shapes or forms assumed by survival curves only reflect different lethal event distributions (e.g., wide or narrow, symmetric or asymmetric with a positive or negative skewness, and unimodal or bimodal; Peleg, 2000).

Deviations from log-linear inactivation kinetics are most often reported for vegetative cells than for spores, especially following prolonged, lower temperature exposures during which vegetative cells continue to metabolize and alter composition and structure (Allwood and Russell, 1970). The shape of a survivor curve, including the presence of tailing or shouldering, is influenced by many factors. In addition to the heterogeneity of thermal resistance seen between different types of microorganisms subjected to identical heat stressing conditions (i.e., the ability of some to withstand higher temperatures for extended periods of time), heat resistance also may be affected by various environmental influences during growth and formation of cells or spores before undergoing heat stress (e.g., metabolic phase, growth temperature and nutrient availability; Brackett *et al.*, 2001; Pflug and Holcomb, 1991). Environmental factors during heat exposure (e.g., carbohydrate availability, a_w and salt concentration, pH and the presence of organic or inorganic compounds) and conditions during cell recovery also may influence the ability of microorganisms to survive or repair damage associated with heat exposure (Pflug and Holcomb, 1991). Additionally, protective mechanisms allowing for an adaptive response to low-pH exposure have been reported among several microorganisms, including *Salmonella* (Foster, 2000). In addition to the protection provided against subsequent low-pH exposure, acid-adapted microorganisms (e.g.,

Salmonella) may exhibit cross-protection against heat, osmotic and oxidative stresses (Foster and Spector, 1995; Lee *et al.*, 1995; Leyer and Johnson, 1993; Ryu and Beuchat, 1998; Wilde *et al.*, 2000). Adaptation resulting in the cross-protection between environmental stresses also may influence heat resistance.

Heat resistance of endospores is influenced by the intrinsic property of the strain, as spores produced by psychrophilic bacteria are generally more susceptible to heat than those produced by thermophiles, and the structure and composition of the spore (Gould, 1989; Pflug and Holcomb, 1991). Normally, cytoplasmic constituents are not intrinsically heat tolerant; however, structure and compositional factors raise their heat tolerance by 35 to 45°C (Warth, 1978). Factors contributing to increased heat tolerance include: (1) structure, as the central membrane-bound cytoplasmic region is surrounded by a thick cortex encapsulated by several protein-based coats; (2) calcium and dipicolinic acid, comprising 2 to 10% of the spore dry weight, plays an indirect role in the establishment and maintenance of resistance and sustained viability; (3) peptidoglycan, the primary constituent of the spore cortex, provides protection against heat stress, and their hydrolysis by endogenous enzymes during germination accompany the resulting increased susceptibility to heat of their vegetative cellular counterparts; (4) core-located basic proteins bind to, and stabilize, spore DNA and may also provide a source of amino acids during germination; (5) mineralization and ion status, spores contain high levels of calcium and often magnesium and manganese, in addition to monovalent cations (e.g., potassium and sodium), that when released result in increased heat sensitivity; and, (6) water content, as the most heat resistant spores possess the lowest core water content

(Beaman *et al.*, 1982; Bender and Marquis, 1985; Gould, 1989; Johnstone *et al.*, 1982; Setlow and Setlow, 1979).

Constitutive heat resistance among vegetative cell populations depends primarily upon the ability to repair damaged DNA or protect DNA from melting or heat-induced depurination, as non-DNA damaged cellular components can be replaced by newly synthesized ones if the DNA remains functional (Gould, 1989). Membrane composition, or the modification of membranes, has been shown to influence heat resistance, as cells containing lower levels of saturated lipids—or, in general, those grown at lower temperature—are more susceptible to heat stress (Beuchat, 1978; Katsui *et al.*, 1981; Russell, 1988). Among thermophiles, heat tolerant proteins have evolved through small changes in their predecessors' primary structures, resulting in newly formed tertiary structures and subsequently increased heat resistance (Amelunxen and Murdock, 1978). Lastly, vegetative cells can adapt to exposures to heat stress through the activation and expression of certain homeostatic or protective genes (Gould, 1989). Sublethal heat exposure may result in the production of heat-shock proteins, and increased heat resistance for transient or prolonged periods of time depending on the duration of protein synthesis (McAlister and Finkelstein, 1980; Yamamori and Yura, 1982). Induction of heat-shock protein synthesis is accompanied by a reduction in cytoplasmic pH, suggesting that the initial antimicrobial effects of heat may be indirect, by impairing membrane functions (e.g., pH homeostasis; Weitzel *et al.*, 1987).

The difficulty in defining the antimicrobial action of heat, mechanistically, results from the many cellular constituent changes that occur during thermal inactivation (Gould, 1989). Determining the exact "event" resulting in cellular death or injury is predictably

difficult; however, major changes have been identified and reviewed among endospores and vegetative cells alike. The primary sites of lethal and sublethal heat-induced damage include the DNA, RNA and ribosomes, cytoplasmic membranes, and specific cellular enzymes (Gould, 1989). Lethal heat exposure by both vegetative cells and spores results in single and double strand breaks in their DNA resulting directly from the heat or, more often, from the accelerated activity of endogenous nucleases (Grecz and Bruszer, 1981; Kadota *et al.*, 1978). Heat-induced DNA damage may result indirectly from oxidation reactions mediated by hydrogen peroxide (H_2O_2) and/or the free radicals derived from it, as heat exposure may inactivate enzymes (e.g., catalase and superoxide dismutase) that provide protection against these damaging agents (Gould, 1989).

Regardless, these DNA aberrations are much more pronounced following dry heating, which requires higher temperatures to achieve inactivation (Kadota *et al.*, 1978). Exposure to mild heat also results in the loss of viability and the accelerated degradation of ribosomal RNA; although, ribosome reassembly does occur during periods of recovery (Flowers and Martin, 1980). Hurst and Hughes (1978) demonstrated that 30S ribosomal subunit degradation was Mg^{2+}-dependent, and suggested that ribosomal destruction results primarily from the loss of Mg^{2+} associated with the increased extracellular "leakage" seen in membrane-damaged cells following heat exposure. Increased membrane permeability or "leakiness" has long been considered a consequence of heat exposure among vegetative cells (Beuchat, 1978). The rapid efflux of amino acids, ions (e.g., K^+) and low molecular weight nucleic acid components impair the various chemiosmotic and transport functions of a cell's outer membranes, and has been

suggested as the fundamental cause of the observed phenotypic expression of "injury" (Gould, 1989; Iandolo and Ordal, 1966).

In addition to DNA, RNA and ribosomes, and cytoplasmic membrane damage, inactivation of key enzymes in the germination pathway also may result from heat exposure. Heat-inactivated spores (e.g., *C. perfringens* and *C. botulinum*) unable to initiate germination and subsequent colony formation were able to do so when recovered on media supplemented with additional amino acids or artificial germinants (e.g., calcium dipicolinate; Adams, 1978; Barach *et al.*, 1975; Edwards *et al.*, 1965; Gurney and Quesnel, 1980, 1981; Scott and Bernard, 1985). Presence of these substrates apparently served to by-pass the inactivated stage of the germination pathway (Gould, 1989). However, in spores that possess two germination pathways, exposure to heat may result in the inactivation of one but not both, allowing for their germination without requiring germinant supplementation (Busta and Adams, 1972).

Overall, evidence suggests that direct or indirect DNA damage is a key lethal event among heat-exposed vegetative cells. However, the phenotype most frequently identified as sublethal injury is probably a result of accumulated damage to other cellular components, especially membranes, resulting in the interference of homeostatic functions (Gould, 1989). As damage accumulates among non-key lethal cellular targets, and the number of functional targets is reduced to a low level, the cell may be rendered unable to initiate growth in the presence of stress (e.g., selective ingredients in a recovery medium), unless the inactivated sites are repaired or replaced. Therefore, as injury accumulates, the probability of a surviving cell or spore initiating growth or germination decreases proportionately (Jensen *et al.*, 1987; Lund *et al.*, 1987). Finally, cellular injury deserves

attention when considering the microbiological status of heated foods in order to prevent underestimations of microbial populations, and to improve upon existing preservation techniques and/or technologies.

Salmonella

Salmonella grow at temperatures ranging from 5.2 to 46.2°C, but prefer a temperature between 35 and 43°C. In addition to the temperature parameters for growth, the temperature at which *Salmonella* are inactivated (thermal inactivation) depends, at least in part, on the *Salmonella* strain, their physiologic state of growth, the composition of the substrate and recovery media, other limiting environmental conditions (e.g., pH, atmosphere, a_w), previous growth and storage conditions, and the presence of other microorganisms (Doyle and Mazzotta, 2000). Comparing published data regarding the thermal resistance of *Salmonella* is difficult due to differences in controlling the aforementioned parameters that influence subsequent heat resistance. This stated, certain serotypes of *Salmonella enterica* have proven to be more resistant to the effects of heat than others, namely *S.* Senftenberg 775W (Ng *et al.*, 1969). Despite the fact that *S.* Senftenberg is not considered an important foodborne pathogen, it is often used as an indicator, or as a "worst case" scenario, organism to verify the effectiveness of thermal processing steps aimed at destroying more common *Salmonella* serotypes (Doyle and Mazzotta, 2000). Among other *Salmonella* serotypes, *S.* Typhimurium and *S.* Enteritidis are often evaluated for their ability to resist or repair damage associated with heat stress. In general, the majority of studies have concluded that *S.* Enteritidis is more heat resistant than *S.* Typhimurium, although in some studies evaluating heat resistance in laboratory

media rather than food, the opposite has been reported (Humphrey *et al.*, 1990; Palumbo *et al.*, 1995; Shah *et al.*, 1991).

Heat resistance of *Salmonella* is influenced by physiologic state, as exponentially growing cells are less resistant to heat stress than are cells in stationary-phase (Humphrey *et al.*, 1995). The ability of *Salmonella* to resist inactivation or death from heat exposure also is increased by growth at elevated temperatures, growth in an environment with limited nutrient availability (e.g., minimal media), or by a previous, transient exposure to sublethal heat (i.e., heat shocking; Bunning *et al.*, 1990; Ng *et al.*, 1969; Shah *et al.*, 1991; Xavier and Ingham, 1997).

Properties of the individual food also influence heat resistance, as *Salmonella* survive better in foods that contain high amounts of solids, or are lower in pH or a_w (Blackburn *et al.*, 1997; Corry, 1972; Ellison *et al.*, 1994). In addition to these properties, the presence of certain additives or preservatives (e.g., EDTA, polyphosphates, hydrogen peroxide, or the lactoperoxidase system) may directly or indirectly result in decreased heat resistance of *Salmonella* (Doyle and Mazzotta, 2000).

The presence of other microorganisms (e.g., members of the genera *Escherichia*, *Citrobacter* or *Pseudomonas*) in sufficient enough numbers ($>10^8$) may result in increased heat resistance among *Salmonella* populations due to the premature expression of stationary-phase genes, or as a result of decreased oxygen due to high numbers of respiring cells, that results in reduced oxidative damage (Doyle and Mazzotta, 2000; Duffy *et al.*, 1995).

CHAPTER III

DISSERTATION RESEARCH INTRODUCTION

In order to address public health issues and concerns regarding food safety in the meat industry, the Food Safety and Inspection Service of the United States Department of Agriculture (FSIS-USDA) mandated industry-wide implementation of HACCP principles and associated microbiological performance criteria (FSIS-USDA, 1996b). In response, the beef industry adopted new technologies, including hot water/steam-vacuuming, steam pasteurization, and ambient temperature or hot water spray washing with or without organic acid solution rinsing (Dickson and Anderson, 1992; Dorsa, 1997; Hardin *et al.*, 1995; Phebus *et al.*, 1997; Smulders and Greer, 1998; Sofos and Smith, 1998).

Since that time, research has demonstrated that the beef industry is producing carcasses of improved microbiological quality (Bacon *et al.*, 2000; Sofos *et al.*, 1999a, b, c). However, surviving bacterial populations on carcass surfaces can proliferate during mild to moderate temperature abuse, and primal cuts and trimmings can be recontaminated during fabrication from employees and improperly sanitized contact surfaces. In fact, cross-contamination occurring directly, between carcasses, primal cuts and trimmings, or indirectly, from repeated contact with employees or contact surfaces, is unavoidable (Gill *et al.*, 1999, 2001b; Upmann *et al.*, 2000). Ultimately, there is a need for information regarding the possibilities for reducing, or controlling the growth of, bacterial populations during the beef carcass fabrication process.

Lastly, the prolific application of decontamination treatments utilizing low-pH or heat stress to inactivate microbial populations associated with the animal-to-muscle food conversion process provides the opportunity for bacterial adaptation and resistance. Additional research comparing the abilities of nonadapted and adapted or "stress hardened" bacteria to resist subsequent lethal and sublethal stress exposures is needed in order to assess the impact of these technologies on the ability of surviving microbiological populations to pose a public health risk.

CHAPTER IV

USE OF A COMMERCIAL STEAM-VACUUMING UNIT TO REDUCE INOCULATED *SALMONELLA* ON CHILLED FRESH BEEF ADIPOSE TISSUE

ABSTRACT

Efficacy of a commercial steam-vacuuming unit to reduce *Salmonella* populations on inoculated, chilled beef carcass adipose tissue was determined. Beef carcass tissue samples (N = 70) were treated as follows: 1) uninoculated (n = 10), without post-chilling steam vacuum application; 2) uninoculated (n = 10), with post-chilling steam vacuum application; 3) inoculated pre-chilling (n = 10), without post-chilling steam vacuum application; and, 4) inoculated pre-chilling (n = 40), with post-chilling steam vacuum application. Following inoculation of the tissue with *Salmonella* (5.2 log colony-forming units (CFU)/cm^2), chilling (24 h; 2.6 ± 0.1°C), and post-chilling steam-vacuuming (1.72 bar steam; 130°C; -0.24 bar vacuum), as required by the assigned treatment, samples (100 cm^2) were sponge-swabbed and the same sample area was excised for microbiological analysis. Despite previous sponge-swabbing of the same area, excision proved to be the most effective sampling method for enumeration of bacterial populations. *Salmonella* populations (3.9 to 4.1 log CFU/cm^2) on chilled tissue were reduced ($P < 0.05$) to between 3.2 and 3.6 log CFU/cm^2 following steam-vacuuming. Under the conditions of this study, and for the inoculum levels tested, results indicated that steam-vacuuming did not achieve reductions of sufficient magnitude (>1.0 log

CFU/cm^2) to support its use for decontamination of chilled beef tissue. The lack of efficacy of steam-vacuuming may have resulted from insufficient application of the unit on the surface of the adipose tissue. Although the manner of application closely simulated use of the device in commercial practice, the firmness of the chilled tissue and duration of contact of steam with bacterial cells that were attached, imbedded or protected by biofilm may not have allowed more bacterial destruction.

INTRODUCTION

The process known as "steam-vacuuming" involves the application of hot water, steam, or a combination of hot water and steam followed by the uptake of condensate and associated contamination by generation of a vacuum (Sofos and Smith, 1998). In April 1996, the application of steam-vacuuming was approved by the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) as an alternative to knife-trimming for the removal of feces, ingesta and other contamination from carcasses, provided that the spot of visible contamination does not exceed 2.5 cm in diameter (FSIS-USDA, 1995, 1996a; Sofos and Smith, 1998). Application of steam-vacuuming has been reported to be at least as effective as knife-trimming, and more consistent in its effectiveness than water washing, for the purpose of spot carcass cleaning/decontamination (Dorsa *et al.*, 1996a; Kochevar *et al.*, 1997).

Two steam/hot water vacuum systems were evaluated (Kochevar *et al.*, 1997) for their efficacy as decontamination treatments when applied to beef carcass surfaces at $\geq$ 82°C, 0.34 to 1.03 bar, with condensate subsequently removed at -0.0093 bar. The application of steam/hot water vacuuming before carcass splitting reduced aerobic plate

counts (APC) and total coliform counts (TCC) by 1.1 to 2.3 and 1.2 to 2.2 log CFU/cm^2, respectively, from initial populations of 4.6 to 5.1 and 2.9 to 3.2 log CFU/cm^2 (Kochevar *et al.*, 1997).

Dorsa *et al.* (1996b) reported that steam-vacuuming reduced ($P < 0.05$) APC, TCC and *Escherichia coli* counts (ECC) on inoculated beef carcass short plates, following an incubation time of 15 min at room temperature, from initial populations of 6.2, 5.0 and 4.8 log CFU/cm^2, respectively, to 3.2, 1.0 and 0.8 log CFU/cm^2. In another study (Dorsa *et al.*, 1996a), steam-vacuuming resulted in 2.0 to 3.0 log CFU/cm^2 reductions in APC and a 5.5 log CFU/cm^2 reduction in *E. coli* O157:H7 populations when beef short plates were inoculated with 5.5 and 7.6 log CFU/cm^2, respectively, and allowed to incubate at room temperature for 15 min.

A 1997 study (Dorsa *et al.*, 1997) evaluated the antimicrobial efficacy of steam-vacuuming and hot-water spray-washing when applied following inoculation of beef short plates and an incubation period of 15 min at room temperature. Following tissue excision, sample stomaching (2 min), and subsequent microbiological analysis, the researchers reported that steam-vacuuming resulted in reductions of 1.6 to 3.0 log CFU/cm^2 of *Listeria innocua*, APC, lactic acid bacteria, *E. coli* O157:H7 and *Clostridium sporogenes* populations (Dorsa *et al.*, 1997). However, following the initial treatment, *L. innocua,* APC and lactic acid bacteria began to grow within 2 days of refrigerated storage and reached populations of at least 7.0 log CFU/cm^2 by 7 days of age (Dorsa *et al.*, 1997).

Studies evaluating the efficacy of steam/hot water vacuuming, applied singly or in combination with other decontamination treatments, have generally concluded that use of

the technology effectively reduces bacterial populations on fresh beef carcass surfaces. However, information is limited regarding the efficacy of this technology when applied to beef carcass surfaces following chilling. Decontamination of chilled carcass surfaces could further contribute to end-product microbiological quality by inactivating bacteria surviving the chilling process, or reducing contamination occurring during the shipment of carcasses to other facilities for subsequent fabrication. The objective of this study was to simulate post-dehiding, carcass surface contamination with *Salmonella* and to determine if steam-vacuuming, applied in a manner similar to the commercial use of this technology as a spot decontaminant, was an effective means of decontamination when applied following a period of chilling, thereby allowing an appropriate amount of time for bacterial attachment, penetration, and/or biofilm formation, as well as tissue "hardening".

MATERIALS AND METHODS

Beef carcass tissue. Samples (N = 70) of adipose tissue (300 to 400 cm^2) were excised from the brisket and rib regions of beef carcasses at a commercial slaughtering facility. Following sample collection, which occurred after the slaughtering/dressing process but immediately before carcass chilling, the excised tissue was placed into coolers and transported (approximately 50 km, in about 45 min) to Colorado State University. Tissue samples (N = 70) were placed onto trays and separated into four treatment groups: (1) uninoculated, without post-chilling steam vacuum application (n = 10); (2) uninoculated, with post-chilling steam vacuum application (n = 10); (3) inoculated, without post-chilling steam vacuum application (n = 10); and, (4) inoculated, with post-chilling steam vacuum application (n = 40). Treatment 3 was an inoculated,

untreated control, and was subsequently compared to treatment 4 for determination of the efficacy of steam-vacuuming in reducing inoculated *Salmonella* populations. The uninoculated controls, without post-chilling steam vacuum application (Treatment 1) and with post-chilling steam vacuum application (Treatment 2), were used to estimate background interference by determining commensal bacterial population levels and their ability to resist the antibacterial effects of streptomycin, as well as determining the efficacy of steam-vacuuming in reducing such populations on previously chilled beef adipose tissue.

Inoculum. A *Salmonella* spp. isolate, confirmed by biochemical testing (API 20E, bioMerieux, Hazelwood, MO), was used to inoculate samples in Treatments 3 (inoculated, without post-chilling steam vacuum application) and 4 (inoculated, with post-chilling steam vacuum application). The isolate was recovered from a beef cattle hide that was sampled after animal stunning and exsanguination, but before hide opening and subsequent hide removal processes, at a commercial slaughtering facility. The *Salmonella* spp. isolate demonstrated a natural ability to resist the antimicrobial effects of streptomycin at a concentration of >600 μg/ml, and was subsequently maintained on trypticase soy agar slants (Difco Laboratories, Sparks, MD) at 4°C for use during this study. The working stock culture was subcultured overnight (18 h), twice in trypticase soy broth containing streptomycin (600 μg/ml; Difco Laboratories) at 37°C, at which time stationary phase cells were diluted (approximately 7.2 x 10^6 CFU/ml) using sterile, 0.1% buffered peptone water (Difco Laboratories). Immediately before inoculation of the adipose tissue samples, a sterile, 100 cm^2 disposable template (USDA Template, International BioProducts, Redmond, WA) was placed onto the adipose tissue and the

outer perimeter was traced with a scalpel to make possible sampling of the enclosed, inoculated area following chilling. Adipose tissue samples were inoculated with 2 ml of inoculum (approximately 7.2 x 10^6 CFU/ml), resulting in the application of 1.44 x 10^7 cells, which were uniformly spread over the 100 cm^2 (1.44 x 10^5 CFU/cm^2 or 5.2 log CFU/cm^2) surface using a sterile glass rod. This admittedly high level of *Salmonella* was used in order to accurately quantify treatment differences following chilling. Inoculated and uninoculated samples were chilled for 24 ± 2 h at 2.6 ± 0.1°C to simulate approximate commercial slaughter plant initial chilling temperatures and times.

Steam Vacuum Application. Following chilling (24 ± 2 h; 2.6 ± 0.1°C), a two-drop (able to apply steam in conjunction with hot water), commercial steam-vacuuming system (BFD Corporation, Aurora, CO) was used to decontaminate the samples of Treatment 2 (uninoculated, with steam vacuum application) and Treatment 4 (inoculated, with steam vacuum application). Steam (1.72 bar; 130°C) was applied to the chilled adipose tissue in four vertical passes (approximately 1.5 to 2.0 sec per pass) using the hand-held application nozzle and with a manner of application that closely simulated use of the device in commercial practice. Resulting condensate was vacuumed (-0.24 bar) away and collected in a 33.1 liter receiving tank. Sterile gloves (International BioProducts) were used to handle each individual sample; following steam vacuum application, gloves were changed in order to reduce the opportunity for cross-contamination between samples.

Microbiological analysis. Sponging and excising sampling methods were used for enumeration of bacteria on inoculated and uninoculated tissue samples. Immediately before sampling, sterile sponges (BioPro Enviro-Sponge Bags, International BioProducts)

were hydrated with 10 ml of sterile, 0.1% buffered peptone water (BioPro, International BioProducts). Sponge sampling of each tissue sample occurred within a 100 cm^2 disposable, sterile template (USDA Template, International BioProducts) and consisted of 10 passes vertically (up-and-down being considered as 1 pass) and 10 passes horizontally (side-to-side being considered as 1 pass) with a pressure equivalent to that which would be used to remove dried blood, as described in the FSIS-USDA Meat and Poultry Inspection regulations (FSIS-USDA, 1996b). Swabbing with sponges was performed aseptically using sterile latex gloves (International BioProducts), which, in addition to the template, were changed between samples. Following sponging, an additional 15 ml of sterile, 0.1% buffered peptone water (Difco Laboratories) was added to the sponge bag, bringing the total volume of buffer to 25 ml.

Previously sponged tissue was then aseptically excised using a sterile scalpel blade and forceps. Excision of the tissue was performed using a sterile 100 cm^2 template (USDA Template, International BioProducts) which, in addition to the scalpel blade and forceps, were changed between samples. Excised tissue (10 cm x 10 cm x < 0.5 cm) was placed into a sample bag where it was weighed and diluted (10^{-1}) using sterile, 0.1% buffered peptone water (Difco Laboratories). Previously sponged tissue was excised and evaluated to determine the microbiological population remaining on the 100 cm^2 surface inasmuch as the time (24 ± 2 h) between inoculation and sampling, although occurring at refrigerated temperatures, may have allowed for increased attachment and tissue penetration of the microorganisms, thereby reducing bacterial enumeration accuracy of the sponge sampling method.

Following tissue sampling, sponge and excision samples were individually agitated (Masticator, IUL Instruments, Barcelona, Spain) for one minute and serial dilutions were made using sterile, 0.1% buffered peptone water (Difco Laboratories). For each dilution tested, an aliquot (0.1 ml) of diluent was dispensed and uniformly spread using a sterile glass rod onto plates of tryptic soy agar (TSA), xylose lysine tergitol 4 (XLT_4) agar, TSA containing streptomycin at a concentration of 600 µg/ml (TSA + streptomycin), and XLT_4 containing streptomycin at a concentration of 600 µg/ml (XLT_4 + streptomycin) (Difco Laboratories). Plates were inverted and incubated for 24 ± 2 h at 35°C, at which point they were removed from the incubator, and colonies were manually counted using a Quebec Darkfield Counter (AO Scientific Instruments, Buffalo, NY). Enumeration on TSA was used to determine the collective, aerobic bacterial population, regardless of streptomycin susceptibility. Conversely, XLT_4 agar, a selective medium, was used to enumerate all *Salmonella* regardless of streptomycin susceptibility. Enumeration on TSA + streptomycin was used to determine the extent of streptomycin resistance within the aerobic bacterial population, while XLT_4 + streptomycin was used to enumerate streptomycin-resistant *Salmonella* only.

Data Analysis. After enumerating the colonies associated with the sponge sampling method and initially reporting the data as CFU/ml of diluent plated, each count was multiplied by 25 (25 ml was the total volume of buffer used) and divided by 100 (100 cm^2 was the total surface area swabbed) to express counts as CFU/cm^2. Similarly, after enumerating the colonies associated with the excision sampling method and initially reporting the data as CFU/ml of sample analyzed, each count was multiplied by the total volume of buffer used (excised sample weight diluted 10^{-1}) and divided by 100 (100 cm^2

was the total surface area excised) to express counts as CFU/cm^2. In addition to individual analysis, bacterial populations (CFU/cm^2) derived from sponge and excision sampling data, for each sample, were combined (sum of sampling counts) to assay the total number of cells present before any sampling occurred. The sponge, excision and combination counts (CFU/cm^2) were transformed to $\log_{10}$ CFU/cm^2 for statistical analyses. Minimum detection limits of counts for sponge sampling, excision sampling, and the combination of sponge and excision sampling were 0.4, approximately 1.2, and approximately 1.3 log CFU/cm^2, respectively, based on maximum sensitivity with no further sample dilution beyond the original buffer volume for sponge swabs (25 ml) or excised tissue (excised sample weight diluted 10^{-1}). Sponge and excision counts falling below the minimum detection limit were recorded as 0.4 and 1.2 log CFU/cm^2, respectively, so that statistical analyses could be performed. Values for the mean plate count ($\bar{x}$) and standard deviation (s) of each enumerated set ($\log_{10}$ CFU/cm^2) were calculated on the assumption of a log-normal distribution of microorganisms (Brown and Baird-Parker, 1982; Gill *et al.*, 1996; Gill and Bryant, 1997; Kilsby and Pugh, 1981).

For each recovery media—TSA, XLT_4, TSA + streptomycin and XLT_4 + streptomycin—bacterial population data derived from both sampling methods were evaluated with repeated measures analysis of variance (AOV) using the model $y = a + x_1 + x_2 + x_1x_2$; least-squares means were computed for bacterial populations for the fixed effects of treatment (x_1; 1 = uninoculated, without post-chilling steam vacuum application; 2 = uninoculated, with post-chilling steam vacuum application; 3 = inoculated, without post-chilling steam vacuum application; and, 4 = inoculated, with post-chilling steam vacuum application), sampling method (x_2; sponge-swabbing and

tissue excision), and treatment x sampling method (x_1x_2), using the Mixed Models procedure of SAS® (SAS, 1999). In most cases, uninoculated treatments (1 and 2) resulted in bacterial populations near the detection limit; therefore, they were removed due to heterogeneity of variance when compared to the inoculated treatments (3 and 4). Due to significant treatment x sampling method interaction for each of the recovery media (TSA: $P = 0.0134$; XLT_4: $P = 0.0021$; TSA + streptomycin: $P = 0.0249$; XLT_4: $P = 0.0019$), only interaction subclass least-squares means are reported. When AOV detected effects ($P < 0.05$), least-squares means were separated using the pairwise t-test of SAS® (SAS, 1999). However, due to interaction and variance that depended on treatment, treatment comparisons were made using the pairwise t-test modified for unequal variance (Satterthwaite degrees of freedom).

RESULTS AND DISCUSSION

No differences ($P > 0.05$) between untreated and treated inoculated samples were observed in bacterial counts obtained by sponging following enumeration on TSA, XLT_4, TSA + streptomycin, or XLT_4 + streptomycin (Tables 4.1-4.4). However, enumeration of excised samples (untreated vs. treated inoculated tissues) reflected reductions ($P < 0.05$) in bacterial populations of 0.5, 0.5, 0.6 and 0.8 log CFU/cm^2 for TSA, XLT_4, TSA + streptomycin, and XLT_4 + streptomycin, respectively, following the application of steam-vacuuming (Tables 4.1-4.4).

There was minimal evidence of background growth and, subsequently, no interference on media containing streptomycin (TSA + streptomycin; XLT_4 + streptomycin), regardless of sampling method, as uninoculated samples produced

detectable bacterial populations close to the detection limits (Tables 4.3 and 4.4). In contrast, recovery on TSA and XLT_4 resulted in average counts of 4.7 and 3.3 log CFU/cm^2, respectively, for the uninoculated without post-chilling steam vacuum application treatment, and corresponding counts of 4.7 and 3.3 log CFU/cm^2, respectively, for the uninoculated with post-chilling steam vacuum treatment (Tables 4.1 and 4.2).

Sponge sampling results for the uninoculated treatments, following recovery on TSA and XLT_4, suggested slightly increased bacterial populations (only numerically) following steam vacuum application. While this trend was not seen in larger bacterial populations associated with the inoculated treatments, steam vacuum application may have facilitated bacterial cell deattachment and subsequent removal during sponge-swabbing. Nonetheless, there were no observed differences between uninoculated treatment means following tissue excision or when bacterial populations obtained using both sampling methods were combined (Tables 4.1-4.4).

Overall, sponge sampling was inferior to the excision sampling method in recovering and accurately enumerating bacterial populations following chilling. The excision sampling method detected differences in inoculated tissue treatments that were not reflected using the sponge sampling method. Furthermore, after combining bacterial population counts obtained using both the sponge and excision sampling methods, for each sample individually, the enumerated total population (mean log value of the summed data) closely mirrored values for plate counts obtained using the excision sampling method alone (Tables 4.1-4.4).

Results from this study confirmed previous research indicating that excision sampling is superior when compared to sponge sampling for the purpose of enumerating total bacterial populations, especially following prolonged exposure and/or tissue chilling (Anderson *et al.*, 1987; Fliss *et al.*, 1991; Gill *et al.*, 2001a; Snijders *et al.*, 1984; Ware *et al.*, 1999). It is possible that the 24 h period of time following inoculation may have allowed for firmer bacterial attachment, penetration, or biofilm formation (Cabedo *et al.*, 1996; Chung *et al.*, 1989; Firstenberg-Eden, 1981), thereby decreasing cell recovery during sampling and subsequently reducing the accuracy of the sponge sampling method. The combination counts, combining enumerations from both sampling methods, revealed that application of steam-vacuuming to post-chilled beef adipose tissue numerically reduced *Salmonella* populations by 0.5 and 0.7 log CFU/cm^2, when compared to the inoculated, untreated controls, leaving 3.6 and 3.2 log CFU/cm^2 of *Salmonella* on the treated beef-tissue surfaces (Tables 4.3 and 4.4). Obviously, residual levels of *Salmonella* of this magnitude on beef products after treatment would not be acceptable.

While previous researchers have demonstrated reductions in total coliform counts of 1.67 log CFU/cm^2 (Kochevar *et al.*, 1997) and of 4.0 log CFU/cm^2 (Dorsa *et al.*, 1996b) on inoculated beef tissue resulting from use of steam vacuum units, in both of those studies, steam-vacuuming was performed on hot (non-chilled) beef tissue leaving bacteria with less time (approximately 15 min) to firmly attach to, and/or penetrate into, the tissue. Results of the present study suggest that, as it was applied in the present study, steam-vacuuming of chilled beef tissue may reduce *Salmonella* populations, but—used singularly—will not remove or destroy enough *Salmonella* (if the population approximates 5.0 log CFU/cm^2) to provide an end-product of suitable microbiological

quality. We believe that steam-vacuuming, performed in rapid up-and-down or side-to-side movements, is analogous to passing a human hand very quickly through an open flame—no damage is done to either bacteria or human flesh if contact time is insufficient to cause protein coagulation/denaturation.

Steam-vacuuming was designed as a spot decontamination treatment and results following whole carcass application may prove to be inconsistent. As has proven to be the case in research designed to decontaminate beef carcasses in association with the harvesting process (slaughtering/dressing), greater reductions in microbial populations are achieved when decontamination treatments are used in combination than when they are used separately and individually (Dorsa *et al.*, 1996b; Dorsa *et al.*, 1997; Graves Delmore *et al.*, 1998). Especially if the microorganisms have been given time and opportunity to attach, to penetrate or to form biofilms on beef-tissue surfaces, application of steam vacuuming alone (especially if vacuuming is performed without allowing adequate contact time between heat and pathogen) will not achieve inactivation of *Salmonella*. Sequential applications of several decontamination processes (e.g., organic acid rinsing, hot-water washing, steam-vacuuming, etc.) may prove more successful in reducing *Salmonella* on the surfaces of beef tissue, especially if application of steam and/or hot water, in spot decontamination, is done carefully and slowly to achieve maximum contact between heat and bacterial cells.

Table 4.1. Least squares means ($\bar{x}$) and standard deviation (s) of the $\log_{10}$ values for plate counts (CFU/cm^2), and the number of samples (n^+) in which bacterial populations were too numerous to count (TNTC[a]) following recovery on tryptic soy agar (TSA).

		Method of Sampling							
		Sponging[b]			Excision[b]			Sponging + Excision	
Treatment	n[c]	$\bar{x}$	s	n^+	$\bar{x}$	s	n^+	$\bar{x}$	s
Uninoculated	10	3.4	0.38	1	4.7	0.06	10	4.7	0.05
Uninoculated + Steam Vacuum	10	3.8	0.07	8	4.6	0.04	8	4.7	0.03
Inoculated	10	4.1^z	0.95	0	5.4^y	0.37	0	5.5	0.41
Inoculated + Steam Vacuum	40	4.1^z	0.64	0	4.9^z	0.45	0	5.0	0.46

[a] Uninoculated sponge samples were TNTC above 3.8 log CFU/cm^2, while inoculated sponge samples were TNTC above 5.8 log CFU/cm^2; uninoculated excision samples were TNTC above approximately 4.7 log CFU/cm^2, while inoculated excision samples were TNTC above approximately 5.7 log CFU/cm^2.

[b] Based on repeated measures ANOVA, excision resulted in greater ($P < 0.05$) bacterial enumeration than the sponge sampling method.

[c] Number of samples (n) analyzed for each treatment.

[yz] Means in the same column with different superscript letters are different ($P < 0.05$).

Table 4.2. Least squares means ($\bar{x}$) and standard deviation (s) of the $\log_{10}$ values for plate counts (CFU/cm^2), and the number of samples (n^-) in which bacterial populations were not detected (ND[a]), following recovery on xylose lysine tergitol 4 (XLT_4) agar.

		Method of Sampling							
		Sponging[b]			Excision[b]			Sponging + Excision	
Treatment	n[c]	$\bar{x}$	s	n^-	$\bar{x}$	s	n^-	$\bar{x}$	s
Uninoculated	10	0.9	0.61	2	3.3	0.76	0	3.3	0.75
Uninoculated + Steam Vacuum	10	1.4	0.61	0	3.3	0.65	0	3.3	0.65
Inoculated	10	2.6[z]	0.63	0	4.1[y]	0.52	0	4.2	0.52
Inoculated + Steam Vacuum	40	2.4[z]	0.51	0	3.6[z]	0.62	0	3.6	0.59

[a] Counts were not detected (ND) at detection limits of 0.4 and approximately 1.2 log CFU/cm^2 for sponging and excision sampling methods, respectively.

[b] Based on repeated measures ANOVA, excision resulted in greater ($P < 0.05$) bacterial enumeration than the sponge sampling method.

[c] Number of samples (n) analyzed for each treatment.

[yz] Means in the same column with different superscript letters are different ($P < 0.05$).

Table 4.3. Least squares means ($\bar{x}$) and standard deviation (s) of the $\log_{10}$ values for *Salmonella* counts (CFU/cm^2), and the number of samples (n$^-$) in which *Salmonella* counts were not detected (ND[a]), following recovery on tryptic soy agar containing streptomycin at a concentration of 600 μg/ml (TSA + Streptomycin).

		Method of Sampling							
		Sponging[b]			Excision[b]			Sponging + Excision	
Treatment	n[c]	$\bar{x}$	s	n$^-$	$\bar{x}$	s	n$^-$	$\bar{x}$	s
Uninoculated	10	0.5	0.19	2	1.4	0.17	4	1.4	0.15
Uninoculated + Steam Vacuum	10	0.6	0.30	3	1.3	0.14	5	1.4	0.15
Inoculated	10	3.0^z	0.57	0	4.1^y	0.26	0	4.1	0.27
Inoculated + Steam Vacuum	40	2.7^z	0.33	0	3.5^z	0.45	0	3.6	0.39

[a] Counts were not detected (ND) at detection limits of 0.4 and approximately 1.2 log CFU/cm^2 for sponging and excision sampling methods, respectively.

[b] Based on repeated measures ANOVA, excision resulted in greater ($P < 0.05$) bacterial enumeration than the sponge sampling method.

[c] Number of samples (n) analyzed for each treatment.

[yz] Means in the same column with different superscript letters are different ($P < 0.05$).

Table 4.4. Least squares means ($\bar{x}$) and standard deviation (s) of the $\log_{10}$ values for *Salmonella* counts (CFU/cm^2), and the number of samples (n^-) in which *Salmonella* counts were not detected (ND[a]), following recovery on xylose lysine tergitol 4 agar containing streptomycin at a concentration of 600 μg/ml (XLT_4 + Streptomycin).

		Method of Sampling							
		Sponging[b]			Excision[b]			Sponging + Excision	
Treatment	n[c]	$\bar{x}$	s	n^-	$\bar{x}$	s	n^-	$\bar{x}$	s
Uninoculated	10	0.4	0.00	10	1.2	0.06	10	1.3	0.05
Uninoculated + Steam Vacuum	10	0.4	0.00	10	1.2	0.05	9	1.3	0.05
Inoculated	10	2.6[z]	0.66	0	3.9[y]	0.33	0	3.9	0.34
Inoculated + Steam Vacuum	40	2.3[z]	0.40	0	3.1[z]	0.46	0	3.2	0.42

[a] Counts were not detected (ND) at detection limits of 0.4 and approximately 1.2 log CFU/cm^2 for sponging and excision sampling methods, respectively.

[b] Based on repeated measures ANOVA, excision resulted in greater ($P < 0.05$) bacterial enumeration than the sponge sampling method.

[c] Number of samples (n) analyzed for each treatment.

[yz] Means in the same column with different superscript letters are different ($P < 0.05$).

CHAPTER V

COMMERCIAL APPLICATION OF A LACTIC ACID SOLUTION TO REDUCE BACTERIAL POPULATIONS ON CHILLED BEEF CARCASSES, SUBPRIMAL CUTS AND TABLE SURFACES DURING FABRICATION

ABSTRACT

Efficacy of a lactic acid solution, applied at three in-plant locations, singly or in combination, in reducing or controlling bacterial populations on beef carcasses and subprimal cuts, and on table surfaces, during carcass fabrication was evaluated. Total plate counts (TPC), total coliform counts (TCC), and *Escherichia coli* counts (ECC) on carcass surfaces immediately before entering fabrication were low, as 9.0, 89.5, and 93.8% of samples had TPC, TCC, and ECC, respectively, below detection limits of 2.2, 0.9, and 0.9 log CFU/100 cm^2. Lactic acid solution rinsing (1.5 to 2.5%; 29.5°C; 182 kPa; 3 sec) of carcasses, immediately before entering the fabrication process, minimally reduced ($P < 0.05$) mean TPC from 3.3 log CFU/100 cm^2 to 3.0 log CFU/100 cm^2, and increased the percentage of non-detectable TCC and ECC from 89.5 and 93.8%, to 92.4 and 94.3%, respectively. Lactic acid solution rinsing of fabrication table surfaces, applied singly or in combination with lactic acid solution rinsing of carcasses, reduced ($P < 0.05$) TPC, TCC and ECC of 5.5, 3.2 and 2.8 log CFU/100 cm^2 by averages of 1.2, 0.8 and 0.7 log CFU/100 cm^2, respectively. Lactic acid solution rinsing of top sirloin butts, when applied alone or in combination with lactic acid solution rinsing of carcasses and fabrication tables, reduced ($P < 0.05$) TPC, TCC and ECC of 5.7 and 5.1, 3.8 and 3.5, and

3.3 and 3.2 log CFU/100 cm^2, respectively, by less than 0.5 log CFU/100 cm^2. Bacterial populations on the surface of carcasses entering the fabrication process remained constant over time, as mean TPC, TCC and ECC were 3.4, 0.9 and 0.9 log CFU/cm^2, respectively, at the start of fabrication (0 h), while corresponding populations after 3 h were 3.5, 1.0 and 0.9 log CFU/cm^2. Conversely, mean TPC, TCC and ECC on surfaces of fabrication tables and top sirloin butts increased by averages of 1.4, 1.1 and 1.0 log CFU/100 cm^2, respectively, to 3.8 and 4.5, 1.9 and 2.6, and 1.8 and 2.2 log CFU/100 cm^2. Overall, the lactic acid solution applications evaluated during this study resulted in minimal ($\leq$ 1 log CFU/100 cm^2) reductions in microbiological populations on the surfaces of carcasses, subprimals and fabrication tables.

INTRODUCTION

As much as one-fourth of the world's food supply is compromised due to deleterious activities of microorganisms, and 72 percent of deaths in the United States, resulting from known foodborne etiology, are attributed to bacteria (Huis in't Veld, 1996; Mead *et al.*, 1999). In response to public health issues and concerns, the Food Safety and Inspection Service of the United States Department of Agriculture (FSIS-USDA) mandated industry-wide implementation of HACCP principles and associated microbiological performance criteria, in an attempt to emphasize the importance of controlling microbiological populations on beef carcass surfaces (FSIS-USDA, 1996b). In response, the beef industry improved existing, and adopted new, strategies and/or technologies that, when employed during the process of converting animals to muscle-foods, subsequently improved overall microbiological carcass quality. Technologies

adopted industry-wide include hot water/steam-vacuuming, steam pasteurization, and ambient temperature or hot water spray washing with or without organic acid solution rinsing (Dickson and Anderson, 1992; Dorsa, 1997; Hardin *et al.*, 1995; Phebus *et al.*, 1997; Smulders and Greer, 1998; Sofos and Smith, 1998).

Detrimental effects of spray washing/rinsing treatments may include: (a) water film formation and increased surface moisture resulting in increased establishment and proliferation of microbes; (b) entrapment or embedding of bacteria, due to excessive application pressure, thereby providing protection against subsequent stress (e.g., other decontamination technologies); (c) reduced competitive inhibition resulting from reductions in commensal microbial populations; (d) emergence of resistance following prolonged and widespread antimicrobial application; and, (e) redistribution or spreading of a localized microbial population over a larger area (Cabedo *et al.*, 1996). However, the majority of research findings supports spray washing/rinsing as an effective means of eliminating, reducing, or controlling the proliferation of pathogenic and nonpathogenic bacterial populations (Cabedo *et al.*, 1996; De Zuniga *et al.*, 1991; Dickson and Anderson, 1992; Dorsa, 1997; Gorman *et al.*, 1995; Hardin *et al.*, 1995; Jay, 2000; Prasai *et al.*, 1991).

The U.S. commercial beef packing industry has expended enormous time and resources, and has succeeded in decreasing microbiological contamination on carcass surfaces. Bacon *et al.* (2000) sampled chilled (18 to 24 h) beef carcasses in five fed cattle and three market cow and bull packing plants and reported that 19.4% of total plate counts (TPC; 62 of 319 samples), 86.2% of total coliform counts (TCC; 275 of 319 samples) and 98.4% of *Escherichia coli* counts (ECC; 315 of 320 samples) were not

detected at detection limits of 2.2, 0.9 and 0.9 log colony-forming units (CFU)/100 cm^2, respectively. Acceptable carcass hygiene results, in part, from strict adherence to good manufacturing practices (GMPs) and effective microbiological intervention (through the applications of multiple-sequential decontamination treatments during the exsanguination/dressing process and subsequent carcass chilling). Traditionally, sustained refrigeration temperatures following the exsanguination/dressing process (i.e., carcass chilling through fabricating, packaging, distributing and retailing) have been considered sufficient to control or reduce proliferation of, and/or toxin production by, foodborne pathogens (Palumbo, 1986). However, with the emergence of psychrotrophic pathogens (e.g., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium botulinum* type E and *Aeromonas hydrophilia*) that grow competitively, refrigeration temperatures alone cannot be deemed sufficient to keep foods safe (ICMSF, 1996; Palumbo, 1986). Furthermore, mesophilic pathogens (e.g., specific *Bacillus cereus* strains, *E. coli*, *Salmonella* and *Staphylococcus aureus*) capable of surviving exposure to low temperatures may proliferate readily in conditions approximating mild temperature abuse (>5 to 10°C; ICMSF, 1996; Palumbo, 1986). While not all of these pathogens are typically considered meatborne, they all have been implicated as etiologic agents in outbreaks involving foods of animal origin, including raw meats (ICMSF, 1996).

Psychrotrophic and surviving mesophilic bacterial populations on carcass surfaces can proliferate during mild to moderate temperature abuse, and primal cuts and trimmings can be further contaminated during fabrication. Employees and improperly sanitized contact surfaces, such as belts, tables, saw blades and cutting boards, serve as potential sources of contamination. Cross-contamination occurring directly, between

carcasses, primal cuts and trimmings, or indirectly, from repeated contact with employees (e.g., hands, knives and meat hooks) or contact surfaces, is probable (Upmann *et al.*, 2000). Gill *et al.* (1999) sampled product immediately before entering and after leaving the fabrication process in four commercial beef packing facilities. Results indicated increased ECC on final fabricated product surfaces, which, following closer inspection of equipment, were attributed to obscurely located detritus associated with large populations of aerobic bacteria, including *E. coli*. When bacteria-harboring equipment was running, even before product passage, bacteria were transferred to meat contact surfaces, ultimately resulting in product contamination (Gill *et al.*, 1999).

In another study (Gill *et al.*, 2001b), surfaces of beef carcasses, primal cuts and tables were swabbed immediately before, during and following carcass fabrication, respectively. TCC and ECC recovered from carcass surfaces were 4.0 and 3.5 log CFU/500 sides, respectively, while corresponding populations on primal cuts were >6.0 and 5.5 log CFU/500 cuts. Because TCC and ECC on primal cut surfaces were higher than those recovered from carcasses (i.e., by approximately 2.0 logs), and were comparable to those recovered from fabrication tables, contamination of primal cuts from table surfaces was implicated (Gill *et al.*, 2001b).

Bacterial populations on the surfaces of tables and primal cuts may be controlled by employing interventions immediately before, during and/or immediately following (before vacuum packaging) beef carcass fabrication. The objective of this study was to determine the efficacy of lactic acid solution applications in reducing or controlling bacterial populations on beef carcasses and subprimal cuts, and on table surfaces, during carcass fabrication.

MATERIALS AND METHODS

Sample collection. Samples (N = 805) were collected during a period of six weeks (A through F) from the surfaces of beef carcasses, fabrication tables and top sirloin butts before, during and immediately following the carcass fabrication process in a commercial fed-beef packing plant. Decontamination treatments evaluated included: (a) post-chilling lactic acid solution (1.5 to 2.5%; 29.5°C; 182 kPa; 3 sec) carcass rinsing, using a commercial spray-wash cabinet located immediately before the chilled carcass scale; (b) lactic acid solution (1.5 to 2.5%; 29.5°C) table rinsing, constantly applied as a fine mist by means of a spray bar mounted to the proximal end of the loin fabrication table; and, (c) lactic acid solution (1.5 to 2.5%; 29.5°C) subprimal rinsing, applied manually in two passes using a compressed air sprayer (8 L; Spray Doc Model 2000P, Gilmour, Somerset, PA) to top sirloin butts removed from the distal end of the loin fabrication table.

Sampling occurred before, during and immediately following the fabrication process at five different in-plant sampling sites, including: (1) carcass (site 1), immediately after entering the fabrication area but before the lactic acid solution carcass rinsing cabinet; (2) carcass (site 2), immediately after the lactic acid solution carcass rinsing cabinet but before the chilled carcass scale; (3) fabrication table (site 3), at the distal end of the loin fabrication line; (4) top sirloin butt (site 4), immediately after being removed from the distal end of the loin fabrication table but before lactic acid solution subprimal rinsing; and, (5) top sirloin butt (site 5), following lactic acid solution subprimal rinsing but before vacuum packaging.

During each week (A through F), at each in-plant sampling site required by the assigned treatment, samples (n = 7) were collected each day (Monday through Friday) every half-hour, for three hours, beginning at the start of the first shift. The sampling protocols for weeks A through F are detailed in Table 5.1. Week A included the collection of samples (n = 35) at each of three in-plant sampling sites with no decontamination treatments applied, in order to develop a complete baseline-data subset of microbiological populations on carcass, fabrication table and top sirloin butt surfaces. Week B included the collection of samples (n = 35) at each of four in-plant sampling sites with the application of lactic acid solution carcass rinsing, in order to determine the efficacy of this decontamination treatment in reducing or controlling bacterial populations on carcasses, and subsequently on fabrication table and top sirloin butt surfaces. Week C included the collection of samples (n = 35) at each of three in-plant sampling sites with the application of lactic acid solution table rinsing, in order to determine the contribution of this decontamination treatment to the microbiological quality of table surfaces and subsequently removed top sirloin butts. Week D included the collection of samples (n = 35) at each of four in-plant sampling sites with lactic acid solution subprimal rinsing, in order to determine the efficacy of this decontamination treatment in reducing or controlling bacterial populations on top sirloin butt surfaces. Week E included the collection of samples (n = 35) at each of four in-plant sampling sites with lactic acid solution rinsing of carcasses and tables, in order to determine the collective contribution of the decontamination treatments to the microbiological quality of table surfaces and subsequently removed top sirloin butts. Week F included the collection of samples (n = 35) at each of five in-plant sampling sites with lactic acid

solution rinsing of carcasses, tables, and subprimals, in order to determine the collective efficacy of all decontamination treatments in reducing or controlling bacterial populations on top sirloin butts (Table 5.1).

Sponge sampling was performed on-line, following procedures described in the FSIS-USDA Meat and Poultry Inspection regulations (FSIS-USDA, 1996b). Immediately before sampling, sterile sponges (BioPro Enviro-Sponge Bags, International BioProducts, Redmond, WA) were hydrated with 10 ml of sterile 0.1% buffered peptone water (BioPro, International BioProducts). Sampling of carcass sides (sites 1 and 2) was performed using a 100 cm^2 disposable, sterile template (USDA Template, International BioProducts) at each of three anatomical locations, which were: (a) flank, where the cutaneous flank muscle comes to within 7.62 cm of the midline; (b) brisket, at a point on the midline level with the elbow; and, (c) rump, where a line from the posterior aspect of the aitch bone to the achilles tendon intersects the cut surface of the round (FSIS-USDA, 1996b). Sampling of table surfaces (site 3) and subprimal cuts (sites 4 and 5) was performed using a 100 cm^2 disposable, sterile template (USDA Template, International BioProducts). Table surfaces were sampled (100 cm^2) at the distal end of the loin fabrication line, while subprimal cuts (top sirloin butts) were sampled (100 cm^2) immediately after being removed from the loin fabrication line, on the surface associated with external adipose tissue.

Sponge-swabbing at each carcass, table, or subprimal cut sampling site, within the 100 cm^2 template area, consisted of 10 passes vertically (up-and-down being considered as 1 pass) and 10 passes horizontally (side-to-side being considered as 1 pass) with a pressure equivalent to that which would be used to remove dried blood (FSIS-USDA,

1996b). Sampling was performed aseptically using sterile, latex gloves (International BioProducts), which, in addition to the template, were changed between samples. Following sample collection, an additional 15 ml of refrigerated (4 to 5°C), sterile 0.1% buffered peptone water (Difco Laboratories, Sparks, MD) was added, increasing the total buffer volume to 25 ml. When daily sample collection was finished (3 h after the commencement of carcass fabrication), excess air was expelled and sponge bags were folded down and packed into shipping coolers for same day delivery to the analytical laboratory (Warren Analytical Laboratory, Greeley, CO).

Microbiological analysis. Sponges and associated buffer were pummeled in a stomacher (Seward Model 400, Tekmar Company, Cincinnati, OH) for 1 min, and analyzed for TPC, TCC and ECC. To determine TPC, appropriate dilutions were plated on Plate Count Agar (PCA; Difco Laboratories) using a spiral plating system (Spiral Systems Instruments, Inc., Cincinnati, OH). Following aerobic incubation at 35 ± 2°C for 48 h, colonies were counted using a laser bacteria colony counter (Model 500A, Spiral Systems Instruments, Inc.) and a computer assisted spiral bio-assay (CASBA) data processor with Bacterial Enumeration Program E20 (Spiral Systems Instruments, Inc.).

To determine TCC and ECC, appropriate dilutions were transferred (1 ml) to Petrifilm™ *E. coli* Count Plates (3M Health Care, St. Paul, MN) and, following a 24 ± 2 h incubation period (Gangar and Curiale, 1999) at 35 ± 2°C, colonies were manually counted using a Quebec Darkfield Counter (AO Scientific Instruments). Colonies closely associated with entrapped gas (approximately one colony diameter), and possessing a bright red or blue to red-blue color, were counted as coliforms (TCC), while the blue to

red-blue colonies closely associated with trapped gas were counted as *E. coli* biotype I (ECC).

Data analysis. Microbiological counts for each enumerated set (TPC, TCC and ECC) were transformed to $\log_{10}$ CFU/100 cm^2 for statistical analyses. Minimum detection limits for TPC, TCC and ECC were 2.2, 0.9 and 0.9 log CFU/100 cm^2 (20, 1 and 1 CFU/ml of diluent), respectively, for carcass samples, and 2.7, 1.4, and 1.4 log CFU/100 cm^2 (20, 1 and 1 CFU/ml of diluent), respectively, for fabrication table and subprimal samples, based on maximum sensitivity of the tests with no further dilution beyond the original buffer volume (25 ml). Undetectable TPC, TCC and ECC were recorded as 2.2, 0.9, and 0.9 log CFU/100 cm^2, respectively, for carcass samples, and 2.7, 1.4, and 1.4 CFU/100 cm^2, respectively, for fabrication table and subprimal samples, so that statistical analysis could be performed. Values for the mean log count and standard deviation of each enumerated set (log CFU/100 cm^2 for TPC, TCC and ECC) were calculated on the assumption of a log-normal distribution of microorganisms (Brown and Baird-Parker, 1982; Kilsby and Pugh, 1981). Data for each enumerated set were evaluated with analysis of variance (AOV) using the model: $y = a + x_1 + x_2 + x_1x_2$. Least-squares means were computed for TPC, TCC and ECC by week (x_1), in-plant sampling site (x_2), and week x in-plant sampling site (x_1x_2) effects using the General Linear Models procedure of SAS® (SAS, 1995). Due to the significant week x in-plant sampling site interaction ($P = 0.0001$), only interaction subclass least squares means are reported (Tables 5.1-5.4). When AOV detected effects ($P \leq 0.05$), mean log values for bacterial populations ($\bar{x}$) on carcass and table surfaces (Tables 5.1 and 5.3) were separated using the pairwise t-test of SAS® (SAS, 1995). Lactic acid solution rinsing of

carcasses (weeks B, E and F) and subprimals (week D) were evaluated by determining mean log value differences (mean log value before treatment application minus mean log value after treatment application) for each enumerated set. Treatment effects (lactic acid solution rinsing of carcasses and subprimals) based on bacterial population differences ($\Delta \overline{x}$; Tables 5.2 and 5.4) were determined using a two-tailed t-test (SAS, 1995).

RESULTS AND DISCUSSION

Mean log TPC, TCC, and ECC for samples from carcass surfaces, collected immediately after entering the fabrication area (in-plant sampling site 1), ranged from 2.9 to 4.0, $\leq$0.9 to 1.0, and $\leq$0.9 log CFU/100 cm^2, respectively, among the six sampling weeks. Of the total carcass samples collected (n = 210) across all sampling weeks, 19 (9.0%), 188 (89.5%) and 197 (93.8%) yielded non-detectable TPC, TCC and ECC, respectively (Table 5.2).

Mean log TPC, TCC, and ECC on carcass surfaces immediately before lactic acid solution carcass rinsing (weeks B, E and F) were 3.3, 1.0 and 0.9 log CFU/100 cm^2, respectively. Lactic acid solution carcass rinsing reduced ($P < 0.05$) TPC, increasing the number of samples below the detection limit from 12.4% (13 of 105) to 21.9% (23 of 105) (Table 5.3). TCC and ECC on carcass surfaces were reduced, but not statistically ($P > 0.05$), following treatment application; however, efficacy of treatments in reducing these microbiological populations may have been masked by extremely high numbers of samples failing to yield detectable TCC or ECC. Nonetheless, the percentage of non-detectable samples increased from 87.6% (92 of 105) and 92.4% (97 of 105) to 92.4% (97 of 105) and 94.3% (99 of 105) for TCC and ECC, respectively (Table 5.3).

Mean log TPC, TCC, and ECC on fabrication table surfaces, without any decontamination treatment, were 5.5 and 5.5, 2.8 and 3.6, and 2.4 and 3.2 log CFU/100 cm^2 during weeks A and D, respectively (Table 5.4). Microbiological populations (TPC, TCC, and ECC) on fabrication table surfaces, following lactic acid solution rinsing of carcasses only (week B), were similar (mean values of 5.2 for TPC, 3.7 for TCC, and 3.5 for ECC) to populations recovered without treatment application (weeks A and D). Lactic acid solution rinsing of fabrication tables (week C) resulted in mean TPC, TCC, and ECC of 4.6, 2.7, and 2.1 log CFU/100 cm^2, respectively, which were lower ($P < 0.05$) than populations (TPC, TCC and ECC) during week D and lower ($P < 0.05$) than TPC and ECC during week A. The combination of lactic acid solution rinsing of carcasses and fabrication table surfaces resulted in mean TPC, TCC, and ECC of 4.1 and 4.2, 2.1 and 2.5, and 1.8 and 2.3 log CFU/100 cm^2 during weeks E and F, respectively (Table 5.4). TPC, TCC, and ECC during week E were lower ($P < 0.05$) than those during weeks A and D. Populations (TPC, TCC, and ECC) during week F were lower ($P < 0.05$) than those during week D, but only TPC were lower ($P < 0.05$) when compared to populations during week A (Table 5.4).

Application of lactic acid solution rinsing to subprimals (week D), resulted in minimal reductions ($P < 0.05$; < 0.5 log CFU/100 cm^2) in TPC, TCC and ECC on top sirloin butts from initial values of 5.7, 3.8 and 3.3 log CFU/100 cm^2, respectively (Table 5.5). Lactic acid solution rinsing of subprimals, applied in combination (as multiple decontamination treatments) with lactic acid solution rinsing of carcasses and fabrication tables (week F), reduced ($P < 0.05$) ECC, but only by 0.3 logs, and had no effect ($P > 0.05$) on mean log TPC and TCC (Table 5.5). Application of multiple decontamination

treatments "upstream" in the fabrication process (i.e., lactic acid solution rinsing of carcasses and fabrication tables) may have masked efficacy of "downstream" treatments (i.e., lactic acid solution subprimal rinsing), due to previous reductions in microbiological populations.

Microbiological populations on carcass surfaces entering the fabrication process did not ($P > 0.05$) depend on sampling time, as mean TPC, TCC, and ECC at the first sampling time (0 min) were 3.4, 0.9, and 0.9 log CFU/100 cm^2, respectively, while corresponding counts after three hours of sampling were 3.5, 1.0, and 0.9 log CFU/100 cm^2, respectively (Table 5.6). In contrast, microbiological populations on surfaces of fabrication tables and top sirloin butts changed over time, as initial (0 min) TPC, TCC, and ECC on fabrication table surfaces were 3.8, 1.9, and 1.8 log CFU/100 cm^2, while corresponding counts after three hours of sampling were 5.2, 3.1, and 2.7 log CFU/100 cm^2, respectively. Initial (0 min) TPC, TCC, and ECC on top sirloin butt surfaces were 4.5, 2.6, and 2.2 log CFU/100 cm^2, respectively, while corresponding counts after three hours were 5.8, 3.6, and 3.2 log CFU/100 cm^2, respectively (Table 5.6). Increased microbiological populations associated with fabrication tables and top sirloin butts, over the 3 h sampling period, suggested bacterial proliferation and/or introduction of additional contamination (e.g., product cross-contamination or environmental contamination).

Application of organic acid solutions to beef carcass surfaces during the exsanguinations/dressing process, as a means of improving microbiological quality, was examined extensively (Cabedo *et al.*, 1996; Dickson and Anderson, 1992; Dorsa, 1997; Gorman *et al.*, 1995; Hardin *et al.*, 1995; Prasai *et al.*, 1991). In general, the majority of

research evidence supports spray washing/rinsing as an effective means of eliminating, reducing, or controlling the proliferation of, pathogenic and nonpathogenic bacterial populations on hot beef carcass surfaces. In contrast, efficacy of organic acids in reducing bacterial populations is reduced significantly when applied to chilled tissue surfaces (Acuff *et al.*, 1987; Brackett *et al.*, 1994; Conner *et al.*, 1990; Dickson and Anderson, 1992; Dixon *et al.*, 1987, 1991). This may be due, in part, to the lower temperatures associated with post-chilling carcass fabrication, since organic acid application temperature influences the extent to which bacterial populations are reduced (Anderson *et al.*, 1988; Anderson and Marshall, 1989, 1990; Dickson and Anderson, 1992), and application to chilled tissue may result in an instantaneous reduction in rinsing solution temperature. Samelis *et al.* (2001a) reported on the ability of *E. coli* O157:H7, *Salmonella* Typhimurium DT104 and *L. monocytogenes* to survive or grow in spray-washing fluids, including lactic acid (2%; 55°C), collected following application to fresh beef top rounds and concluded that pathogen survival in acidic spray-washings was better at 4°C than at 10°C (Samelis *et al.*, 2001a).

Castillo *et al.* (2001a) evaluated the decontamination efficacy of a lactic acid solution applied to chilled, outside beef rounds inoculated with *E. coli* O157:H7 and *S.* Typhimurium (7.0 to 7.3 log CFU/cm^2). Before chilling, inoculated samples were exposed to a water wash (1.5 L hand wash applied for 90 s; 5.0 L cabinet wash applied for 9 s at 35°C), resulting in 3.3 to 3.4 log CFU/cm^2 reductions in inoculated populations, or a water wash followed by a pre-chilling lactic acid solution rinse (250 ml; 2% l-lactic acid; 15 s; 55°C), resulting in 5.2 log CFU/cm^2 reductions in inoculated populations. Samples were chilled for 24 h at 4°C, at which time the post-chilling lactic acid solution

treatment (500 ml; 4% l-lactic acid; 30 s; 55°C) was applied. Post-chilling lactic acid solution application reduced existing inoculated bacterial populations by an additional 1.9 to 2.4 and 1.6 to 2.0 log cycles for the pre-chill water wash and water wash + lactic acid solution rinse treatment groups, respectively. The researchers concluded that significant reductions in bacterial populations could be achieved with the application of a 4% lactic acid solution (500 ml; 55°C) for 30 s to chilled beef carcass sides (Castillo *et al.*, 2001a). In a follow-up study (Castillo *et al.*, 2001b), researchers evaluated the antimicrobial efficacy of a lactic acid solution rinse (4% l-lactic acid; 55°C; 35 s) when applied to chilled beef carcasses (n = 40) in a commercial packing facility. The decontamination solution was applied using a handheld compressed-air sprayer, and a 50 to 60 min dwell time was allowed before subsequent sponge sampling of carcass surfaces (clod, brisket and neck) occurred. The researchers reported 3.0 to 3.3 log CFU/100 cm^2 reductions in aerobic plate counts (APC), and increased numbers ($P < 0.05$) of undetectable TCC and ECC (Castillo *et al.*, 2001b). Factors contributing to the reportedly greater reductions in populations may have included higher application concentrations (i.e., 4% as compared to 0.5 to 2.5%) and increased exposure time (i.e., 35 s).

For a given organic acid solution, the extent to which bacterial populations are reduced following application may differ between fresh (hot) and chilled beef tissue surfaces. Typically, bacterial contamination on fresh or hot beef carcass tissue has been recently transferred, while that on chilled beef carcass tissue has had time (24 to 72 h) to attach, penetrate and/or form biofilms. Bacterial attachment to surfaces (e.g., meat) occurs in two consecutive, but distinct stages. Initial attachment results from the interaction of reversible forces associated with van der Waals' attractive or repulsive

energies (Marshall *et al.*, 1971). More importantly, the second, time-dependent phase involves irreversible attachment through exopolymer (polysaccharide) formation. There is a linear relationship between the concentration of bacteria attached to a surface, and the time allowed for the attachment process to occur (Cabedo *et al.*, 1996; Firstenberg-Eden, 1981). It is this second phase involving glycocalyx development, and subsequent biofilm formation of a microcolony, which provides additional protection against environmental stresses, promoting bacterial survival (Schwach and Zottola, 1982). Cabedo *et al.* (1996) reported that, as the time of beef carcass tissue exposure to fecal contamination increases, the number of bacteria removed during subsequent spray washing/rinsing treatments decreases.

Application of lactic acid solutions in this study resulted in minimal ($\leq$ 1 log CFU/100 cm^2) reductions in microbiological populations on the surfaces of carcasses, subprimals and fabrication tables. The lower efficacy of lactic acid in decreasing contamination of cold, compared to hot, carcasses reported in the literature, may be due to more complete bacterial attachment, lower carcass temperatures occurring after chilling, and lower bacterial populations. While none of the decontamination strategies evaluated during this study completely prevented or significantly reduced ($>$ 1 log CFU/100 cm^2) contamination on fabrication table and subprimal surfaces, there was evidence, albeit minimal, of additional reductions through multiple applications of a decontamination treatment. Information from this study regarding methods to lower or control proliferation of bacterial populations on beef carcass and subprimal surfaces, and on fabrication tables, suggests reduced antimicrobial efficacy of currently used

decontamination treatments, or at least of lactic acid solution rinsing, when applied during beef carcass fabrication.

Table 5.1. Decontamination treatments applied and number of samples collected during each week, by in-plant sampling site before, during and immediately following beef carcass fabrication.

		In-Plant Sampling Sites[a]				
		Carcass		Fabrication Table	Top Sirloin Butt	
Week	Decontamination Treatments Applied[b]	Site 1	Site 2	Site 3	Site 4	Site 5
A	None	35	---	35	35	---
B	Carcass Rinsing	35	35	35	35	---
C	Table Rinsing	35	---	35	35	---
D	Subprimal Rinsing	35	---	35	35	35
E	Carcass + Table Rinsing	35	35	35	35	---
F	Carcass + Table + Subprimal Rinsing	35	35	35	35	35
Total		210	105	210	210	70

Total number of samples collected: 805.

[a] Sampling occurred at five different in-plant sampling sites, including: (1) carcass (site 1), immediately after entering the fabrication area but before the lactic acid solution carcass rinsing cabinet; (2) carcass (site 2), immediately after the lactic acid solution carcass rinsing cabinet; (3) fabrication table (site 3), at the distal end of the loin line; (4) top sirloin butt (site 4), immediately after being removed from the distal end of the loin fabrication table; and, (5) top sirloin butt (site 5), following lactic acid solution subprimal rinsing but before vacuum packaging.

[b] Decontamination treatments included: (a) post-chilling lactic acid solution carcass rinsing, immediately before the chilled carcass scale; (b) lactic acid solution fabrication table rinsing, applied by means of a spray bar mounted to the proximal end of the loin fabrication table; and, (c) lactic acid solution subprimal rinsing, applied to top sirloin butts removed from the distal end of the loin fabrication table.

Table 5.2. Least squares means ($\bar{x}$) and standard deviation (s) of the $\log_{10}$ values of total plate counts (TPC), total coliform counts (TCC), and *Escherichia coli* counts (ECC) (CFU/100 cm^2), and the number of samples (n (%)) in which counts were not detected, on carcasses immediately prior to fabrication, by sampling week.

	Microbiological Counts on Carcass Surfaces								
	Total Plate Counts			Total Coliform Counts			*Escherichia coli* Counts		
Week*	$\bar{x}$	s	n	$\bar{x}$	s	n	$\bar{x}$	s	n
A	4.0^{a}	1.40	5 (14.3)	0.9^{b}	0.10	32 (91.4)	0.9^{a}	0.06	34 (97.1)
B	3.1^{bc}	0.81	5 (14.3)	1.0^{a}	0.56	29 (82.9)	0.9^{a}	0.33	33 (94.3)
C	3.3^{b}	0.63	1 (2.9)	0.9^{b}	0.12	31 (88.6)	0.9^{a}	0.01	33 (94.3)
D	3.9^{a}	0.65	0 (0.0)	0.9^{ab}	0.26	33 (94.3)	0.9^{a}	0.26	33 (94.3)
E	3.7^{a}	1.37	2 (5.7)	0.9^{b}	0.18	32 (91.4)	0.9^{a}	0.01	33 (94.3)
F	2.9^{c}	0.67	6 (17.1)	0.9^{b}	0.14	31 (88.6)	0.9^{a}	0.14	31 (88.6)
Total	3.5	1.00	19 (9.0)	0.9	0.28	188 (89.5)	0.9	0.18	197 (93.8)

*Number of samples analyzed during each week: 35 for each enumerated set (TPC, TCC and ECC); total number of samples analyzed: 210 for each enumerated set.

Detection limits for TPC, TCC, and ECC were 2.2, 0.9, and 0.9 log CFU/100 cm^2, respectively.

^{abc} Means in the same column bearing a common superscript letter are not different ($P > 0.05$).

Table 5.3. Least squares means ($\Delta \bar{x}$)[a] and standard deviation (s) of the difference in log_{10} values of total plate counts (TPC), total coliform counts (TCC) and *Escherichia coli* counts (ECC) (CFU/100 cm^2), the number[b] of samples in which counts were not detected before (n_1 (%)) and after (n_2 (%)) treatment application, and the $\Delta \bar{x}$ test statistic (T), P-value, and 95% confidence interval (CI) associated with lactic acid solution carcass rinsing.

	Decontamination Effects of Lactic Acid Solution Carcass Rinsing[c]						
Microbiological Counts On Carcass Surfaces	$\Delta\bar{x}$	s	n_1 (%)	n_2 (%)	T	P-value	CI
TPC	0.22	0.98	13 (12.4)	23 (21.9)	2.26	0.03	0.03 - 0.40
TCC	0.06	0.35	92 (87.6)	97 (92.4)	1.77	0.08	-0.01 - 0.10
ECC	0.03	0.21	97 (92.4)	99 (94.3)	1.25	0.22	-0.02 - 0.07

[a] $\Delta \bar{x}$ is the difference in least squares means for each enumerated set (TPC, TCC and ECC) between sampling sites 1 and 2 (site 1 minus site 2).

[b] Number of samples analyzed at each of the two in-plant sampling sites: 105 for each enumerated set.

[c] Mean log values for TPC, TCC and ECC on carcass surfaces immediately before treatment application were 3.3, 1.0 and 0.9 log CFU/100 cm^2, respectively.

Detection limits for TPC, TCC, and ECC were 2.2, 0.9, and 0.9 log CFU/100 cm^2, respectively.

Table 5.4. Least squares means ($\bar{x}$) and standard deviation (s) of the $\log_{10}$ values of total plate counts (TPC), total coliform counts (TCC), and *Escherichia coli* counts (ECC) (CFU/100 cm^2) on table surfaces, by sampling week.

	Microbiological Counts on Table Surfaces*					
	Total Plate Counts		Total Coliform Counts		*Escherichia coli* Counts	
Week*	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
A	5.5^{a}	1.08	2.8^{b}	0.81	2.4^{c}	0.77
B	5.2^{a}	1.12	3.7^{a}	0.91	3.5^{a}	0.92
C	4.6^{b}	1.00	2.7^{b}	0.66	2.1^{de}	0.58
D	5.5^{a}	0.81	3.6^{a}	0.79	3.2^{b}	0.78
E	4.1^{c}	1.13	2.1^{c}	0.99	1.8^{e}	0.83
F	4.2^{bc}	1.16	2.5^{b}	0.90	2.3^{cd}	0.89

* Lactic acid solution rinsing of tables alone (as a decontamination treatment) occurred during week C, and lactic acid solution rinsing of tables, in combination (as multiple decontamination treatments) with lactic acid solution carcass rinsing occurred during weeks E and F; number of samples analyzed during each week: 35 for each enumerated set (TPC, TCC and ECC).

^{abcd} Means in the same column bearing a common superscript letter are not different ($P > 0.05$).

Table 5.5. Least squares means ($\Delta\bar{x}$)[a] and standard deviation (s) of the difference in log_{10} values of total plate counts (TPC), total coliform counts (TCC) and *Escherichia coli* counts (ECC) (CFU/100 cm^2), and the $\Delta\bar{x}$ test statistic (T), P-value, and 95% confidence interval (CI) associated with lactic acid solution subprimal rinsing in each of two decontamination systems.

Microbiological Counts On Subprimal Surfaces	Decontamination Effects of Lactic Acid Solution Subprimal Rinsing in Two Systems[b]*: Applied as the Only Decontamination Treatment[c] $\Delta\bar{x}$	s	T	P-value	CI	Applied Following Carcass and Table Rinsing[d] $\Delta\bar{x}$	s	T	P-value	CI
TPC	0.34	0.74	2.76	0.01	0.09 - 0.60	-0.07	0.78	-0.54	0.59	-0.34 - 0.20
TCC	0.41	0.57	4.33	0.00	0.22 - 0.61	0.15	0.83	1.09	0.29	-0.14 - 0.44
ECC	0.32	0.73	2.62	0.02	0.07 - 0.58	0.32	0.86	2.17	0.04	0.02 - 0.62

[a] $\Delta\bar{x}$ is the difference in least squares means for each enumerated set (TPC, TCC and ECC) between sampling sites 4 and 5 (site 4 minus site 5).

[b] Lactic acid solution rinsing of top sirloin butts alone (as a decontamination treatment) occurred during week D; lactic acid solution rinsing of top sirloin butts, in combination (as multiple decontamination treatments) with lactic acid solution carcass and table rinsing, occurred during week F.

[c] Mean log values for TPC, TCC and ECC on subprimal surfaces immediately before treatment application were 5.7, 3.8 and 3.3 log CFU/100 cm^2, respectively.

[d] Mean log values for TPC, TCC and ECC on subprimal surfaces immediately before treatment application were 5.1, 3.5 and 3.2 log CFU/100 cm^2, respectively.

* Number of samples analyzed for each system (week), at each of the two in-plant sampling sites: 35 for each enumerated set.

Table 5.6. Least squares means (standard deviations) of the $\log_{10}$ values of total plate counts (TPC), total coliform counts (TCC), and *Escherichia coli* counts (ECC) (CFU/100 cm^2) for each sampling time[a] and by in-plant sampling site, averaged across all weeks.

Sampling Time (min)[c]	In-Plant Sampling Site[b]								
	Carcass (Site 1)			Table (Site 3)			Subprimal (Site 4)		
	TPC	TCC	ECC	TPC	TCC	ECC	TPC	TCC	ECC
1 (0)	3.4 (0.82)	0.9 (0.25)	0.9 (0.25)	3.8 (1.12)	1.9 (0.85)	1.8 (0.76)	4.5 (1.30)	2.6 (1.27)	2.2 (1.02)
2 (30)	3.5 (1.16)	0.9 (0.13)	0.9 (0.06)	4.9 (0.98)	2.6 (0.91)	2.3 (0.87)	5.2 (1.23)	2.9 (1.14)	2.7 (1.11)
3 (60)	3.3 (0.86)	0.9 (0.15)	0.9 (0.15)	4.9 (1.06)	3.0 (0.95)	2.6 (0.95)	5.7 (1.02)	3.2 (1.09)	2.8 (1.03)
4 (90)	3.5 (1.07)	0.9 (0.20)	0.9 (0.01)	4.9 (1.20)	3.1 (0.98)	2.8 (0.95)	5.6 (0.92)	3.1 (1.15)	2.8 (1.10)
5 (120)	3.5 (0.94)	0.9 (0.14)	0.9 (0.01)	5.2 (1.17)	3.3 (0.93)	2.9 (1.01)	5.7 (1.08)	3.4 (1.06)	3.1 (1.04)
6 (150)	3.7 (1.21)	0.9 (0.15)	0.9 (0.14)	5.0 (1.31)	3.1 (1.10)	2.8 (1.08)	5.8 (1.02)	3.8 (0.97)	3.4 (1.02)
7 (180)	3.5 (1.23)	1.0 (0.60)	0.9 (0.36)	5.2 (1.03)	3.1 (0.94)	2.7 (0.96)	5.8 (0.85)	3.6 (0.82)	3.2 (0.89)

[a] Sampling time 1 occurred with the commencement of fabrication; subsequent samples were taken every half-hour over a three-hour period of time.

[b] Detection limits for TPC, TCC, and ECC were 2.2, 0.9, and 0.9 log CFU/100 cm^2, respectively, for samples taken from carcass surfaces; corresponding detection limits for samples taken from table or subprimal surfaces were 2.7, 1.4, and 1.4 log CFU/100 cm^2, respectively.

[c] Number of samples analyzed at each time: 30 for each enumerated set (TPC, TCC and ECC).

CHAPTER VI

PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY OF *SALMONELLA* ISOLATED FROM BEEF ANIMAL HIDES AND CARCASSES

ABSTRACT

This study determined the prevalence of *Salmonella* on beef animal hides and carcasses, and antimicrobial susceptibility profiles against a panel of thirteen antibiotics. In each of eight commercial packing facilities, of which five processed primarily heifers and steers and the remaining three processed primarily cows and bulls, hide and carcass sponge swab samples were obtained immediately before hide removal and before carcass chilling, respectively. Overall, prevalence of *Salmonella* on external surfaces (hides) of cattle was 15.4% (49/319), while prevalence on carcass surfaces immediately before chilling was 1.3% (4/320). From 53 total *Salmonella*-positive hide and carcass samples, 526 biochemically-confirmed isolates were obtained to determine antimicrobial susceptibility profiles. Of 53 *Salmonella*-positive samples, individually, 24 (45.3%), 17 (32.1%), 17 (32.1%), 11 (20.8%), 8 (15.1%), 8 (15.1%), 8 (15.1%), 4 (7.5%), and 2 (3.8%) samples yielded at least one isolate resistant to amoxicillin/clavulanic acid, tetracycline, streptomycin, sulfonamide, ampicillin, ampicillin/sulbactam, chloramphenicol, gentamicin, and trimethoprim/sulfamethoxazole, respectively. None of the *Salmonella*-positive samples yielded an isolate resistant to ceftriaxone, ciprofloxacin, enrofloxacin, or levofloxacin. While none of the samples yielded an isolate

simultaneously resistant to three or four antimicrobials, a total of 8 samples yielded at least one isolate resistant to five or more antimicrobials tested. Included among the 18 B group-positive samples were 3 samples that, individually, yielded at least one *Salmonella* Typhimurium DT104 var. Copenhagen isolate resistant to at least six antimicrobials tested. Results from this study supported current prudent therapeutic and subtherapeutic antimicrobial use recommendations.

INTRODUCTION

Foodborne diseases are estimated to cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year in the United States alone (Mead *et al.*, 1999). Nontyphoidal strains of the genus *Salmonella* are estimated to be responsible for over 1.4 million illnesses, and subsequently account for 9.7% of total foodborne illnesses, 25.6% of total hospitalizations, and 30.6% of deaths caused by known foodborne pathogens (Mead *et al.*, 1999). *Salmonella* are primarily associated with the intestinal tracts of animals and humans and, although human illness has been associated with exposure to other vehicles of transmission (e.g., pets, reptiles and contaminated water), it is estimated that 95% of salmonellosis cases involve foodborne transmission (Mead *et al.*, 1999; Tauxe, 1991). Foods most often associated with the transmission of *Salmonellae* include those of animal origin, such as beef, pork, poultry, eggs, raw milk and milk products such as dried milk, ice cream and cheese; however, other foods including fresh fruits, unpasteurized fruit juices, and vegetables, such as sprouts, have also served as vehicles of transmission (Bryan, 1983; ICMSF, 1996; Jay, 2000; Tauxe, 1991). Symptoms of salmonellosis, in humans, consist of diarrhea, fever, headache,

nausea, abdominal pain, vomiting, and, to a lesser extent, bloody stools (Akkina *et al.*, 2000; Poppe *et al.*, 1998). Antimicrobial therapy is not recommended as a course of action to combat the uncomplicated, self-limiting gastroenteritis typically associated with nontyphoidal *Salmonella* infection. However, this type of treatment may be lifesaving for individuals experiencing an invasive infection, as bacteremia occurs in 3 to 10 percent of culture-confirmed cases, potentially resulting in severe illness leading to disability or even death (Angulo *et al.*, 2000; Glynn *et al.*, 1998; Lee *et al.*, 1994a; Tauxe, 1991).

Emergence of antimicrobial resistant *Salmonella* is a problem not solely confined to the United States, where it has been on the rise since 1979 and 1980 when 16% of isolates were found to be resistant to at least one antimicrobial agent. Subsequently, in 1989 and 1990, the level of reported resistance increased to 29%, and in 1996, 37% of isolates were found to be resistant to at least one antimicrobial agent (Dabney *et al.*, 1997; Lee *et al.*, 1994a; MacDonald *et al.*, 1987; Riley *et al.*, 1984). Furthermore, in 1996, the United States experienced its first pentadrug-resistant outbreak involving *Salmonella enterica* serovar Typhimurium definitive phage type 104 (*S.* Typhimurium DT104), previously described in the United Kingdom, which exhibited resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (CDC, 1997). In 1998, a stool sample from a young boy in Nebraska yielded a *S.* Typhimurium (variant Copenhagen) isolate resistant to ampicillin, chloramphenicol, streptomycin, sulfisoxozole, tetracycline, kanamycin, broad-spectrum cephalosporins (i.e., cephalothin), extended-spectrum cephalosporins (i.e., ceftriaxone and ceftiofur), aztreonam, cefoxitin, gentamicin and tobramycin (Fey *et al.*, 2000; Gordon, 2000). Although the exact means of transmission was not determined, pulsed-field gel electrophoresis and plasmid profile

analysis results were used to suggest that the child acquired the pathogen from cattle located on the family farm.

While the emergence of *S.* Typhimurium that is resistant to several antimicrobials (including aminoglycosides, chloramphenicol, penicillins, sulfonamides, tetracycline, trimethoprim, etc.) has been documented (Aarestrup *et al.*, 1997; Davies *et al.*, 1978; Dunne *et al.*, 2000; Finlayson and Jackson, 1978; Glynn *et al.*, 1998), current concerns focus on the emergence of cephalosporin (e.g. ceftriaxone; Dunne *et al.*, 2000; Herikstad *et al.*, 1997a; Shannon and French, 1998) and fluoroquinolone (e.g. ciprofloxacin; Campo *et al.*, 1997; Herikstad *et al.*, 1997b; Hof *et al.*, 1991; Piddock *et al.*, 1990; Threlfall *et al.*, 1998; Walker *et al.*, 2000) resistance, as these antimicrobials remain the recommended treatments to combat invasive *Salmonella* infection in humans (Angulo *et al.*, 2000; Dutta *et al.*, 1995; Glynn *et al.*, 1998; Herikstad *et al.*, 1997a, 1997b; Poppe *et al.*, 1998).

The objectives of this study were to: (1) determine the prevalence of *Salmonella* on beef animal hides and carcasses; (2) determine the antimicrobial susceptibility profiles of the derived isolates to the five antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline) of concern regarding the emergence of penta-resistant *S.* Typhimurium DT104, and to eight additional antimicrobials (amoxicillin/clavulanic acid, ampicillin/sulbactam, ceftriaxone, ciprofloxacin, enrofloxacin, gentamicin, levofloxacin, and sulfamethoxazole/trimethoprim) which, with the exception of enrofloxacin, play a role in the treatment of human infections; and, (3) determine if *S.* Typhimurium DT104 was present among confirmed *Salmonella* spp. isolates.

MATERIALS AND METHODS

Sample collection. Samples (n = 80) were collected between June and October in each of eight geographically dispersed commercial beef packing plants, of which five processed primarily fed heifers and steers, and the remaining three processed primarily market cows and bulls. For those plants that processed primarily fed heifers and steers, designated as plants 1 through 5, slaughter chain speeds ranged from 250 to 350 carcasses per hour, while corresponding slaughter chain speeds for those plants (6 through 8) that processed primarily market cows and bulls ranged from 135 to 174 carcasses per hour. Final chain speeds, located between the hot scale and the cooler used for initial carcass chilling, were approximately 400 and 220 carcasses per hour for plants 1 through 5 and for plants 6 through 8, respectively.

Carcass decontamination treatments, applied between the two in-plant sampling locations, in four of the heifer/steer plants (1 through 4) included: (a) steam-vacuuming (104 to 110°C, 138 to 345 kPa steam, negative 7 to 12 mm of Hg vacuum) of spot contamination following hide removal; (b) pre-evisceration carcass washing (29 to 38°C water at 193 to 331 kPa, 6–8 sec); (c) pre-evisceration application of organic acid solution rinsing (1.6 to 2.6% acetic acid solution, 43 to 60°C, 317 to 324 kPa, 2 to 4 sec), immediately following pre-evisceration carcass washing; (d) thermal pasteurizing (71 to 77°C water, 69 to 228 kPa, 10 to 14 sec), after evisceration, carcass splitting, and associated processes; (e) post-evisceration carcass washing (16 to 32°C water, 483 to 897 kPa, 10 to 14 sec); and, (f) post-evisceration application of organic acid solution rinsing (1.6 to 2.6% acetic acid solution, 43 to 60°C, 317 to 324 kPa, 2 to 4 sec), immediately following post-evisceration carcass washing, but before chilling. The carcass

decontamination system in the fifth heifer/steer plant (plant 5) was the same as those in the other four plants, except that carcasses were not rinsed with an acetic acid solution at either the pre-evisceration or post-evisceration sites. In the cow/bull plants (6 through 8), the carcass decontamination system consisted of: (a) steam-vacuuming, (b) thermal pasteurizing, (c) post-evisceration carcass washing, and (d) post-evisceration lactic acid solution rinsing.

Samples (N = 640) were collected during the slaughtering/dressing process at two in-plant locations: (1) hide-on (site 1; n = 320), which occurred post-exsanguination but before hide opening and subsequent hide removal, and (2) carcass (site 2; n = 320), which occurred immediately following post-evisceration carcass washing and any associated organic acid solution rinsing, but before chilling. At each of the in-plant sampling locations (sites 1 and 2), 40 samples were collected in each of the eight commercial beef packing plants. Carcasses from animals sampled hide-on at site 1 were visually tracked until completion of the dehiding process, at which point one of the two sides of each carcass was tagged to facilitate identification for subsequent sampling at site 2. Sampling at both in-plant locations (sites 1 and 2) was performed by sponge-swabbing and followed procedures described in the FSIS-USDA Meat and Poultry Inspection regulations (FSIS-USDA, 1996b).

Immediately before sampling, sterile sponges (BioPro Enviro-Sponge Bags, International BioProducts, Redmond, WA) were hydrated with 10 ml of 0.1% sterile, buffered peptone water (BioPro, International BioProducts). Sampling was performed using a 100 cm^2 disposable, sterile template (USDA Template, International BioProducts) at each of three anatomical locations, resulting in a total sampling area of 300 cm^2. The

three anatomical locations sampled included: (1) flank, at a point where the medial border of the cutaneous flank muscle came within 7.62 cm of the midline, (2) brisket, at a midline point, level with the elbow, and (3) rump, at a point where a line from the posterior aspect of the aitch bone to the achilles tendon intersected the cut surface of the round (FSIS-USDA, 1996b). Sponging at each site, within the 100 cm^2 template area, consisted of 10 passes vertically (up-and-down being considered as 1 pass) and 10 passes horizontally (side-to-side being considered as 1 pass). Samples were collected aseptically using sterile, latex gloves (Basic™, International BioProducts), which, in addition to the template, were changed between samples. Following sampling, an additional 15 ml of refrigerated 0.1% sterile, buffered peptone water (Difco Laboratories, Sparks, MD) was added to the sponge, bringing the total volume of buffer to 25 ml. After excess air was expelled and the sponge bags were closed, the samples were placed with icepacks and a cardboard divider (to prevent direct contact with the icepacks) into shipping coolers for overnight delivery to the laboratory (Warren Analytical Laboratory, Greeley, CO) for analysis.

Microbiological analysis. Following arrival of samples at the laboratory, the sponges and associated buffer were agitated in a stomacher (Seward Model 400, Tekmar Company, Cincinnati, OH) for 1 min, at which point sterile lactose broth (LB) (Mikrobiologie, EM Science, Gibbstown, NJ) was added to the homogenate to create a 10:1 lactose broth to sample weight ratio. Following sample enrichment for 24 h at 35 ± 2°C, 1 ml of the sample was added to each of 9 ml of cysteine selenite broth (CSB) and tetrathionite broth (TTB; Difco Laboratories), to which 0.1 ml of 1.0% Brilliant Green solution and 0.2 ml iodine solution had been added. Inoculated CSB tubes were

incubated at 35 ± 2°C for 24 h, while TTB tubes were held at 42 ± 0.5°C for 24 h in a water bath. After selective enrichment, for samples retrieved from plants 1 through 7, 1 ml of TTB and CSB culture was combined and added to each 9 ml of M Broth (Difco Laboratories), to which 1 ml of 0.0001% novobiocin solution had been added. Following a second enrichment at 42 ± 0.5°C for 5 h in a water bath, the samples were vortexed and an enzyme-linked immunosorbent assay (ELISA) test was used to screen for the presence of *Salmonella* species (Salmonella-Tek™, Organon Teknika Corporation, Durham, NC). After selective enrichment for samples derived from plant 8, 1 ml of TTB and 1 ml of CSB were combined and added to each 9 ml of M Broth (Difco Laboratories). Following a second enrichment at 42 ± 0.5°C for 6 h in a water bath, the samples were vortexed and an ELISA test was used to screen for the presence of *Salmonella* spp. (Biocontrol® Assurance EIA, BioControl Systems, Inc., Bothell, WA).

Presumptive-positive samples were subsequently transferred from CSB, TTB, and M Broth onto three types of selective media (bismuth sulfite, hektoen enteric, and xylose lysine deoxycholate; Difco Laboratories) and incubated for 24 h at 35 ± 2°C, at which time they were transported (45 min) to Colorado State University where up to 15 suspect colonies from any of the three types of media were tested biochemically (API 20E, bioMerieux, Hazelwood, MO).

Antimicrobial susceptibility. Antimicrobial susceptibility of the isolates was determined by the disk-diffusion method in accordance with the standards of the National Committee for Clinical Laboratory Standards (Bauer *et al.*, 1966; NCCLS, 1999a, 1999b). Confirmed *Salmonella* spp. isolates were transferred onto tryptic soy agar plates containing 5% sheep blood (Difco Laboratories). Following incubation for 24 h at 35° C,

the BBL Prompt™ system was used to produce a standardized inoculum of $1x10^8$ colony forming units (CFU)/ml (Becton Dickinson Microbiology Systems, Sparks, MD). After standardization, the inoculum was uniformly spread onto Mueller Hinton agar (Difco Laboratories), at which time the antimicrobial-containing discs were dispensed and tamped into place (BBL™ Sensi-Disc™, Becton Dickinson). Antimicrobial susceptibility testing was conducted using the following antimicrobials (concentration): the combination of amoxicillin and clavulanic acid (20/10 μg), ampicillin (10 μg), the combination of ampicillin and sulbactam (10/10 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), gentamicin (10 μg), levofloxacin (5 μg), streptomycin (10 μg), sulfonamides (trisulfapyramidine, triple sulfa) (250 μg), tetracycline (30 μg), and the combination of trimethoprim and sulfamethoxazole (1.25/23.75 μg). Following an incubation period of 17 ± 1 h at 35° C, the plates were removed and calipers were used to measure the respective zones of inhibition for each of the antimicrobials tested (NCCLS, 1999b).

***Salmonella* Typhimurium DT104 determination.** In addition to antimicrobial susceptibility testing, confirmed *Salmonella* spp. isolates were serologically screened using polyvalent O and O group B typing antisera (Difco Laboratories) in order to identify serogroup B isolates (Ewing, 1986; Popoff and Le Minor, 1997). All serogroup B isolates were subsequently transferred to nutrient agar slants (Difco Laboratories) and shipped overnight to the National Veterinary Services Laboratory (NVSL/USDA, Ames, IA) for serotyping. Following serotyping, all *S.* Typhimurium were submitted for phage typing to determine if they were definitive type 104 (DT104) by NVSL/USDA.

RESULTS AND DISCUSSION

During sample analysis, the post-selective enrichment protocol changed for samples derived from plant 8 as a result of changes in the laboratory standard procedure for presumptive-positive *Salmonella* spp. testing. Even though both specificity and sensitivity were equal to methods used for presumptive-positive *Salmonella* spp. testing of samples derived from plants 1 through 7, antibody attribute differences may have introduced serotypic selection bias. In all cases, positive ELISA results based upon sample absorbency were interpreted as presumed positive for the presence of *Salmonella* spp.; but a negative result was considered conclusive, as the sample did not contain detectable levels of *Salmonella* antigen.

Samples were collected at site 1 to determine the prevalence of *Salmonella* on external (hide) surfaces of incoming cattle, as this is the primary source of bacteria subsequently transferred to the underlying, sterile carcass surface and because errors in slaughtering/dressing are the primary vehicles of carcass contamination (Castillo *et al.*, 1998; Kochevar *et al.*, 1997). Overall, the prevalence of *Salmonella* on external surfaces (hides) of cattle was 15.4% (49/319), while the prevalence on carcasses immediately before chilling was 1.3% (4/320; Table 6.1). In two of the plants (plants 3 and 5), all of the samples collected from both sampling sites were *Salmonella*-negative. In the remaining plants, the prevalence of *Salmonella*-positive samples obtained at sampling site 1 (hide-on) ranged from 10.0 to 47.5%, while the prevalence of *Salmonella*-positive samples obtained at sampling site 2 (carcass) ranged from 0.0 to 7.5% (Table 6.1). *Salmonella*-positive samples were obtained at sampling site 2 in two of the eight commercial packing plants, yielding 3 and 1 positive carcass samples (Table 6.1).

Selection of specific antimicrobials, and desired concentrations, were based upon recent information regarding the emergence of various drug-resistant *S.* Typhimurium DT104 strains (Akkina *et al.*, 2000; Dunne *et al.*, 2000; Fey *et al.*, 2000; Walker *et al.*, 2000), previously published recommendations for routine testing by veterinary diagnostic laboratories (NCCLS, 1999b), and manufacturers' guidelines (BBL™ Sensi-Disc™, Becton Dickinson). Of 53 total *Salmonella*-positive samples (yielding 526 confirmed isolates), individually, 24 (45.3%), 17 (32.1%), 17 (32.1%), 11 (20.8%), 8 (15.1%), 8 (15.1%), 8 (15.1%), 4 (7.5%), and 2 (3.8%) samples yielded at least one isolate resistant to amoxicillin and clavulanic acid (amoxicillin/clavulanic acid), tetracycline, streptomycin, sulfonamide, ampicillin, ampicillin and sulbactam (ampicillin/sulbactam), chloramphenicol, gentamicin, and trimethoprim and sulfamethoxazole (trimethoprim/sulfamethoxazole), respectively (Table 6.2). None of the *Salmonella*-positive samples yielded an isolate resistant to ceftriaxone, ciprofloxacin, enrofloxacin, or levofloxacin. Of the 53 samples, 6 and 1 yielded 13 and 2 isolates of intermediate resistance to ceftriaxone and ciprofloxacin, respectively, although isolates derived from the same sample, with identical susceptibility profiles, were probably clones (Table 6.2). There were no isolates of intermediate resistance to enrofloxacin or levofloxacin.

Although 24 and 17 samples yielded 65 and 43 isolates resistant to amoxicillin/clavulanic acid and streptomycin, respectively, minimal intermediate resistance was evident as only 2 and 4 samples yielded 3 and 4 isolates of intermediate resistance (Table 6.2). Conversely, intermediate resistance to ampicillin/sulbactam, sulfonamides, and especially tetracycline, individually, was evident. Despite the

ambiguity associated with the intermediate level of resistance, the frequency of this level among samples and isolates when evaluated across all antimicrobials did not depend specifically on the frequency of resistant samples and isolates.

Of the 53 *Salmonella*-positive samples, all 135 isolates from 18 (40.0%) of the samples (both from the exterior of the hide and from the carcass surface) were susceptible to all 13 antimicrobials (Table 6.3). Twenty-two total samples (41.5%; hide plus carcass surfaces) produced 93 isolates resistant to at least one of the antimicrobials tested. Of the 7 *Salmonella*-positive samples obtained in plant 8, all 7 samples yielded a total of 67 isolates solely resistant to tetracycline, thereby accounting for 93.1% of all isolates demonstrating that single resistant profile. Across all plants, 5 samples (9.4%; all from the exterior of the hide) were simultaneously resistant to two antimicrobials, yielding 9 isolates resistant to sulfonamides and tetracycline, 2 isolates resistant to amoxicillin/clavulanic acid and ampicillin/sulbactam, 1 isolate resistant to amoxicillin/clavulanic acid and streptomycin, 1 isolate resistant to amoxicillin/clavulanic acid and tetracycline, and 1 isolate resistant to ampicillin/sulbactam and streptomycin. While none of the samples was resistant to three or four antimicrobials specifically, one hide sample produced a single isolate resistant to five antimicrobials (ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline). One hide sample also produced a single isolate resistant to six antimicrobials, possessing the same resistance profile as the penta-resistant isolate combined with additional resistance to trimethoprim/sulfamethoxazole. Two hide samples were resistant to seven antimicrobials, yielding 10 isolates possessing the same resistant profile as the penta-resistant isolate and with 9 of the isolates demonstrating additional resistance to

amoxicillin/clavulanic acid and ampicillin/sulbactam, and 1 isolate demonstrating additional resistance to amoxicillin/clavulanic acid and trimethoprim/sulfamethoxazole. Lastly, 8 isolates from 4 hide samples were resistant to eight antimicrobials, including amoxicillin/clavulanic acid, ampicillin, ampicillin/sulbactam, chloramphenicol, gentamicin, streptomycin, sulfonamides and tetracycline (Tables 6.3 and 6.4).

Eighteen samples, individually, yielded at least one serogroup B isolate. Following serotyping and any phage-typing of serogroup B isolates, 9 samples yielded three serotypes that were susceptible to all of the antimicrobials tested (Table 6.4). One sample yielded isolates (*S.* Reading) solely resistant to tetracycline, while another sample yielded isolates (*S.* Agona) simultaneously resistant to sulfonamides and tetracycline. One sample yielded an isolate (*S.* Typhimurium DT104 var. Copenhagen) resistant to six antimicrobials; two samples yielded isolates (*S.* Typhimurium DT104 var. Copenhagen) resistant to seven antimicrobials; and, four samples yielded isolates (*S.* Reading) resistant to eight antimicrobials (Tables 6.3 and 6.4). The one sample yielding one isolate resistant to five antimicrobials was not serogroup B; therefore, its serotype was not subsequently determined.

Ampicillin and chloramphenicol resistance was, individually, restricted to the 8 samples (15.1%) resistant to five or more antimicrobials (Tables 6.2 and 6.4). Those antimicrobials employing the combination of a ß-lactam with a ß-lactamase inhibitor, as with amoxicillin/clavulanic acid and ampicillin/sulbactam, did not result in greater isolate susceptibility. In fact, 8 samples yielded 20 isolates resistant to both ampicillin and the combination of ampicillin and sulbactam (Table 6.2). All isolates screened, however, were susceptible to the extended-spectrum cephalosporin (ceftriaxone) and

fluoroquinolones (ciprofloxacin and levofloxacin), suggesting that these antimicrobials would be effective in the treatment of an invasive *Salmonella* infection involving an organism identified during this study.

Overall, the prevalence of *Salmonella* on beef animal hides and carcasses was 15.4 and 1.3%, respectively. Of the 49 positive samples obtained from beef animal hides, 34 (69.4%) yielded at least one isolate resistant to at least one of the antimicrobials tested. Although this was influenced by the high level of tetracycline resistance among *Salmonella* recovered in plant 8, the only plant in the study located in the southeast, data collected in this study suggested that for every 1000 beef animal hides sampled, approximately 107 (10.7%) would contain *Salmonella* resistant to at least one of the thirteen antimicrobials tested. Additionally, 3 samples yielded 11 *S.* Typhimurium var Copenhagen DT104 isolates that were, individually, resistant to at least six of the antimicrobials tested.

Prevalence of *Salmonella* on the external surfaces (hides) of cattle upon entry into commercial packing facilities serves as an indication of contamination that potentially could be transferred to underlying, otherwise sterile, carcass surfaces during the dehiding process. This information is not an indication of the number of animals previously carrying/shedding the pathogen. A previous study, analyzing fecal samples (N = 5,049) obtained from 187 cow-calf operations in 22 states, determined that 1.4% of samples were positive for *Salmonella* (Dargatz *et al.*, 2000). Furthermore, of the 78 isolates identified from the 70 positive samples, 68 (87.2%) were classified as susceptible to the 17 antimicrobials tested, which, among others, included amoxicillin/clavulanic acid, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, streptomycin,

tetracycline and trimethoprim/sulfamethoxazole (Dargatz *et al.*, 2000). However, under feedlot and marketing conditions, increased animal stress may contribute to an increase in shedding, which, coupled with increased animal density, would facilitate animal-to-animal spread both internally and externally (i.e., on hides; Corrier *et al.*, 1990). Some 5.5% of fecal samples obtained from feedlots was positive for *Salmonella* (Fedorka-Cray *et al.*, 1998). Additionally, seasonal variation in shedding patterns may contribute to a higher prevalence of *Salmonella* on animal hides and perhaps on carcasses, especially if samples are collected during months (June through October) typically associated with warmer weather (Williams, 1975).

Emergence of antimicrobial resistance, resulting from a series of societal, technological, environmental and microbial changes, has become a global concern. Public health concerns revolve around increased morbidity and mortality resulting from failing antimicrobial treatment regimen and the increased costs of healthcare required for the development of new, efficacious antimicrobial drugs (Cohen, 1997; Tollefson *et al.*, 1999). In contrast to human medicine, antimicrobial use in veterinary medicine serves three purposes: therapy, prophylaxis or promotion of growth or efficiency of growth. The growth and/or efficiency of growth promotion basis of antimicrobial application depends on the mode of action of the substance (i.e., inhibition of cell wall, protein and DNA synthesis, as well as modulation of cation permeability), and is most often used during the production of swine, broilers, veal calves and beef cattle (Helmuth and Protz, 1997). Although alternative practices have been suggested, prudent therapeutic and subtherapeutic antimicrobial use during food animal production is currently recommended and should be adhered to until such a time that consensus regarding the

risk to public health is reached and additional recommendations are made. Furthermore, antimicrobial use is not an appropriate solution to poor hygiene standards, nor is it a suitable method of pathogen eradication (Helmuth and Protz, 1997; Wierup, 2000).

The Center for Veterinary Medicine of the Food and Drug Administration (CVM-FDA) released a document entitled: "A Proposed Framework for Evaluating and Assuring the Human Safety of the Microbial Effects of Antimicrobial New Animal Drugs Intended for use in Food-Producing Animals." The "Framework Document" suggests that evaluation of how development of resistant bacteria, to antimicrobial drugs used in food-producing animals, impacts human health depends primarily upon the role that the drug plays in human medicine, and the potential for human exposure to the resistant bacteria (CVM-FDA, 1998). Based on these factors, antimicrobials administered to food-producing animals could be categorized, thereby facilitating the process of evaluating long-term availability of safe and effective antimicrobial drugs for the treatment of human disease (CVM-FDA, 1998).

Table 6.1. Prevalence of *Salmonella* on beef animal hides and carcasses in eight commercial beef slaughtering facilities.

	In-Plant Sampling Location*			
	Hide-On (Site 1)		Carcass (Site 2)	
Plant	Positive Samples	Prevalence (%)	Positive Samples	Prevalence (%)
1	19	47.5	3	7.5
2	4	10.0	0	0.0
3	0	0.0	0	0.0
4	9	23.1	0	0.0
5	0	0.0	0	0.0
6	4	10.0	0	0.0
7	7	17.5	0	0.0
8	6	15.0	1	2.5
Total	49	15.4	4	1.3

* Number of samples analyzed at each location in each plant = 40; but only 39 samples were reported at site 1 in Plant 4 due to laboratory error during analysis.

Table 6.2. Number of *Salmonella*-positive samples and corresponding isolates[a], and their level of resistance to each antimicrobial tested.

	Level of Resistance					
	Resistant		Intermediate		Susceptible	
Antimicrobial	Samples (%)[b]	Isolates	Samples	Isolates	Samples	Isolates
Aminoglycosides						
Gentamicin	4 (7.5)	10	0	0	52	516
Streptomycin	17 (32.1)	43	4	4	51	479
β-Lactams						
Cephalosporin:						
Ceftriaxone	0 (0.0)	0	6	13	52	513
Penicillin:						
Ampicillin	8 (15.1)	20	0	0	51	506
β-Lactams/ β-Lactamase Inhibitors						
Penicillins:	24 (45.3)	65	2	3	50	458
Amoxicillin and clavulanic acid	8 (15.1)	20	14	20	50	486
Ampicillin and sulbactam						
Chloramphenicol	8 (15.1)	20	0	0	51	506
Fluoroquinolones						
Ciprofloxacin	0 (0.0)	0	1	2	53	524
Enrofloxacin	0 (0.0)	0	0	0	53	526
Levofloxacin	0 (0.0)	0	0	0	53	526
Sulfonamide						
Trisulfapyramidine	11 (20.8)	35	10	11	51	480
Tetracycline	17 (32.1)	102	35	143	51	281
Trimethoprim/ Sulfonamide						
Trimethoprim and sulfamethoxazole	2 (3.8)	2	0	0	53	524

[a] A total of 526 isolates from 53 positive samples was tested.
[b] Percentage of positive samples yielding at least one resistant isolate.

Table 6.3. Plants in which sampling occurred and the number of *Salmonella*-positive samples, and corresponding isolates[a], demonstrating resistance to a maximum number of antimicrobials tested, by in-plant sampling location.

Number of Antimicrobials	In-Plant Sampling Location					
	Hide-On (Site 1)			Carcass (Site 2)		
	Plant(s)	Samples	Isolates	Plant(s)	Samples	Isolates
0	1, 2, 3, 4, 5, 6, 7	15	127	1	3	8
1	1, 4, 6, 7, 8	21	91	8	1	2
2	4, 7, 8	5	14			
3						
4						
5	1	1	1			
6	2	1	1			
7	2	2	10			
8	1	4	8			

[a] A total of 526 isolates from 53 positive samples was tested.

Table 6.4. Number of samples yielding serogroup B isolates, and their corresponding antimicrobial resistance pattern by *Salmonella* serotype.

Salmonella Serotype	Samples	Isolates	Antimicrobial Resistance Pattern[a]
4, 12:Nonmotile	1	1	Susceptible to all antimicrobials
Saint-Paul	1	4	Susceptible to all antimicrobials
Agona	7	52	Susceptible to all antimicrobials
Reading	1	4	T
Agona	1	9	SuT
Typhimurium DT104 var. Copenhagen	1	1	ACSSuT + Trimethoprim/sulfamethoxazole
Typhimurium DT104 var. Copenhagen	1	8	ACSSuT + Amoxicillin/clavulanic acid and Ampicillin/sulbactam
Typhimurium DT104 var. Copenhagen	1	1	ACSSuT + Amoxicillin/clavulanic acid and Ampicillin/sulbactam
		1	ACSSuT + Amoxicillin/clavulanic acid and Trimethoprim/sulfamethoxazole
Reading	4	8	ACSSuT + Amoxicillin/clavulanic acid, Ampicillin/sulbactam and Gentamicin

[a] ACSSuT: common abbreviations for ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline.

CHAPTER VII

COMPARATIVE ANALYSIS OF ACID RESISTANCE BETWEEN SUSCEPTIBLE AND MULTI-ANTIMICROBIAL RESISTANT *SALMONELLA* STRAINS CULTURED UNDER STATIONARY-PHASE ACID TOLERANCE-INDUCING AND NONINDUCING CONDITIONS

ABSTRACT

Acid resistance of each of five antimicrobial susceptible strains of *Salmonella* and five strains simultaneously resistant to a minimum of six antimicrobials was evaluated. Induction of a stationary-phase acid tolerance response (ATR) was attempted by both transient low-pH acid-shock and acid-adaptation. For acid-shock induction, strains were grown for 18 h in pH 8.0 minimal (EG) medium, exposed to sublethal acid stress (pH 4.3) for 2 h and, subsequently, both shocked and nonshocked cultures were acid-challenged (pH 3.0) for 4 h. Acid-adaptation was achieved by growing strains for 18 h in tryptic soy broth containing 1.0% glucose (TSB+G; pH 7.3), while nonadapted cultures were grown for 18 h in glucose-free tryptic soy broth (TSB-G; pH 7.3). Acid-adapted and nonadapted inocula were acid-challenged (pH 2.3) for 4 h. Growth of *Salmonella* in EG medium, TSB-G, or TSB+G altered media pH to 5.9 to 6.1, 8.1 to 8.3 and 4.7 to 4.8, respectively, and resulted in initial (0 h), non-challenged mean strain populations of 8.5 to 8.7, 8.4 to 8.8, and 8.2 to 8.3 log CFU/ml. After 4 h of acid-challenge, mean strain populations were 3.0 to 4.8 and 2.5 to 3.7 log CFU/ml for previously acid-shocked susceptible and resistant *Salmonella*, respectively, while corresponding counts for

nonshocked strains were 4.3 to 5.5 and 3.9 to 4.9 log CFU/ml. Following 4 h of acid-exposure, acid-adapted cultures of susceptible and resistant strains had mean populations of 6.1 to 6.4 and 6.4 to 6.6 log CFU/ml, respectively, while corresponding counts of nonadapted cultures were 1.8 to 2.0 and 1.8 to 1.9 log CFU/ml. A low-pH inducible ATR was not achieved through transient acid shock, while an ATR was evident following acid-adaptation, as adapted populations were 4.2 to 4.8 logs higher than the nonadapted populations following acid-exposure. Results from this study suggested no association between antimicrobial susceptibility and the ability of the *Salmonella* strains evaluated to survive low-pH stress.

INTRODUCTION

Bacteria may face transient or prolonged exposures to potentially lethal or sublethal pH conditions occurring in both natural and host environments (Foster and Spector, 1995). Survival under such conditions ultimately depends on the presence of adaptive mechanisms and the ability of the organism to mount an appropriate molecular response by regulating the expression of regulons and/or stimulons in order to avoid or repair damage associated with low-pH exposure (Foster, 2000; Neidhardt, 1987). Although protective mechanisms allowing for an adaptive response to low-pH exposure have been reported among other *Salmonella* serotypes, *S.* Typhimurium has been the primary subject of acid survival investigation due to its well-defined genetics and its ability to survive a wide-range of acid stresses found in both intra- and extra-host environments (Foster, 1995; Leyer and Johnson, 1992). *Salmonella* Typhimurium possess two distinct low-pH inducible systems referred to as acid tolerance responses

(ATR), which are distinguished based on the growth phase during which induction occurs (Foster, 2000). Induction of the log-phase ATR occurs when exponentially growing cells are suddenly exposed to low pH resulting in the production of 50 acid shock proteins (ASP; Foster, 2000). The *rpoS* and *fur* genes, encoding the alternative sigma factor σ^S, and the iron-regulatory protein Fur, respectively, in addition to the two-component signal transduction system PhoPQ, all play a regulatory role in log-phase acid tolerance (Foster, 2000; Hall and Foster, 1996; Lee *et al.*, 1995).

In natural and host environments, salmonellae are not always engaged in exponential growth when they encounter acid stress. A second, pH-dependent system known as the stationary-phase ATR is induced when stationary-phase cells are exposed to low pH (Lee *et al.*, 1994b). This system is distinct from the pH-independent general stress response system that is induced by entry into stationary phase, as the general stress response system requires stationary-phase induction of the alternative sigma factor, σ^S, while the acid-induced stationary-phase ATR does not (Foster, 2000). Stationary-phase ATR requires the regulatory gene *ompR* and results in the production of 15 ASP (most of which are distinct from log-phase ASP) that afford a higher level of acid resistance than does the log-phase ATR (Bang *et al.*, 2000; Lee *et al.*, 1994b).

In addition to withstanding low pH exposure, acid-adapted *S.* Typhimurium may exhibit cross-protection against heat, osmotic and oxidative stresses (Lee *et al.*, 1995; Leyer and Johnson, 1993; Wilde *et al.*, 2000). A strong correlation between the ability to mount an ATR and enhanced virulence also has been suggested (Idziak and Suvanmongkol, 1971; Wilmes-Riesenberg *et al.*, 1996). Stress cross-protection associated with the ATR may prove important during pathogenesis, as cells undergoing

acid-adaptation prior to ingestion by the host or by acid shock in the stomach may be better prepared to endure environmental stresses subsequently confronted in the stomach or intestinal and intracellular environments, respectively (Idziak and Suvanmongkol, 1971; Wilmes-Riesenberg *et al.*, 1996).

Emergence of antibiotic resistant bacterial pathogens, resulting from a series of societal, technological, environmental and/or microbial changes, has evoked global concern regarding potential for increased morbidity and mortality resulting from failing antimicrobial treatment regimens, and increased costs of health care (Cohen, 1997; Tollefson *et al.*, 1999). In the United States, antimicrobial resistance among *Salmonella* has been on the rise since 1979-1980 when 16% of isolates were found to be resistant to at least one antimicrobial agent, to 1996, when 37% of isolates were found to be resistant to at least one antimicrobial agent (Dabney *et al.*, 1997; MacDonald *et al.*, 1987).

In 1996, the United States experienced its first documented pentadrug-resistant outbreak involving *S.* Typhimurium definitive phage type 104 (*S.* Typhimurium DT104; CDC, 1997), and in 1998, *S.* Typhimurium (variant Copenhagen) isolated from a young boy in Nebraska was found to be resistant to ampicillin, chloramphenicol, streptomycin, sulfisoxozole, tetracycline, kanamycin, broad-spectrum cephalosporins (i.e., cephalothin), extended-spectrum cephalosporins (i.e., ceftriaxone and ceftiofur), aztreonam, cefoxitin, gentamicin and tobramycin (Fey *et al.*, 2000).

More recently, 502 strains of *Salmonella* isolated from numerous domestic and imported foods and related samples, between 1999 and 2000, were evaluated for their susceptibility to 11 antimicrobial agents (Kiessling *et al.*, 2002). Of the cultures screened (N = 502), 49.2% (n = 247) were resistant to at least one of the antimicrobials tested, and

of those, 31.2% (n = 77) were multi-antimicrobial resistant. Multi-antimicrobial resistant *Salmonella* were isolated from numerous types of foods, including sprouts, spices, frozen seafood, freshwater fish, ice cream, meat and bone meal, snails, herbs, cheese and lettuce (Kiessling *et al.*, 2002). Presence of susceptible or antimicrobial resistant *Salmonella* in these foods is a public health concern, especially among those that may not receive the additional processing (e.g., cooking) required to inactivate viable microorganisms.

Currently, knowledge regarding the relationship between antimicrobial susceptibility of foodborne pathogens and tolerance to food-related stresses is lacking. Therefore, the objective of this study was to evaluate acid resistance between susceptible and multi-antimicrobial resistant strains of *Salmonella*, with or without previous low-pH induction of an ATR, to determine if an association existed between antibiotic susceptibility and the ability to avoid or repair damage associated with low-pH exposure.

MATERIALS AND METHODS

***Salmonella* strains.** *Salmonella* strains used in this study are listed in Table 7.1, and were selected based on their source and antimicrobial resistance profiles. With the exception of *S.* Typhimurium DT104 (ATCC 700408), all strains were derived from beef animal hide surface samples (n = 40) collected in each of four commercial beef packing plants, three of which primarily processed heifers and steers and the other of which processed cows and bulls (Chapter VI). External hide surfaces of animals (N = 160) were sampled following stunning and exsanguination, but before hide opening and subsequent hide removal. Sampling involved sponge-swabbing (BioPro Enviro-Sponge Bags, International BioProducts, Redmond, WA) according to procedures for carcasses

described in the FSIS-USDA Meat and Poultry Inspection regulations (FSIS-USDA, 1996b). Sponge-swab samples were analyzed for *Salmonella* using an enzyme-linked immunosorbent assay (ELISA; Salmonella-Tek™, Organon Teknika Corporation, Durham, NC). Presumptive-positive samples were transferred onto selective media (bismuth sulfite, hektoen enteric, and xylose lysine deoxycholate; Difco Laboratories, Sparks, MD) and, following incubation (24 h at 35 ± 2°C), suspect colonies were biochemically confirmed (API 20E, bioMerieux, Hazelwood, MO).

Antimicrobial susceptibility of confirmed *Salmonella* isolates was determined before, and confirmed after, acid-challenge studies by the disk-diffusion method as described by the National Committee for Clinical Laboratory Standards (Bauer *et al.*, 1966; NCCLS, 1999a, 1999b). The BBL Prompt™ system was used for inoculum standardization ($1x10^8$ colony forming units (CFU)/ml; Becton Dickinson Microbiology Systems, Sparks, MD), on which, after being uniformly spread onto Mueller Hinton agar (Difco Laboratories), antimicrobial-containing discs were dispensed and tamped into place (BBL™ Sensi-Disc™, Becton Dickinson). The following antimicrobials (concentration) were included in the screened panel: the combination of amoxicillin and clavulanic acid (20/10 μg); ampicillin (10 μg); the combination of ampicillin and sulbactam (10/10 μg); ceftriaxone (30 μg); chloramphenicol (30 μg); ciprofloxacin (5 μg); enrofloxacin (5 μg); gentamicin (10 μg); levofloxacin (5 μg); streptomycin (10 μg); sulfonamides (trisulfapyramidine, triple sulfa) (250 μg); tetracycline (30 μg); and, the combination of trimethoprim and sulfamethoxazole (1.25/23.75 μg; Chapter VI). Serotyping and subsequent phage-typing of *S.* Typhimurium was performed by the National Veterinary Services Laboratory (NVSL/USDA, Ames, IA).

Assay of low-pH inducible stationary-phase ATR. Cultures were maintained at 4°C with monthly transfers on tryptic soy agar (TSA; Difco Laboratories) slants. Culture media included minimal E medium containing 0.4% glucose (EG medium; Fisher Chemicals, Fisher Scientific, Fair Lawn, NJ; Vogel and Bonner, 1956), glucose-free tryptic soy broth (TSB-G), and tryptic soy broth with 0.25% (TSB) and 1.00% (TSB+G) glucose (Difco Laboratories). The pH of EG medium (pH 8.0) used for growth of *Salmonella* strains was adjusted with 5N NaOH, while the pH values of EG medium used for acid-shocking (pH 4.3), and EG medium and TSB used for acid-challenge (pH 3.0 and 2.3, respectively), were adjusted with 5N HCl (Fisher Chemicals).

Acid-shock-induced stationary-phase ATR was evaluated as previously reported (Bang *et al.*, 2000; Lee *et al.*, 1994b) with slight modification. Briefly, working stock cultures were subcultured twice, each time for 18 h at 37°C in 9 ml of pH 8.0 EG medium. *Salmonella* were subsequently grown in 6 ml of EG medium (18 h; pH 8.0; 37°C; 130 shakes/min), at which time three, 1.5 ml aliquots were removed. Cells contained in the aliquots (1.5 ml) were harvested by centrifugation (3 min; 13,000 x g; 4°C) and washed in an equal volume of EG medium (pH 7.0). Following a second centrifugation, the cells were resuspended to 3 to 5 x 10^8 cells/ml in each of 9 ml of EG medium at pH 7.0 (to serve as an unchallenged control), pH 4.3 (for acid-induction of the ATR), and pH 3.0 (for noninduced ATR acid-challenge). Acid-induced suspensions (pH 4.3) were held statically for 2 h at 37°C, at which time they were again harvested and washed in an equal volume of EG medium (pH 4.3), then resuspended in 9 ml of pH 3.0 EG medium for acid-challenge.

Alternatively, acid-induced stationary-phase ATR also was evaluated by culturing *Salmonella* in the presence of glucose (Buchanan and Edelson, 1996; Wilde *et al.*, 2000). Working stock cultures also were subcultured twice, each time for 18 h at 37°C in 9 ml of pH 7.3 TSB. *Salmonella* were subsequently grown (18 h; 37°C; 130 shakes/min) in 6 ml of pH 7.3 TSB+G (1.00% glucose) or TSB-G (0.00% glucose), at which time two, 1.5 ml aliquots were removed. Cells contained in the aliquots (1.5 ml) were harvested by centrifugation (3 min; 13,000 x g; 4°C) and washed in an equal volume of pH 7.3 TSB. Following a second centrifugation, cells were resuspended to 2 to 6 x 10^8 cells/ml in each of 9 ml of TSB (0.25% glucose) at pH 7.3 to serve as an unchallenged control, and pH 2.3 for acid-challenge.

Acid-challenge experiments involved four treatments: (a) acid-shocked = cells that were exposed to pH 4.3 in EG medium for 2 h before acid-challenge; (b) nonshocked = direct acid-challenge of cells at pH 3.0 in EG medium without transient exposure to pH 4.3; (c) acid-adapted = cells that were grown in TSB+G before suspension in pH 2.3 TSB for acid-challenge; and, (d) nonadapted = cells that were grown in TSB-G before suspension in pH 2.3 TSB for acid-challenge. All acid-challenge experiments were performed statically and in triplicate, during which aliquots were collected at timed intervals for the acid-shocked and nonshocked (0.5, 1, 2 and 4 h), and acid-adapted and nonadapted (0, 0.5, 1, 2 and 4 h) treatments. Additionally, aliquots also were collected at timed intervals (0, 0.5, 1, 2 and 4 h) for non-challenged controls. Viable cell counts were enumerated using a spiral plating system (Spiral Plater Model D, Spiral Systems Instruments, Inc., Cincinnati, OH) to plate appropriate dilutions made using sterile, 0.1% buffered peptone water on tryptose phosphate agar supplemented with 0.1% (wt/vol)

sodium pyruvate (TPAP; Difco Laboratories; Leyer and Johnson, 1992). Following aerobic incubation at 35°C for 48 h, colonies were counted using a laser bacteria colony counter (model 500A, Spiral Systems Instruments, Inc.) and a computer assisted spiral bio-assay (CASBA) data processor with Bacterial Enumeration Program E20 (Spiral Systems Instruments, Inc.).

Data analysis. *Salmonella* population data (CFU/ml) obtained during each of three experimental replicates was transformed to $\log_{10}$ CFU/ml for statistical analyses. The minimum detection limit was 1.3 log CFU/ml (20 CFU/ml) based on maximum sensitivity of the spiral plating system with a sample volume of 0.0492 ml plated for viable cell enumeration. Following incubation, plates without detectable colonies were recorded as 1.3 log CFU/ml so that statistical analysis could be performed. Data were evaluated with analysis of variance (AOV) for each individual treatment (acid-shocked, nonshocked, acid-adapted and nonadapted), at each of four individual acid-challenge times (0.5, 1, 2 and 4 h), and means were computed for *Salmonella* populations by strain using the General Linear Models procedure of SAS® (SAS, 2001). When AOV detected effects ($P < 0.05$), mean log values for *Salmonella* strains were separated using Tukey's Studentized Range (HSD) test (SAS, 2001).

RESULTS AND DISCUSSION

Fermentative growth (18 h; 37°C; 130 shakes/min) of *Salmonella* strains in EG medium (6 ml; pH 8.0; 0.4% glucose) reduced the medium pH to approximately 5.9 to 6.1. Comparatively, growth (18h; 37°C; 130 shakes/min) of *Salmonella* strains in TSB (6

ml; pH 7.3) with (1.00%) or without (0.00%) a fermentable carbohydrate (i.e., glucose) resulted in pH values of approximately 4.7 to 4.8 and 8.1 to 8.3, respectively.

Acid-shocked and nonshocked cultures. Mean populations of nonshocked/non-challenged (N/N) *Salmonella* grown in EG medium increased from 8.5 to 8.6 and 8.6 to 8.7 log CFU/ml for susceptible and antimicrobial resistant strains, respectively, to 9.0 to 9.1 and 9.0 to 9.2 after 4 h incubation (Table 7.2). For both the acid-shocked and nonshocked cultures, suspension of cells in the acid-challenge medium (pH 3.0) following washing and centrifugation resulted in similar increases in challenge pH of 0.2 ± 0.05 units (data not shown in tabular form). For the acid-shocked treatment, surviving populations were *Salmonella* strain-dependent ($P < 0.05$) following 0.5, 1 and 4 h of acid-challenge. Mean populations ranged from 7.7 to 8.4 and 8.2 to 8.3 log CFU/ml for susceptible and multi-antimicrobial resistant *Salmonella* strains, respectively, following 0.5 h of acid-challenge (Table 7.2). With the exception of one strain (i.e., *S.* Anatum; susceptible 2; 7.7 log CFU/ml), there were no differences ($P > 0.05$) between acid-shocked *Salmonella* populations (8.2 to 8.4 log CFU/ml) following 0.5 h of acid-challenge. After 1 h of acid challenge, mean acid-shocked *Salmonella* populations ranged from 7.2 to 8.3 and 7.6 to 8.1 log CFU/ml for susceptible and multi-antimicrobial resistant strains, respectively (Table 7.2). Similar to the 0.5 h acid-challenge, susceptible 2 was the most sensitive *Salmonella* strain, with a surviving population of 7.2 log CFU/ml. Mean populations following 4 h of acid-challenge ranged from 3.0 to 4.8 and 2.5 to 3.7 log CFU/ml for susceptible and multi-antimicrobial resistant *Salmonella* strains, respectively (Table 7.2). With the exception of one strain (i.e., *S.* Agona; susceptible 5; 4.8 log CFU/ml) that was more resistant ($P < 0.05$) than four others (i.e.,

susceptible 1, resistant 2, resistant 3 and resistant 4), surviving populations were not different ($P > 0.05$) and ranged from 2.5 to 3.8 log CFU/ml for the remaining nine strains following 4 h of acid-challenge. For the nonshocked treatment, surviving populations were not ($P > 0.05$) *Salmonella* strain-dependent, regardless of acid-challenge time, as mean populations following 2 h of acid-challenge ranged from 6.6 to 7.2 and 6.3 to 7.0 log CFU/ml for antimicrobial susceptible and resistant strains, respectively, while corresponding populations following 4 h of acid-challenge ranged from 4.3 to 5.5 and 3.9 to 4.9 log CFU/ml (Table 7.2).

These results indicated that a low-pH inducible ATR was not evident following transient acid shock, as nonshocked populations were 0.5 to 1.9 and 0.5 to 2.0 log CFU/ml greater than acid-shocked populations following acid-challenges for 2 and 4 h, respectively (Table 7.2). In contrast, Lee *et al.* (1994b) reported that, following a 4 h acid-challenge, stationary-phase cells from pH 4.3 EG medium that were resuspended in pH 3.0 EG medium survived at least 1,000-fold better than stationary phase cells from a pH 7.3 EG medium. Additionally, they reported that shocking stationary phase cells at pH 4.3 for 2 h prior to pH 3.0 acid-challenge resulted in maximum protection against acid stress (Lee *et al.*, 1994b). Differences in challenge pH between results of this study and those of Lee *et al.* (1994b) may have contributed to this discrepancy in results.

Centrifugation and washing of the nonshocked and acid-shocked cells with EG media of corresponding pH values (i.e., pH 7.0 and pH 4.3, respectively), rather than using media of challenge pH values (pH 3.0), elevated the pH of the challenge media (3.2 ± 0.05). This resulted in a weakened challenge environment and did not result in complete inactivation of nonshocked cells after 4 h of acid-challenge, as was previously

reported (Lee *et al.*, 1994b), but still may have been sufficient to result in the increased inactivation rate among previously stressed, perhaps pre-exhausted, acid-shocked cells.

Another experimental difference between this study and that of Lee *et al.* (1994b) involved the recovery media, as Lee *et al.* (1994b) used Luria-Bertani medium (LB media; Miller, 1972) for the enumeration of viable populations following low-pH exposure, while in the present study, we used tryptose phosphate agar supplemented with 0.1% sodium pyruvate (TPAP). Leyer and Johnson (1992) evaluated the conditions necessary for the recovery of acid-exposed (EG medium; pH 5.8; adjusted using 10 N HCl), logarithmic-phase *S.* Typhimurium, following low-pH (lactic acid; 125 mM; pH 3.85) exposure in E buffer (i.e., EG medium without glucose). They concluded that recovery following sublethal injury was poor using LB medium, and subsequently used tryptose phosphate agar supplemented with 0.1% (wt/vol) sodium pyruvate (TPAP) for enumeration of stressed bacterial populations (Leyer and Johnson, 1992). The addition of sodium pyruvate to both LB and EG media reportedly increased recovery by approximately 1000-fold, which may suggest that low-pH stressed cells are hypersensitive to oxygen radicals, and therefore that sodium pyruvate acted as a protective agent, subsequently preventing an underestimation of the actual viable population (Leyer and Johnson, 1992; McDonald *et al.*, 1983).

Lastly, it has been reported that the extent to which a successfully mounted ATR protects a given bacterium depends on serotype and strain (Leyer and Johnson, 1992). Heterogeneity of constitutive acid resistance among the *Salmonella* strains evaluated in this study, coupled with differences in the extent to which they mount a successful ATR, may, at least in part, have contributed to the disparities in acid-challenge surviving

populations that appeared irrespective of antimicrobial susceptibility. While various serotypes were used in this study, Lee *et al.* (1994b) only reported on the stationary-phase ATR of strains of *S.* Typhimurium LT2.

Acid-adapted and nonadapted cultures. Initial (time = 0 h) mean populations of non-challenged (N) *Salmonella* grown in TSB with 1.00% glucose (TSB+G; acid-adapted) ranged from 8.2 to 8.3 log CFU/ml for both susceptible and multi-antimicrobial resistant strains, while corresponding populations after 4 h of incubation ranged from 8.7 to 8.9 and 8.8 to 9.3 log CFU/ml, respectively (Table 7.3). For both the acid-adapted and nonadapted treatments, resuspension of harvested cells in challenge media (pH 2.3) increased the pH to 2.6 ± 0.1 units (data not shown in tabular form).

Acid-adapted *Salmonella* populations following 0.5 and 1 h of acid-challenge were strain-dependent ($P < 0.05$). After 0.5 h of acid-challenge, one strain (i.e., *S.* Typhimurium DT104; resistant 1) was more susceptible than two others (i.e., resistant 2 and resistant 3); however, there were no differences ($P > 0.05$) in mean surviving populations between the remaining nine *Salmonella* strains (Table 7.3). Similarly, following 1 h of acid-challenge, one strain (i.e., *S.* Anatum; susceptible 2; 7.6 log CFU/ml) was more susceptible than seven others, while there were no differences ($P > 0.05$) in mean surviving populations between eight of the remaining nine *Salmonella* strains. Acid-adapted surviving populations were not ($P > 0.05$) *Salmonella* strain-dependent following the remaining acid-challenge times, as populations ranged from 7.3 to 7.7 and 7.4 to 7.7 log CFU/ml for susceptible and multi-antimicrobial resistant *Salmonella* strains following 2 h of acid-challenge, respectively, while corresponding

populations, following 4 h of acid-challenge, ranged from 6.1 to 6.4 and 6.4 to 6.6 log CFU/ml, respectively (Table 7.3).

Initial (time = 0 h) mean populations for non-challenged (N) *Salmonella* grown in TSB with 0.00% glucose (TSB-G; nonadapted) ranged from 8.5 to 8.8 and 8.4 to 8.7 log CFU/ml for antimicrobial susceptible and resistant strains, respectively, while populations after 4 h of incubation ranged from 9.0 to 9.2 log CFU/ml for both susceptible and resistant strains (Table 7.4). Nonadapted populations following 0.5, 1 and 4 h of acid-challenge did not ($P > 0.05$) depend on *Salmonella* strain. However, after 2 h of acid-challenge, one susceptible (i.e., *S.* Anatum; susceptible 2) and one antimicrobial resistant (i.e., *S.* Typhimurium DT104; resistant 1) strain were more sensitive ($P < 0.05$) than six of the others, as mean populations were 3.2 and 2.9 log CFU/ml, respectively, while mean populations of the remaining eight strains did not differ ($P > 0.05$) from each other and ranged from 4.1 to 4.8 log CFU/ml (Table 7.4). Again, acid resistance may, at least in part, be serotype- and/or strain-dependent (Leyer and Johnson, 1992).

These results indicated that, in contrast to acid-shocking, an induced ATR was evident following acid-adaptation, as acid-adapted surviving populations were 2.4 to 4.5 and 4.2 to 4.8 log CFU/ml higher than corresponding nonadapted populations following acid-challenge for 2 and 4 h, respectively (Tables 7.3 and 7.4). These findings supported previous research indicating that the presence of a fermentable carbohydrate in a growth medium is an effective means of inducing acid tolerance (Buchanan and Edelson, 1996; Wilde *et al.*, 2000). Buchanan and Edelson (1996) reported increased acid resistance among each of seven *Escherichia coli* O157:H7 and two non-O157:H7 strains following

growth (37°C; 18 h) in TSB containing 1.00% (wt/vol) dextrose. Wilde *et al.* (2000) reported RpoS-independent induction of stationary-phase acid tolerance among *S.* Enteritidis PT4 isolates following overnight growth in TSB, nutrient broth, and tryptone soya broth, all containing 0.25% glucose.

The decline of pH, over time, associated with food fermentation may provide a means by which microorganisms become acid-adapted. Many foods (e.g., fermented dairy products and fermented sausages) rely on this process for their production and development of associated organoleptic characteristics (Jay, 2000). A study evaluating nine different commercially available brands of semi-dry sausage reported pH values ranging from 4.5 to 5.2 with a mean of 4.87, and for summer-type sausages, pH values of 4.5 to 4.7 have been reported following a fermentation period of 72 h (Jay, 2000). During this study, growth of *Salmonella* strains in TSB (pH 7.3) containing 1.00% glucose resulted in comparable pH values of approximately 4.7 to 4.8. Furthermore, acid-adaptation through the gradual pH decline over time associated with fermentative growth provided protection to subsequent acid stress, allowing for substantially increased survival (up to 4.8 log CFU/ml difference between acid-adapted and nonadapted cultures).

In addition to the production of fermented foods, transient or prolonged bacterial exposure to sublethal pH also may occur during the food-animal to muscle-food conversion process. Efficacies of organic acid solution rinsing and ambient to hot water washing in reducing bacterial populations on carcass surfaces has been extensively researched (Dickson and Anderson, 1992; Dorsa, 1997; Gorman *et al.*, 1995; Hardin *et al.*, 1995) and, as a result, these technologies have been implemented throughout the

commercial red meat and poultry industries as a means of improving the microbiological quality of carcasses. The combination of low-pH solution carcass rinsing and ambient or hot water carcass washing provides the opportunity for diluted, sublethal pH microenvironments within a slaughtering facility and, following bacterial exposure to these microenvironments, a means by which acid tolerance may be developed (Samelis *et al.*, 2001b). Successful induction of a stationary-phase ATR among bacteria exposed to the declining and reduced pHs associated with fermented food manufacturing or organic acid solution use during food production, may result in an increased ability to endure otherwise fatal low-pH exposures associated with the stomach or intestinal and intracellular environments (Idziak and Suvanmongkol, 1971; Wilmes-Riesenberg *et al.*, 1996).

Multi-antimicrobial resistant *Salmonella* has been isolated from various foods that undergo minimal processing before consumption, including raw vegetables (green onions, clover and alfalfa sprouts), herbs and spices (cilantro, coriander, cumin, rosemary, oregano, thyme and chili powder), orange juice, ice cream, and various types of cheeses (Kiessling *et al.*, 2002). Presence of *Salmonella* in those products is a public health concern, especially regarding the possibility of increased morbidity and mortality resulting from antimicrobial resistant bacterial infections and the subsequent inability to successfully treat them (Cohen, 1997; Tollefson *et al.*, 1999). Under the conditions of the present study, results comparing the acid resistance between susceptible and multi-antimicrobial resistant *Salmonella* strains in minimal and complex media acidified with HCl, following preparation under acid tolerance-inducing and noninducing conditions, suggested that antimicrobial resistance did not afford low-pH cross-protection. This lack

of cross-protection between antimicrobial resistance and the ability to survive low-pH stress exposure may result from the expression of different genes coding for adaptive or resistant mechanisms. Therefore, the previous induction of a stationary-phase ATR afforded increased acid resistance, but the ability of *Salmonella* to survive low-pH exposure appeared to be independent of antimicrobial susceptibility.

Table 7.1. *Salmonella* isolates used during comparative analysis of stationary-phase acid tolerance, identified by serotype, serogroup, source and antimicrobial resistance pattern.

Salmonella Serotype[a]	O group	Source[b]	Antimicrobial Resistance Pattern[c]
Susceptible:			
(1) Saint-Paul	B	Fed Plant 2	Susceptible to all antimicrobials
(2) Anatum	E_1	Fed Plant 3	Susceptible to all antimicrobials
(3) Mbandaka	C_1	Fed Plant 1	Susceptible to all antimicrobials
(4) Agona	B	Non-fed Plant 1	Susceptible to all antimicrobials
(5) Agona	B	Non-fed Plant 1	Susceptible to all antimicrobials
Resistant:			
(1) Typhimurium DT104	B	ATCC 700408	ACSSuT + Trimethoprim/sulfamethoxazole
(2) Reading	B	Fed Plant 1	ACSSuT + Amoxicillin/clavulanic acid, Ampicillin/sulbactam and Gentamicin
(3) Reading	B	Fed Plant 1	ACSSuT + Amoxicillin/clavulanic acid, Ampicillin/sulbactam and Gentamicin
(4) Typhimurium DT104 (var. Copenhagen)	B	Fed Plant 2	ACSSuT + Amoxicillin/clavulanic acid and Ampicillin/sulbactam
(5) Typhimurium DT104 (var. Copenhagen)	B	Fed Plant 2	ACSSuT + Amoxicillin/clavulanic acid and Trimethoprim/sulfamethoxazole

[a] Susceptible and multi-antimicrobial resistant *Salmonella* strains are subsequently referred to as Susceptible 1 through Susceptible 5 and Resistant 1 through Resistant 5 in tables 7.2-7.4.

[b] *Salmonella* strains used during this study were derived from animal hide samples (N = 160) collected in four commercial beef packing plants, of which three processed primarily heifers and steers (fed plants) and the remaining one processed primarily cows and bulls (non-fed plant).

[c] ACSSuT: common abbreviations for ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline.

Table 7.2. Means (standard deviation) of *Salmonella*[a] counts (log CFU/ml) for each of ten strains and three treatment combinations (nonshocked/non-challenged[b], N/N; nonshocked/challenged[c], N/C; and, acid-shocked/challenged[d], S/C), at each acid-challenge time following recovery on tryptose phosphate agar containing 0.1% sodium pyruvate (TPAP).

		Acid-Challenge Time (h)[e]				
Strain[e]	Treatment	0.0	0.5	1.0	2.0	4.0
Susceptible 1	N/N	8.6 (0.11)	8.7 (0.06)	8.7 (0.03)	8.8 (0.02)	9.0 (0.06)
	N/C		8.6 (0.04)	8.3 (0.19)	6.9 (1.04)	4.5 (0.29)
	S/C		8.3^{w} (0.11)	7.8^{wxy} (0.15)	5.7 (0.66)	3.0^{x} (0.51)
Susceptible 2	N/N	8.6 (0.18)	8.6 (0.06)	8.8 (0.08)	8.4 (0.51)	9.1 (0.01)
	N/C		8.7 (0.04)	8.3 (0.11)	6.8 (0.54)	4.4 (0.30)
	S/C		7.7^{x} (0.37)	7.2^{z} (0.09)	5.1 (0.93)	3.4^{wx} (0.16)
Susceptible 3	N/N	8.5 (0.18)	8.5 (0.04)	8.8 (0.13)	8.7 (0.08)	9.1 (0.07)
	N/C		8.6 (0.04)	8.5 (0.40)	6.6 (0.44)	4.3 (1.15)
	S/C		8.2^{w} (0.05)	8.0^{wxy} (0.14)	5.9 (0.60)	3.8^{wx} (0.07)
Susceptible 4	N/N	8.6 (0.05)	8.6 (0.05)	8.7 (0.05)	8.7 (0.03)	9.1 (0.03)
	N/C		8.6 (0.07)	8.3 (0.24)	6.8 (0.51)	4.8 (0.22)
	S/C		8.4^{w} (0.04)	8.1^{wx} (0.12)	5.8 (0.95)	3.7^{wx} (0.04)
Susceptible 5	N/N	8.6 (0.03)	8.7 (0.07)	8.7 (0.07)	8.7 (0.13)	9.1 (0.01)
	N/C		8.6 (0.06)	8.3 (0.13)	7.2 (0.57)	5.5 (0.10)
	S/C		8.4^{w} (0.27)	8.3^{w} (0.08)	5.8 (0.99)	4.8^{w} (0.40)
Resistant 1	N/N	8.6 (0.06)	8.6 (0.01)	8.7 (0.05)	8.8 (0.09)	9.2 (0.05)
	N/C		8.6 (0.03)	8.2 (0.08)	6.5 (0.28)	4.9 (0.51)
	S/C		8.3^{w} (0.10)	7.8^{xy} (0.12)	5.9 (0.36)	3.4^{wx} (1.05)
Resistant 2	N/N	8.6 (0.03)	8.7 (0.03)	8.7 (0.10)	8.8 (0.11)	9.2 (0.06)
	N/C		8.7 (0.07)	8.6 (0.18)	7.0 (0.45)	3.9 (1.10)
	S/C		8.2^{w} (0.11)	8.1^{wx} (0.22)	5.4 (0.92)	2.5^{x} (0.12)
Resistant 3	N/N	8.6 (0.04)	8.7 (0.06)	8.8 (0.09)	8.8 (0.02)	9.2 (0.08)
	N/C		8.6 (0.13)	8.3 (0.31)	6.5 (0.89)	4.5 (0.33)
	S/C		8.2^{wx} (0.14)	7.6^{y} (0.12)	5.2 (0.84)	2.5^{x} (0.20)
Resistant 4	N/N	8.7 (0.05)	8.6 (0.12)	8.6 (0.06)	8.5 (0.39)	9.0 (0.11)
	N/C		8.7 (0.03)	8.4 (0.15)	6.9 (0.45)	4.5 (0.12)
	S/C		8.2^{w} (0.08)	7.9^{wxy} (0.22)	5.0 (0.79)	3.2^{x} (0.81)
Resistant 5	N/N	8.6 (0.12)	8.6 (0.04)	8.7 (0.10)	8.8 (0.03)	9.1 (0.04)
	N/C		8.7 (0.11)	8.1 (0.12)	6.3 (0.22)	4.8 (0.77)
	S/C		8.2^{w} (0.07)	8.0^{wxy} (0.14)	5.8 (1.00)	3.7^{wx} (0.90)

[a] *Salmonella* strains were prepared overnight (18 h; 37°C) in EG medium (0.4% glucose) at pH 8.0, adjusted with 5N NaOH.

[b] N/N (nonshocked/non-challenged) strains were resuspended in EG medium (0.4% glucose) at pH 7.0.

[c] N/C (nonshocked/challenged) strains were resuspended in EG medium (0.4% glucose) at pH 3.0, adjusted with 5N HCL.

[d] S/C (acid-shocked/challenged) strains were shocked in EG medium (0.4% glucose) at pH 4.3, adjusted with 5N HCL, for 2 h, then resuspended in EG medium at pH 3.0.

[e] Analysis of variance determined that N/C surviving populations did not ($P > 0.05$) depend on *Salmonella* strain regardless of acid-challenge time, however, S/C surviving populations were *Salmonella* strain-dependent ($P < 0.05$) at the 0.5, 1.0 and 4.0 h acid-challenge times.

[wxyz] Means in the same column, within a treatment, bearing a common superscript letter are not different ($P > 0.05$).

Table 7.3. Means (standard deviation) of *Salmonella*[a] counts (log CFU/ml) for each of ten acid-adapted strains and two treatment combinations (non-challenged (N)[b] and challenged (C)[c]), at each acid-challenge time following recovery on tryptose phosphate agar containing 0.1% sodium pyruvate (TPAP).

		Challenge Time (h)[d]				
Strain[d]	Treatment	0.0	0.5	1.0	2.0	4.0
Susceptible 1	N	8.3 (0.15)	8.3 (0.05)	8.4 (0.03)	8.6 (0.08)	8.7 (0.25)
	C	8.4 (0.04)	8.4^{xy} (0.07)	8.4^{xy} (0.10)	7.5 (0.16)	6.4 (0.78)
Susceptible 2	N	8.2 (0.16)	8.2 (0.14)	8.4 (0.04)	8.6 (0.07)	8.8 (0.31)
	C	8.3 (0.06)	8.4^{xy} (0.10)	7.6^{z} (0.27)	7.3 (0.48)	6.2 (0.66)
Susceptible 3	N	8.3 (0.15)	8.4 (0.01)	8.4 (0.06)	8.6 (0.02)	8.9 (0.34)
	C	8.3 (0.06)	8.4^{xy} (0.04)	7.9^{yz} (0.06)	7.5 (0.40)	6.1 (0.72)
Susceptible 4	N	8.3 (0.14)	8.3 (0.13)	8.4 (0.11)	8.5 (0.03)	8.9 (0.21)
	C	8.3 (0.06)	8.3^{xy} (0.13)	8.2^{xy} (0.28)	7.7 (0.19)	6.4 (0.57)
Susceptible 5	N	8.3 (0.06)	8.3 (0.04)	8.4 (0.09)	8.5 (0.01)	8.8 (0.14)
	C	8.4 (0.06)	8.5^{xy} (0.06)	8.1^{xy} (0.22)	7.5 (0.29)	6.4 (0.91)
Resistant 1	N	8.2 (0.05)	8.3 (0.05)	8.3 (0.04)	8.4 (0.03)	8.8 (0.04)
	C	8.1 (0.02)	8.3^{y} (0.06)	8.1^{xyz} (0.20)	7.4 (0.32)	6.4 (0.71)
Resistant 2	N	8.3 (0.08)	8.4 (0.02)	8.4 (0.02)	8.5 (0.11)	9.2 (0.18)
	C	8.3 (0.07)	8.5^{x} (0.10)	8.4^{xy} (0.04)	7.6 (0.14)	6.4 (0.58)
Resistant 3	N	8.3 (0.14)	8.4 (0.05)	8.5 (0.03)	8.6 (0.06)	9.3 (0.50)
	C	8.4 (0.01)	8.5^{x} (0.08)	8.4^{xy} (0.07)	7.7 (0.21)	6.6 (0.30)
Resistant 4	N	8.2 (0.09)	8.3 (0.07)	8.3 (0.10)	8.5 (0.04)	9.0 (0.14)
	C	8.3 (0.10)	8.4^{xy} (0.04)	8.3^{xy} (0.04)	7.6 (0.15)	6.5 (0.59)
Resistant 5	N	8.3 (0.11)	8.3 (0.02)	8.4 (0.04)	8.6 (0.07)	8.9 (0.08)
	C	8.2 (0.14)	8.4^{xy} (0.04)	8.4^{x} (0.14)	7.5 (0.25)	6.4 (0.81)

[a] *Salmonella* strains were prepared overnight (18 h; 37°C) in tryptic soy broth containing 1.0% glucose (TSB+G) at pH 7.3.

[b] N (non-challenged) strains were resuspended in tryptic soy broth containing 0.25% glucose (TSB) at pH 7.3.

[c] C (challenged) strains were resuspended in tryptic soy broth containing 0.25% glucose (TSB) at pH 2.3, adjusted with 5N HCL.

[d] Analysis of variance determined that surviving populations were *Salmonella* strain-dependent ($P < 0.05$) at the 0.5 and 1.0 h acid-challenge times.

[xyz] Means in the same column, within a treatment, bearing a common superscript letter are not different ($P > 0.05$).

Table 7.4. Means (standard deviation) of *Salmonella*[a] counts (log CFU/ml) for each of ten nonadapted strains and two treatment combinations (non-challenged (N)[b] and challenged (C)[c]), at each acid-challenge time following recovery on tryptose phosphate agar containing 0.1% sodium pyruvate (TPAP).

		Acid-Challenge Time (h)[d]				
Strain[d]	Treatment	0.0	0.5	1.0	2.0	4.0
Susceptible 1	N	8.6 (0.10)	8.6 (0.13)	8.6 (0.03)	9.0 (0.11)	9.0 (0.08)
	C	8.5 (0.21)	8.1 (0.28)	7.3 (0.01)	4.8^{x} (0.42)	2.0 (0.70)
Susceptible 2	N	8.6 (0.15)	8.7 (0.33)	8.7 (0.02)	9.1 (0.44)	9.0 (0.01)
	C	8.5 (0.11)	8.1 (0.28)	7.4 (0.86)	3.2^{yz} (0.31)	1.9 (0.56)
Susceptible 3	N	8.8 (0.22)	8.9 (0.11)	9.0 (0.01)	9.0 (0.16)	9.2 (0.03)
	C	8.7 (0.22)	8.0 (0.22)	7.3 (0.21)	4.1^{xyz} (0.51)	1.9 (0.59)
Susceptible 4	N	8.5 (0.15)	8.8 (0.34)	8.7 (0.01)	9.0 (0.37)	9.1 (0.03)
	C	8.4 (0.17)	8.2 (0.39)	7.7 (0.37)	4.5^{x} (0.08)	1.8 (0.52)
Susceptible 5	N	8.6 (0.11)	8.8 (0.30)	8.8 (0.03)	9.1 (0.53)	9.1 (0.08)
	C	8.6 (0.14)	8.2 (0.19)	7.7 (0.45)	4.6^{x} (0.29)	2.0 (0.68)
Resistant 1	N	8.4 (0.15)	8.8 (0.38)	8.6 (0.01)	9.1 (0.54)	9.0 (0.03)
	C	8.3 (0.10)	7.6 (0.03)	7.5 (0.44)	2.9^{z} (0.65)	1.8 (0.52)
Resistant 2	N	8.6 (0.16)	8.6 (0.17)	9.1 (0.03)	9.0 (0.17)	9.1 (0.03)
	C	8.5 (0.19)	8.2 (0.15)	7.6 (0.37)	4.7^{x} (0.29)	1.9 (0.63)
Resistant 3	N	8.6 (0.25)	8.8 (0.27)	8.8 (0.04)	8.9 (0.12)	9.2 (0.06)
	C	8.6 (0.11)	8.4 (0.34)	7.6 (0.68)	4.4^{xy} (0.31)	1.8 (0.52)
Resistant 4	N	8.7 (0.23)	8.8 (0.33)	9.2 (0.01)	8.9 (0.04)	9.1 (0.04)
	C	8.6 (0.27)	8.2 (0.28)	7.5 (0.36)	4.2^{xyz} (0.58)	1.9 (0.56)
Resistant 5	N	8.6 (0.22)	8.8 (0.31)	8.8 (0.01)	8.9 (0.05)	9.2 (0.06)
	C	8.8 (0.17)	8.2 (0.31)	7.6 (0.35)	4.5^{x} (0.58)	1.8 (0.52)

[a] *Salmonella* strains were prepared overnight (18 h; 37°C) in tryptic soy broth containing 0.0% glucose (TSB-G) at pH 7.3.

[b] N (non-challenged) strains were resuspended in tryptic soy broth containing 0.25% glucose (TSB) at pH 7.3.

[c] C (challenged) strains were resuspended in tryptic soy broth containing 0.25% glucose (TSB) at pH 2.3, adjusted with 5N HCL.

[d] Analysis of variance determined that surviving populations were *Salmonella* strain-dependent ($P < 0.05$) at the 2.0 h acid-challenge time.

^{xyz} Means in the same column, within a treatment, bearing a common superscript letter are not different ($P > 0.05$).

CHAPTER VIII

THERMAL INACTIVATION OF SUSCEPTIBLE AND MULTI-ANTIMICROBIAL RESISTANT *SALMONELLA* GROWN IN THE ABSENCE OR PRESENCE OF GLUCOSE

ABSTRACT

Heat resistance of composites of five susceptible or five multi-antimicrobial resistant *Salmonella* strains, grown to stationary-phase in glucose-free tryptic soy broth supplemented with 0.6% yeast extract (TSBYE-G; nonadapted), or in regular (0.25% glucose) TSBYE or in TSBYE-G with 1.00% added glucose (TSBYE+G; acid-adapted), was determined at 55, 57, 59 and 61°C. Composite cultures were heated in sterile, 0.1% buffered peptone water (50 µl) in heat-sealed capillary tubes, which were immersed in a thermostatically controlled, circulating water-bath. At predetermined time intervals, duplicate capillary tubes (three replicates) were removed and viable cell populations were enumerated on tryptic soy agar supplemented with 0.6% yeast extract and 1.0% sodium pyruvate. Decimal reduction times (*D*-values) were calculated from survival curves having r^2 values of > 0.90. $D_{59°C}$-values increased ($P < 0.05$) from 0.50 to 0.58 to 0.66 min for TSBYE-G, TSBYE, and TSBYE+G cultures, respectively. $D_{61°C}$-values of antimicrobial susceptible *Salmonella* increased ($P < 0.05$) from 0.14 to 0.19 as glucose level increased from 0.00 to 1.00%, respectively, while $D_{61°C}$-values of multi-antimicrobial resistant *Salmonella* were higher (0.21 min; $P < 0.05$) and did not differ ($P > 0.05$) between TSBYE-G or TSBYE+G cultures. When averaged across glucose levels

and temperatures, there were no differences ($P > 0.05$) in *D*-values between susceptible and multi-antimicrobial resistant *Salmonella* inocula. Collectively, *D*-values ranged from 4.23 to 5.39, 1.47 to 1.81, 0.50 to 0.66, and 0.16 to 0.20 min for *Salmonella* inactivated at 55, 57, 59 and 61°C, respectively. Temperature coefficients of inactivation (z_D-values) were 1.20, 1.48 and 1.49°C for *Salmonella* grown in TSBYE+G, TSBYE and TSBYE-G, respectively, while corresponding activation energies of inactivation were 497, 493 and 494 kJ/mol. Results from this study suggested a cross-protective effect of acid-adaptation on thermal inactivation, but no association between antimicrobial susceptibility and the ability of *Salmonella* to survive heat stress.

INTRODUCTION

Exposure to heat has long been recognized as a primary method for preserving foods, typically resulting in the production of either a pasteurized or commercially sterile food product, or the more classic "cooking of food" in preparation for consumption (Cassens, 1994). Characteristic survival models describe the effects of heat stress on bacterial populations in terms of decimal reduction time (*D*-value); that is, thermal inactivation is generally known to increase exponentially with an increase in temperature (Gould, 1989).

Various factors may influence heat resistance of microorganisms. Inherently, different types of microorganisms subjected to identical heat stressing conditions react differently, as some are able to withstand higher temperatures for extended periods of time (Pflug and Holcomb, 1991; Brackett *et al.*, 2001). Heat resistance also is affected by various environmental influences during growth and formation of cells or spores prior

to heat stress, such as, among others, metabolic phase, growth temperature, and nutrient availability (Pflug and Holcomb, 1991). Heat shocking *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes* and *Aeromonas hydrophila* cells during exponential growth resulted in increased thermal resistance (Foegeding and Leasor, 1990; Juneja *et al.*, 1998; Katsui *et al.*, 1982; Schuman *et al.*, 1997). Additionally, protective mechanisms allowing for an adaptive response to low-pH exposure have been reported among several microorganisms, including *Salmonella* (Foster, 2000). Among *Salmonella* serotypes, *S.* Typhimurium has received the majority of attention regarding its acid adaptive capabilities (Foster and Spector, 1995; Leyer and Johnson, 1992). *Salmonella* Typhimurium possesses two growth phase dependent, low-pH inducible systems, each capable of mounting an acid tolerance response (ATR; Foster, 2000; Foster and Spector, 1995).

In addition to the protection provided against subsequent low-pH exposure, acid-adapted *S.* Typhimurium may exhibit cross-protection against heat, osmotic and oxidative stresses (Foster and Spector, 1995; Lee *et al.*, 1995; Leyer and Johnson, 1993; Wilde *et al.*, 2000). This stress-induced cross-protection phenomenon also has been reported among other bacteria, including *E. coli* O157:H7 and *L. monocytogenes* (Buchanan and Edelson, 1999; Farber and Pagotto, 1992; Rowe and Kirk, 1999; Ryu and Beuchat, 1999). Lastly, environmental factors during heat exposure, such as carbohydrate availability, a_w and salt concentration, pH and the presence of organic or inorganic compounds, in addition to conditions during cell recovery, influence the ability of microorganisms to survive or repair damage associated with heat stress (Pflug and Holcomb, 1991). For

example, Juneja *et al.* (2001) reported that increased resistance among *Salmonella* occurs when the organism is subjected to thermal stress in ground meat versus chicken broth.

In recent years, recognition, or emergence of antimicrobial resistant bacterial pathogens has resulted in worldwide concern focused on potential for increased morbidity and mortality resulting from failing antimicrobial treatments (Cohen, 1997; Tollefson *et al.*, 1999). In the United States, the prevalence of *Salmonella* resistant to at least one antimicrobial has increased from 16% in 1980 to 37% in 1996, when the first outbreak involving a multi-antimicrobial resistant strain of *Salmonella* (*S.* Typhimurium DT104) occurred (CDC, 1997; Dabney *et al.*, 1997; MacDonald *et al.*, 1987). More recently, antimicrobial resistant *Salmonella* have been isolated from ground meats sampled at retail supermarkets in the Northeastern United States (White *et al.*, 2001). White *et al.* (2001) reported a prevalence of *Salmonella* in ground chicken, turkey, pork and beef of 35, 24, 16 and 6%, respectively. Although samples were collected in only one U.S. city, of the 200 ground meat samples analyzed, 20% (n = 41) were *Salmonella*-positive, yielding 45 isolates representing thirteen serotypes. Antimicrobial susceptibility of the 45 isolates to a panel of 17 antimicrobials used by the National Antimicrobial Resistance Monitoring System (NARMS) was determined (Tollefson *et al.*, 1998). Of the 45 isolates, 84% (n = 38) were resistant to at least one of the antimicrobials screened, and 53% (n = 24) were resistant to at least three. Among the multi-antimicrobial resistant isolates were five isolates of *S.* Agona and two isolates of *S.* Typhimurium DT208 simultaneously resistant to nine and twelve antimicrobials, respectively. While all of the *Salmonella* isolates were susceptible to ciprofloxacin, 16% were resistant to ceftriaxone, the drug of choice for combating salmonellosis in children (White *et al.*, 2001).

In addition to its presence in ground meats, multi-antimicrobial resistant *Salmonella* has been isolated from numerous other domestic and imported foods, including sprouts, spices, frozen seafood, freshwater fish, ice cream, meat and bone meal, herbs, cheese and lettuce (Kiessling *et al.*, 2002). In a study evaluating the antimicrobial susceptibility of 502 *Salmonella* strains isolated from various foods and associated samples between 1999 and 2000, 49.2% (n = 247) were resistant to at least one of the antimicrobials tested, and of those, 31.2% (n = 77) were multi-antimicrobial resistant (Kiessling *et al.*, 2002).

Presence of viable susceptible and antimicrobial resistant bacterial pathogens at the retail level demonstrates the importance of safe product handling and proper preparation during cooking. Information regarding the relationship between antimicrobial susceptibility and the ability to resist heat stress is lacking. Therefore, the objective of this study was to determine if heat resistance differences existed between inocula of susceptible and multi-antimicrobial resistant *Salmonella* strains prepared under acid-tolerance inducing and noninducing conditions.

MATERIALS AND METHODS

Salmonella. Strains of *Salmonella* used during this study are listed in Table 8.1, which, with the exception of *S.* Typhimurium DT104 (ATCC 700408), were all isolated from animal hides following stunning and exsanguination, but before hide opening and subsequent hide removal, in four commercial beef packing plants (Chapter VI). Following biochemical confirmation (API 20E, bioMerieux, Hazelwood, MO) of the *Salmonella* isolates, antimicrobial susceptibility was determined (Chapter VI) using the

disk-diffusion method in accordance with experimental standards and procedures established by the National Committee for Clinical Laboratory Standards (Bauer *et al.*, 1966; NCCLS, 1999a, 1999b). Antimicrobial selection, including concentration, was based upon the emergence of drug-resistant *S.* Typhimurium DT104 (Akkina *et al.*, 2000; Fey *et al.*, 2000; Walker *et al.*, 2000), previously published recommendations for routine testing by veterinary diagnostic laboratories (NCCLS, 1999b), and manufacturer's recommendations (BBL™ Sensi-Disc™, Becton Dickinson, Sparks, MD). Briefly, cell suspensions were standardized ($1x10^8$ colony forming units) using the BBL Prompt™ system (BBL Prompt™, Becton Dickinson), and following spread-plating onto Mueller Hinton agar (Difco Laboratories, Becton Dickinson), antimicrobial-containing discs (BBL™ Sensi-Disc™, Becton Dickinson) were dispensed and tamped into place. Following incubation (17 ± 1 h; 35° C), the plates were removed and the diameters of the zones of inhibition were measured for each antimicrobial. Susceptibility to the following antimicrobials (concentration) was determined: amoxicillin and clavulanic acid (20/10 μg), ampicillin (10 μg), ampicillin and sulbactam (10/10 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), gentamicin (10 μg), levofloxacin (5 μg), streptomycin (10 μg), sulfonamides (trisulfapyramidine, triple sulfa) (250 μg), tetracycline (30 μg), and the combination of trimethoprim and sulfamethoxazole (1.25/23.75 μg; Chapter VI). Cultures were maintained at 4°C with monthly transfers on tryptic soy agar (TSA; Difco Laboratories) slants. Serotype confirmation and subsequent phage typing of *S.* Typhimurium was performed at the National Veterinary Services Laboratory (NVSL/USDA, Ames, IA).

Culture preparation. Culture media used in this study included glucose-free tryptic soy broth (pH 7.3) supplemented with 0.6% yeast extract (TSBYE-G), commercial TSBYE containing 0.25% glucose (TSBYE), or glucose-free TSBYE supplemented with 1.00% glucose (TSBYE+G; Difco Laboratories). Working stock cultures were subcultured twice, each time for 18 h at 37°C in 9 ml of TSBYE, at which time *Salmonella* strains, individually, were grown for 18 h at 37°C in 9 ml of TSBYE-G, TSBYE and TSBYE+G to produce stationary-phase cells exhibiting nonadapted and induced acid tolerant characteristics (Buchanan and Edelson, 1996; Wilde *et al.*, 2000). Following overnight incubation (37°C), a homogeneous 0.2 ml aliquot was removed from each culture. Cells were harvested (3 min; 13,000 x g) and washed in an equal volume of sterile, 0.1% (wt/vol) buffered peptone water (Difco Laboratories). After a second washing, cell fractions of each strain were combined and resuspended to approximately 10^8 cells/ml in sterile, 0.1% buffered peptone water, thereby producing six, five-strain culture composites; three each of susceptible and multi-antimicrobial resistant *Salmonella*, each grown in the presence (0.25 or 1.00%) or absence of the fermentable carbohydrate (i.e., glucose).

Heat resistance. Thermal inactivation experiments were performed following previously reported procedures evaluating thermal resistance of *Salmonella* (Brackett *et al.*, 2001; Jung and Beuchat, 2000), *L. monocytogenes* (Foegeding and Leasor, 1990; Lou and Yousef, 1996) and *A. hydrophila* (Schuman *et al.*, 1997) in liquid whole eggs, egg fractions, and phosphate buffer. Capillary tubes (Kimax-51, 0.8 to 1.1 by 90 mm, Kimble Products, Vineland, NJ) used in thermal inactivation experiments were sterilized by placing 10 each into screw-capped test tubes (16 by 125 mm) and autoclaving. Each

culture composite was dispensed (50 µl) into capillary tubes using a 50 µl syringe (Hamilton Gastight® #1705, Hamilton Company, Reno, NV) with a 22-gauge, 10.2 cm hypodermic needle with a deflected (septum) point (Popper & Sons, Inc., New Hyde Park, NY). Capillary tubes were then heat-sealed manually using a propane torch, during which care was taken to avoid heating the cell suspension. Immediately after sealing, capillary tubes were suspended in a thermostatically controlled, circulating water bath (Isotemp 2013S; Fisher Scientific, Pittsburgh, PA). During heating, capillary tubes were completely submerged in the water bath using a suspended, screen-covered test tube rack. For each cell suspension, at each challenge temperature, duplicate capillary tubes were removed at equally spaced time intervals for each of the following challenge temperatures: (1) at 55°C, samples were removed at 0, 3, 6, 9, 12, 15 and 18 min.; (2) at 57°C, samples were removed at 0, 45, 90, 135, 180, 225 and 270 s; (3) at 59°C, samples were removed at 0, 30, 60, 90, 120, 150 and 180 s; and, (4) at 61°C, samples were removed at 0, 10, 20, 30, 40 and 50 s. After heating, capillary tubes were cooled in a beaker containing an ice-water mixture, and then sanitized by immersion in sodium hypochlorite (500 ppm; pH 6.5). Residual sanitizer was removed by two sequential immersions in sterile, distilled water. Following rinsing, each capillary tube was aseptically transferred using sterile forceps into a 16 by 125 mm test tube containing 9 ml of sterile, 0.1% buffered peptone water. Capillary tubes were finely crushed using a sterile glass rod while contained within the 16 by 125 mm test tubes. A homogeneous sample was then removed and serial dilutions were made using sterile, 0.1% buffered peptone water. Viable cell populations were enumerated by plating (0.1 ml) appropriate dilutions, in duplicate, on tryptic soy agar supplemented with 0.6% yeast extract

(TSAYE) and 1.0% sodium pyruvate (TSAYE+P; Difco Laboratories). Plates were incubated at 28 ± 1°C for 72 h, at which time colonies were counted manually and recorded as colony-forming units per ml (CFU/ml).

Data analysis. Thermal inactivation experiments were performed in triplicate, during which duplicate samples (capillary tubes) were removed and analyzed at each sampling time. Resulting *Salmonella* populations (CFU/ml) were transformed to $\log_{10}$ CFU/ml for statistical analyses. The minimum detection limit for plated sample volumes (0.1 ml) was 3.3 log CFU/ml (1 CFU/ml), based on maximum sensitivity without further dilution of the capillary tube-contained sample (50 µl) following crushing. Counts for samples without detectable colonies (< 1) were recorded as 3.2 log CFU/ml (0.9 CFU/ml) so that statistical analysis could be performed. For each experimental replicate, involving each challenge temperature, glucose level, and *Salmonella* culture composite, survival curves were constructed by plotting the log of surviving counts versus their corresponding challenge times (Figure 8.1). Following linear regression analysis using the REG procedure of SAS® (SAS, 2001), only survivor curves with more than five values in the straight portion and having a coefficient of determination (r^2) greater than 0.90 were used (Table 8.2; Juneja and Eblen, 2000; Juneja *et al.*, 2001). Decimal reduction time (*D*-value) was calculated as the negative reciprocal of the slope of the linear regression line of the survivor curve ($D = -\text{slope}^{-1}$; Foegeding and Leasor, 1990; Jung and Beuchat, 2000; Lou and Yousef, 1996; Schuman *et al.*, 1997).

Data (*D*-values) were evaluated with analysis of variance (AOV) at each challenge temperature (55, 57, 59 and 61°C) using the model: $y = a + x_1 + x_2 + x_1x_2$. Least-squares means were computed for *D*-values by glucose level (x_1), *Salmonella*

culture composite (x_2), and glucose level x *Salmonella* culture (x_1x_2) effects using the General Linear Models procedure of SAS® (SAS, 2001). At 55°C, the glucose level fixed effect was essentially significant ($P = 0.0511$), while *Salmonella* culture composite was not ($P > 0.05$; Table 8.3). Not one of the fixed effects was significant ($P > 0.05$) at 57°C; at 59°C, the glucose level fixed effect was significant ($P < 0.05$). At 61°C, glucose level, *Salmonella* culture composite, and the glucose level x *Salmonella* culture composite interaction were significant at $\alpha = 0.05$ (Table 8.3). Due to the interaction ($P < 0.05$), subclass least squares means were reported for *Salmonella* challenged at 61°C (Table 8.4).

Data also were evaluated to determine *D*-value differences between challenge temperatures. Preliminary AOV indicated no difference ($P = 0.7179$) between *Salmonella* culture composites when evaluated across all glucose levels and challenge temperatures. Subsequently, *Salmonella* culture composites were pooled, data were evaluated with AOV using the model: $y = a + x_1 + x_2 + x_1x_2$. Least-squares means were computed for *D*-values by glucose level (x_1), challenge temperature (x_2), and glucose level x challenge temperature (x_1x_2) effects using the General Linear Models procedure of SAS® (SAS, 2001). Glucose level, challenge temperature, and the glucose level x challenge temperature interaction were significant ($P < 0.05$). Due to the interaction ($P < 0.05$), subclass least squares means were reported and used for subsequent z_D-value and activation energy of inactivation determinations (Table 8.5). However, due to heterogeneity of variance, interaction subclass least squares means were separated using models including the following challenge temperature comparisons: (1) 55 and 57°C; (2) 57 and 59°C; and, (3) 59 and 61°C. When AOV detected effects ($P < 0.05$) within or

between challenge temperatures (Tables 8.4 and 8.5), *D*-values were separated using the pairwise t-test of SAS® (SAS, 2001).

Log_{10} *D*-values were plotted versus their corresponding temperatures, and z_D-values were calculated as the negative reciprocal of the slope of the linear regression line of the thermal death time curve (z_D-value = -slope^{-1}). Activation energies of inactivation (E_a) were also determined by constructing Arrhenius plots [log k vs. temperature^{-1} in Kelvin, where the rate constant (k) = 2.3/*D*], from which the slopes of the lines were used to calculate E_a, where E_a = -2.3(R)(slope), where R = the gas constant, 8.31 J K^{-1} mol^{-1} (Foegeding and Leasor, 1990; Schuman *et al.*, 1997).

RESULTS AND DISCUSSION

Salmonella were grown in the presence (0.25 or 1.00%) or absence of a fermentable carbohydrate (i.e., glucose) in order to evaluate heat tolerance of stationary-phase, susceptible and multi-antimicrobial resistant cultures with and without prior acid-adaptation (Buchanan and Edelson, 1996; Wilde *et al.*, 2000). Wilde *et al.* (2000) reported RpoS-independent induction of stationary-phase acid tolerance among *S.* Enteritidis PT4 isolates following overnight growth in TSB, nutrient broth, and tryptone soya broth, containing 0.25% glucose. During the current study, overnight growth (18h; 37°C) of *Salmonella* strains in TSBYE (9 ml; pH 7.3) containing 0.00, 0.25 or 1.00% glucose resulted in ultimate pH values of approximately 8.1 to 8.3, 7.2 to 7.4 and 4.7 to 4.8, respectively, due to differences in fermentable carbohydrate availability. Following thermal stress, viable populations were recovered and enumerated on TSAYE supplemented with 1.0% sodium pyruvate, which has been reported to increase recovery

of low-pH stressed cells by approximately 1000-fold due to the protective action afforded against oxygen radicals (Leyer and Johnson, 1992; McDonald *et al.*, 1983). Furthermore, plates were incubated at 28°C for 72 h prior to counting colonies, as sub-optimum temperatures have been reported to enhance repair of heat damage (Juneja and Eblen, 2000; Juneja *et al.*, 2001; Katsui *et al.*, 1982).

Analysis of variance for glucose level (0.00, 0.25 and 1.00%) and culture type (susceptible and multi-antimicrobial resistant) main effects, at each of the four challenge temperatures (i.e., 55, 57, 59 and 61°C), indicated that thermal resistance (*D*-value) depended ($P < 0.05$) on previous growth in the presence or absence of glucose (acid-adaptation) at 59°C, and on previous acid-adaptation and culture composite ($P < 0.05$) at 61°C (Table 8.3). $D_{61°C}$-values of antimicrobial susceptible *Salmonella* increased ($P < 0.05$) from 0.14 to 0.19 as glucose level increased from 0.00 to 1.00%, respectively, while for the multi-antimicrobial resistant *Salmonella* strain composite, corresponding *D*-values did not differ ($P > 0.05$) as growth in both TSBYE-G and TSBYE+G resulted in decimal reduction times of 0.21 min (Table 8.4).

Additionally, multi-antimicrobial resistant *Salmonella* grown in the absence or presence of 1.00% glucose were slightly more resistant to heat (61°C) compared to the susceptible culture composite. At 61°C, thermal resistance of susceptible and multi-antimicrobial resistant composite cultures was not different ($P > 0.05$) when grown in the presence of 0.25% glucose (Table 8.4). At 59°C, when averaged across culture composites, decimal reduction times increased ($P < 0.05$) from 0.50 min for *Salmonella* grown in the absence of glucose, to 0.58 min for *Salmonella* grown in 0.25% glucose; $D_{59°C}$-values also increased ($P < 0.05$) from 0.58 min following growth in 0.25% glucose,

to 0.66 min for *Salmonella* grown in the presence of 1.00% glucose, respectively (Table 8.5). This data suggested that heat resistance increased with previous acid-adaptation.

Data also were analyzed to determine *D*-value differences among challenge temperatures. After pooling culture composite data due to a lack of difference ($P = 0.7179$) between susceptible and resistant *Salmonella*, both glucose level and challenge temperature fixed effects and the glucose level x challenge temperature interaction were significant ($P < 0.05$). Decimal reduction times decreased ($P < 0.05$) as challenge temperature increased from 55 to 57°C, from 57 to 59°C and from 59 to 61°C, for *Salmonella* grown in 0.00, 0.25 or 1.00% glucose (Table 8.5). Collectively, *D*-values ranged from 4.23 to 5.39, 1.47 to 1.81, 0.50 to 0.66, and 0.16 to 0.20 min for *Salmonella* inactivated at 55, 57, 59 and 61°C, respectively (Table 8.5). For each of the glucose levels (0.00, 0.25 and 1.00%), corresponding z_D-values, or the change in temperature required to effect a 10-fold change in the decimal reduction time, were 1.49, 1.48 and 1.20°C, respectively, while corresponding activation energies of inactivation (E_a) were 494, 493 and 497 kJ/mol (Table 8.5).

Data suggested a positive relationship between acid-adaptation and the ability to resist thermal stress. Decimal reduction times associated with heating at 59 and 61°C increased ($P < 0.05$) following previous acid-adaptation, and despite greater variability, a similar relationship also was suggested at 55°C ($P = 0.0511$; Table 8.3). Researchers have reported that, in addition to withstanding low-pH exposure, acid-adapted *S.* Typhimurium exhibit cross-protection to heat, osmotic and oxidative stresses (Leyer and Johnson, 1993; Wilde *et al.*, 2000). Leyer and Johnson (1993) reported that acid-adaptation during logarithmic growth alters cellular resistance to a wide range of

environmental stresses, including temperature and salt, by inducing the synthesis of specific outer membrane proteins, subsequently resulting in increased outer surface hydrophobicity in addition to intracellular proton homeostatic activities. They further reported ≥10-fold differences in survivor counts between acid-adapted and nonadapted *S.* Typhimurium following prolonged exposure to 50.0, 55.0 and 57.5°C, and enumeration on tryptose-phosphate agar containing at least 0.1% sodium pyruvate (Leyer and Johnson, 1993). Wilde *et al.* (2000) evaluated heat- and acid-tolerance among *S.* Enteritidis PT4 strains, possessing normal and mutant *rpo*S alleles, following fermentative stationary-phase growth in complex media containing glucose. Using blood agar to enumerate viable cells, induction of the RpoS-independent ATR resulted in significantly ($P < 0.001$) higher tolerance to heat among cells grown in tryptic soy broth, or in nutrient broth (No. 2) containing 0.25% glucose, when compared to cells grown in the absence of glucose (Wilde *et al.*, 2000). It is worth noting that, much like the physiological state of a bacterium has been shown to influence stress survival (Lee *et al.*, 1994b), so may nutrient availability. Cells grown under laboratory conditions, in a medium containing an abundant supply of nutrients, may not accurately simulate the physiology of cells found in the food environment (Leyer and Johnson, 1993). The lack of available nutrients has been found to induce heat-shock protein production in *S.* Typhimurium and *E. coli*, resulting in starvation-induced thermal cross-protection (Jenkins *et al.*, 1988; Leyer and Johnson, 1993).

The relationship between acid-adaptation and the ability to resist thermal stress may prove important during the production/manufacturing of foods, as low-pH, which may be a characteristic associated with some foods, and heat stresses are the basis of

many technologies utilized as means of improving microbiological quality. A potential scenario that may be associated with the parameters of this study involves the production of fermented food products (e.g., ripened cheeses and fermented sausages). Commercially available brands of semi-dry and summer-type sausages have been found to have pH values ranging from 4.5 to 5.2 and from 4.5 to 4.7, respectively (Jay, 2000). During this study, overnight growth of *Salmonella* strains in TSBYE (pH 7.3) containing 1.00% glucose resulted in pH values of approximately 4.7 to 4.8. The gradual decline of pH over time associated with the fermentation process may provide an environment in which microorganisms capable of inducing low-pH protective mechanisms become tolerant.

Microorganisms also may encounter sublethal, low-pH stress during the animal-to-muscle food conversion process. Application of organic acids to decontaminate beef carcass surfaces has been examined and, in general, data support the use of spray washing/rinsing as an effective means of reducing microbial populations. As a result, acid solution rinsing is used throughout the commercial beef industry during the slaughtering/dressing process (Dickson and Anderson, 1992; Dorsa, 1997; Gorman *et al.*, 1995; Hardin *et al.*, 1995; Prasai *et al.*, 1991). Despite the recognized antimicrobial efficacy associated with organic acid solution rinsing of carcasses, there exists the opportunity for sublethal, transient low-pH exposure and subsequent acid tolerance development among associated bacterial populations (e.g., *Salmonella*). Occurrence of localized, sublethal pH microenvironments within a slaughtering facility is not only plausible, but probable considering that in-plant organic acid solution rinsing almost always occurs immediately following ambient temperature carcass washing (water); a

matter of previous discussion (Samelis *et al.*, 2001b). Residual effects of organic acids on the pH of microenvironments within commercial slaughtering facilities are not fully understood, but may provide a means by which acid tolerant bacteria are not only developed, but are reintroduced to carcass surfaces.

Regardless of the scenario, acid-adaptation resulting from exposure(s) to sublethal low-pH stress may result in cross-protection against heat stress, subsequently increasing pathogen survival in fresh or fermented, undercooked/underprocessed foods (Leyer and Johnson, 1993; Ryu and Beuchat, 1998). Furthermore, the ability of *Salmonella* to regulate expression of genes coding for adaptive or resistant mechanisms in order to mount an ATR may prove important during pathogenesis, as acid-adapted cells may survive subsequent acid stress exposure(s) associated with food ingestion (i.e., stomach, intestinal and intracellular environments), especially among individuals with compromised gastrointestinal tracts, ultimately resulting in infection (Idziak and Suvanmongkol, 1971; Wilmes-Riesenberg *et al.*, 1996). Results of this study comparing the thermal tolerance of susceptible and multi-antimicrobial resistant *Salmonella* when challenged following preparation under acid tolerant-inducing and noninducing conditions suggested no association between antibiotic susceptibility and the ability to survive or repair damage associated with heat stress. The apparent lack of cross-protection between antimicrobial resistance and the ability to resist heat stress may be due to molecular response differences initiated by each type of stress, as the antibiotic stress response does not appear to coincide with the same general stress responses initiated by other unfavorable environmental conditions (e.g., low-pH and low nutrient availilability). Additional research is required to further understand the relationship

between antimicrobial susceptibility, stationary-phase ATR induction and sensitivity to environmental stresses, and how these relationships are intertwined with current food manufacturing/processing practices.

Table 8.1. Serotypes and antimicrobial susceptibility patterns of susceptible and multi-antimicrobial resistant *Salmonella* strains used in this study.

Salmonella Serotype	Antimicrobial Susceptibility Pattern[b]
Susceptible[a]:	
Saint-Paul	Susceptible to all antimicrobials
Anatum	Susceptible to all antimicrobials
Mbandaka	Susceptible to all antimicrobials
Agona	Susceptible to all antimicrobials
Agona	Susceptible to all antimicrobials
Resistant[a]:	
Typhimurium DT104	ACSSuT + Trimethoprim/sulfamethoxazole
Reading	ACSSuT + Amoxicillin/clavulanic acid, Ampicillin/sulbactam and Gentamicin
Reading	ACSSuT + Amoxicillin/clavulanic acid, Ampicillin/sulbactam and Gentamicin
Typhimurium DT104 (var. Copenhagen)	ACSSuT + Amoxicillin/clavulanic acid and Ampicillin/sulbactam
Typhimurium DT104 (var. Copenhagen)	ACSSuT + Amoxicillin/clavulanic acid and Trimethoprim/sulfamethoxazole

[a] With the exception of *S.* Typhimurium DT104 ATCC 700408, all *Salmonella* strains used during this study were derived from beef animal hide samples (N = 160) collected in four commercial packing plants.

[b] ACSSuT: common abbreviations for ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline.

Table 8.2. Survivor curve data [slopes and coefficients of determination (r^2)] for each of three replicate experiments by challenge temperature, glucose level and *Salmonella* culture composite.

			Replicate Experiment[a]					
			1		2		3	
(°C)[b]	Glucose (%)[c]	*Salmonella* Composite[d]	slope	r^2	slope	r^2	slope	r^2
55	0.00	S	-0.27730	0.99	-0.29411	0.98	-0.21195	0.96
		R	-0.24069	0.91	-0.24951	0.97	-0.18237	0.95
	0.25	S	-0.23852	0.97	-0.17035	0.99	-0.25218	0.98
		R	-0.29607	0.95	-0.19350	0.97	-0.27198	0.96
	1.00	S	-0.17224	0.97	-0.17182	0.97	-0.18965	0.93
		R	-0.17382	0.99	-0.18782	0.99	-0.22844	0.99
57	0.00	S	-0.00654	0.94	-0.01631	0.95	-0.01613	0.95
		R	-0.00674	0.98	-0.00893	0.94	-0.00881	0.90
	0.25	S	-0.01362	0.98	-0.01036	0.94	-0.01093	0.94
		R	-0.01021	0.98	-0.01112	0.95	-0.01229	0.92
	1.00	S	-0.00671	0.97	-0.01262	0.97	-0.01387	0.95
		R	-0.00748	0.98	-0.01030	0.98	-0.01102	0.98
59	0.00	S	-0.03376	0.90	-0.03506	0.91	-0.03581	0.91
		R	-0.02993	0.91	-0.03285	0.94	-0.03211	0.93
	0.25	S	-0.03206	0.97	-0.03155	0.94	-0.02433	0.95
		R	-0.02930	0.97	-0.02918	0.97	-0.02641	0.92
	1.00	S	-0.02652	0.96	-0.02700	0.96	-0.02616	0.96
		R	-0.02367	0.98	-0.02408	0.99	-0.02497	0.99
61	0.00	S	-0.11854	0.94	-0.12556	0.94	-0.11572	0.93
		R	-0.08484	0.97	-0.08083	0.97	-0.07544	0.97
	0.25	S	-0.11540	0.96	-0.10980	0.96	-0.10036	0.99
		R	-0.09940	0.98	-0.09623	0.97	-0.10219	0.97
	1.00	S	-0.08340	0.96	-0.09632	0.93	-0.09133	0.95
		R	-0.07682	0.97	-0.07951	0.98	-0.08761	0.99

[a] Data for each replicate were derived from duplicate samples analyzed at each predetermined, challenge temperature-dependent sampling time.

[b] Heat resistance of *Salmonella* was determined at each of four challenge temperatures (55, 57, 59 and 61°C).

[c] Five susceptible and five multi-antimicrobial resistant strains were grown to stationary-phase with (0.25 or 1.00%) or without (0.00%) glucose.

[d] Cells were combined producing two composites consisting of susceptible (S) or multi-antimicrobial resistant (R) *Salmonella*.

Table 8.3. Analysis of variance (AOV)[a] for glucose level and culture composite fixed effects, and the glucose level*culture composite interaction, for each challenge temperature.

	Challenge Temperature (°C)[b]							
	55		57		59		61	
Fixed Effect	Test Statistic	P-value	Test Statistic	P-value	Test Statistic	P-value	Test Statistic	P-value
Glucose level[c]	3.85	0.0511	0.68	0.5269	19.23	0.0002	15.36	0.0005
Composite[d]	0.16	0.6994	0.93	0.3545	4.01	0.0682	45.09	< 0.0001
Glucose level*Composite	1.12	0.3568	0.41	0.6738	0.50	0.6178	11.41	0.0017

[a] Mean *D*-values were calculated from triplicate trials and analysis of variance (AOV) was performed using the General Linear Models procedure of SAS®.

[b] Heat resistance of *Salmonella* was determined at each of four challenge temperatures (55, 57, 59 and 61°C).

[c] Five susceptible and five multi-antimicrobial resistant strains were grown to stationary-phase with (0.25 or 1.00%) or without (0.00%) glucose.

[d] Cells were combined producing two composites consisting of susceptible or multi-antimicrobial resistant *Salmonella*.

Table 8.4. Decimal reduction times[a] and standard deviations of *Salmonella* grown to stationary-phase with or without glucose and challenged at 61°C, by *Salmonella* culture composite.

Glucose level (%)[b]	*Salmonella* Culture Composite[c]	*D*-value (min)	Standard Deviation
0.00	S	0.14^{w}	0.01
	R	0.21^{z}	0.01
0.25	S	0.15^{wx}	0.01
	R	0.17^{xy}	0.01
1.00	S	0.19^{y}	0.01
	R	0.21^{z}	0.01

[a] Decimal reduction times (*D*-values; min) were calculated using the survivor curves from triplicate trials.

[b] *Salmonella* were grown overnight with (0.25 or 1.00%) or without (0.00%) glucose.

[c] Cells were combined producing two composites consisting of susceptible (S) or multi-antimicrobial resistant (R) *Salmonella*.

[wxyz] *D*-values in the same column with a common superscript letter are not different ($P > 0.05$).

Table 8.5. Decimal reduction times[a] (standard deviation), z_D-values, and activation energies of inactivation (E_a) of *Salmonella* grown to stationary-phase with or without glucose.

	Challenge Temperature (°C)					
Glucose level (%)[b]	55	57	59	61	z_D-value (°C)[c]	E_a (kJ/mol)[d]
0.00	4.23^{z} (0.77)	1.81^{y} (0.67)	0.50^{x} (0.03)	0.17^{x} (0.04)	1.49	494
0.25	4.38^{z} (0.95)	1.47^{y} (0.16)	0.58^{x} (0.06)	0.16^{x} (0.01)	1.48	493
1.00	5.39^{z} (0.55)	1.73^{y} (0.51)	0.66^{x} (0.04)	0.20^{x} (0.02)	1.20	497

[a] Decimal reduction times (*D*-values; min) were calculated using the survivor curves from triplicate trials.

[b] *Salmonella* were grown overnight with (0.25 or 1.00%) or without (0.00%) glucose.

[c] z_D-values, or the temperature change (°C) necessary to effect a 10-fold change in the *D*-value, were calculated using the thermal death time curves ($\log_{10}$ *D*-values plotted vs. temperature).

[d] Arrhenius plots were constructed and activation energies of inactivation were calculated from the slopes using the equation: $E_a = -2.3(R)(\text{slope})$, where $R = 8.31\ J\ K^{-1}\ mol^{-1}$.

[xyz] *D*-values in the same row with a common superscript letter are not different ($P > 0.05$).

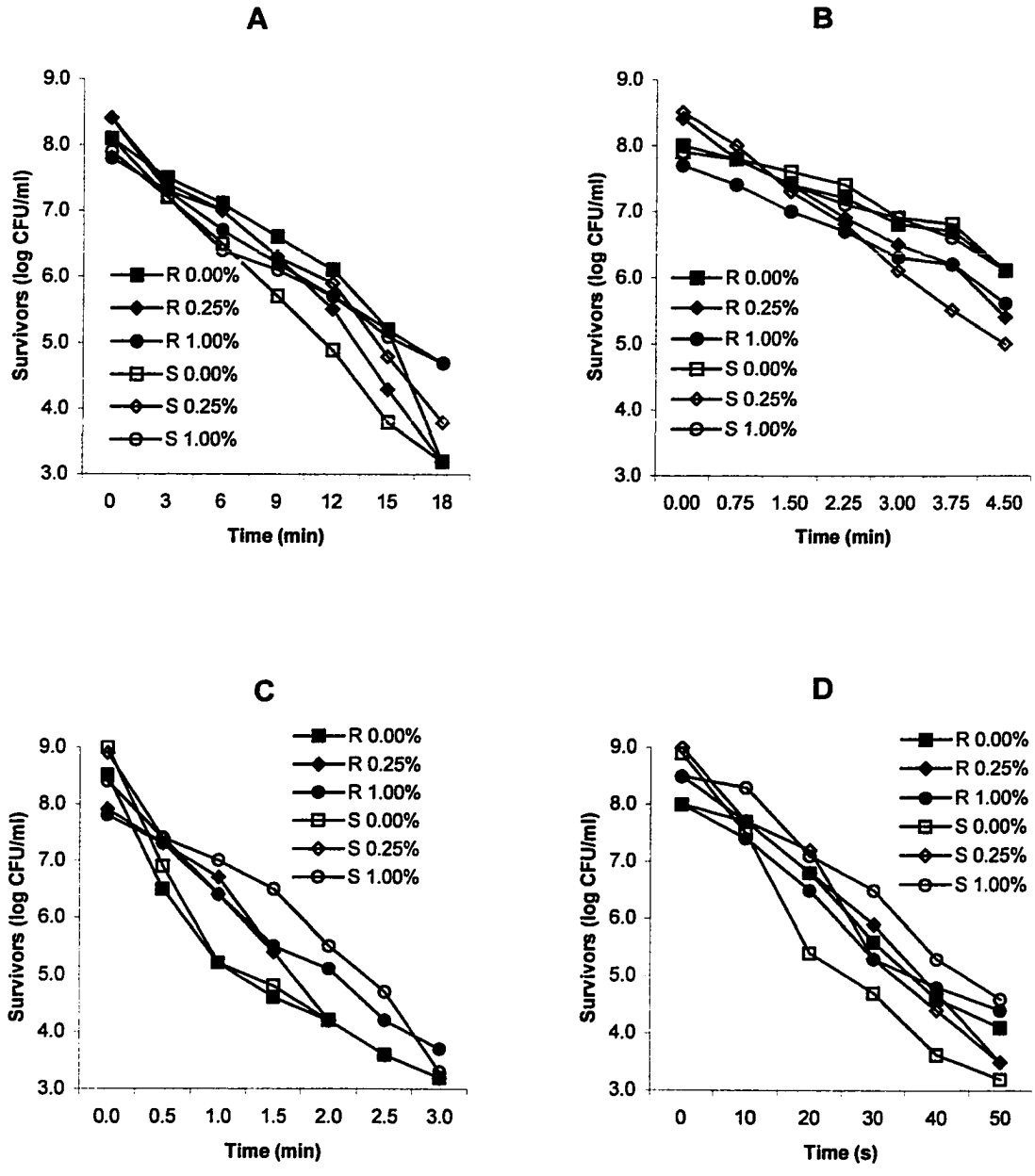

Figure 8.1. Survivor curves of susceptible (S) and multi-antimicrobial resistant (R) *Salmonella* cultures prepared by overnight growth in the absence (0.00%) or presence (0.25% or 1.00%) of glucose, and subsequent exposure to 55 (A), 57 (B), 59 (C) or 61°C (D). For each challenge temperature, values represent survivor populations from only one of three experimental replicates.

CHAPTER IX

DISSERTATION SUMMARY

Results of studies included within this dissertation regarding methods to improve microbiological quality of chilled beef carcass and subprimal surfaces, and on fabrication tables, suggested reduced antimicrobial efficacy ($\leq$ 1 log CFU/100 cm^2 reduction) of currently used decontamination treatments, or at least of steam-vacuuming and lactic acid solution rinsing, when applied during beef carcass fabrication. These findings are in contradiction to the majority of data supporting steam-vacuuming and spray washing/rinsing as effective means of eliminating, reducing, or controlling the prevalence of, pathogenic and nonpathogenic bacterial populations on hot beef carcass surfaces. The antimicrobial efficacy of these treatments may be reduced significantly when applied to chilled tissue surfaces, in part due to lower temperatures associated with tissue chilling and carcass fabrication, in addition to the altered texture and characteristics of the tissue itself. Additional stress tolerance may result from increased bacterial exposure time, as bacterial contamination on fresh or hot beef carcass tissue has been recently transferred, while that on chilled beef carcass tissue has had time (24 to 72 h) to attach, penetrate and/or form biofilms. Furthermore, it has been reported previously that as the time of beef carcass tissue exposure to fecal contamination increases, the number of bacteria removed during subsequent spray washing/rinsing treatments decreases.

Study results also validated concerns regarding increased antimicrobial resistance among foodborne bacterial populations, as data collected suggested that for every 1000 beef animal hides sampled, approximately 107 (10.7%) would contain *Salmonella* resistant to at least one of the thirteen antimicrobials screened. Technologies adopted industry-wide for the purpose of removing and/or inactivating microbiological populations transferred to carcass sufaces during hide removal and subsequent slaughtering/dressing processes have included hot water/steam-vacuuming, steam pasteurization, and ambient temperature or hot water spray washing with or without organic acid solution rinsing. Study results comparing the stress tolerance capabilities of susceptible and multi-antimicrobial resistant *Salmonella* strains suggested no association between antibiotic susceptibility and the ability to employ strategies aimed at avoiding or repairing damage associate with low-pH or thermal stress exposures.

Additional research is required in order to understand more completely the relationships between: (1) antimicrobial treatment efficacy and time allowed for attachment, penetration and/or biofilm formation at refrigeration temperatures; and, (2) stationary-phase acid tolerance following transient low-pH exposure and subsequent sensitivity to environmental stresses associated with current food manufacturing/processing practices. This information would aid government and industry personnel in producing end products of improved microbiological quality.

REFERENCES

Aarestrup, F. M., N. E. Jensen, and D. L. Baggesen. 1997. Clonal spread of tetracycline-resistant *Salmonella typhimurium* in Danish dairy herds. Vet. Rec. 140:313-314.

Acuff, G. R., C. Vanderzant, J. W. Savell, D. K. Jones, D. B. Griffin, and J. G. Ehlers. 1987. Effect of acid decontamination of beef subprimal cuts on the microbiological and sensory characteristics of steaks. Meat Sci. 19:217-226.

Adams, D. M. 1978. Heat injury of bacterial spores. Adv. Appl. Microbiol. 23:245-261.

Akkina, J. E., A. T. Hogue, F. J. Angulo, R. Johnson, K. E. Petersen, P. K. Saini, P. J. Fedorka-Cray, and W. D. Schlosser. 2000. Epidemiologic aspects, control, and importance of multiple-drug resistant *Salmonella* Typhimurium DT104 in the United States. J. Am. Vet. Med. Assoc. 214:790-798.

Alberts, B., D. Bray, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 1998. Essential cell biology: An introduction to the molecular biology of the cell. Garland Publishing, Inc., New York, NY.

Allwood, M. C., and A. D. Russell. 1970. Mechanisms of thermal injury in non-sporulating bacteria. Adv. Appl. Microbiol. 12:89-119.

Alpuche-Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller. 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. 89:10079-10083.

Amelunxen, R. E., and A. L. Murdock. 1978. Life at high temperatures: Mechanisms and molecular aspects, p. 217-278. *In* D. J. Kushner (ed.), Microbial life in extreme environments. Academic Press, New York, NY.

Anderson, M. E., H. E. Huff, H. D. Naumann, and R. T. Marshall. 1988. Counts of six types of bacteria on lamb carcasses dipped or sprayed with acetic acid at 25° or 55°C and stored vacuum packaged at 0°C. J. Food Prot. 51:874-877.

Anderson, M. E., H. E. Huff, H. D. Naumann, R. T. Marshall, J. Damare, R. Johnston, and M. Pratt. 1987. Evaluation of swab and tissue excision methods for

recovering microorganisms from washed and sanitized beef carcasses. J. Food Prot. 50:741-743.

Anderson, M. E., and R. T. Marshall. 1989. Interaction of concentration and temperature of acetic acid solution on reduction of various species of microorganisms on beef surfaces. J. Food Prot. 52:312-315.

Anderson, M. E., and R. T. Marshall. 1990. Reducing microbial populations on beef tissues: Concentration and temperature of lactic acid. J. Food Safety 10:181-190.

Anderson, W. F., P. J. McClure, A. C. Baird-Parker, and M. B. Cole. 1996. The application of a log-logistic model to describe the thermal inactivation of *C. botulinum* 213B at temperatures below 121.1°C. J. Appl. Bacteriol. 80:283-290.

Andriole, V. T. 1998. Quinolones, p. 275-289. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Angelotti, R. 1978. Cooking requirements for cooked beef and roast beef. Federal Register 43:30791-307793.

Angulo, F. J., K. R. Johnson, R. V. Tauxe, and M. L. Cohen. 2000. Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: Implications for the use of fluoroquinolones in food animals. Microb. Drug Resist. 6:77-83.

Archer, D. L. 1997. Preservation microbiology and safety: Evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Tech. 7:91-95.

Arioli, V., M. Berti, G. Carniti, E. Randisi, E. Rossi, and R. Scotti. 1981. Antibacterial activity of DL 473, a new semisynthetic rifamycin derivative. J. Antibiot. (Tokyo) 34:1026-1032.

Ashkenazi, S., T. G. Cleary, B. E. Murray, A. Wanger, and L. K. Pickering. 1988. Quantitative analysis and partial characterization of cytotoxin production by *Salmonella* strains. Infect. Immun. 56:3089-3094.

Augustin, J. C., V. Carlier, and J. Rozjier. 1998. Mathematical modeling of the heat resistance of *L. monocytoghenes*. Appl. Microbiol. 84:185-191.

Bacon, R. T., K. E. Belk, J. N. Sofos, R. P. Clayton, J. O. Reagan, and G. C. Smith. 2000. Microbial populations on animal hides and beef carcasses at different stages of slaughter in plants employing multiple-sequential interventions for decontamination. J. Food Prot. 63:1080-1086.

Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park. 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. 182:2245-2252.

Barach, J. T., R. S. Flowers, and D. M. Adams. 1975. Repair of heat-injured *Clostridium perfringens* spores during outgrowth. Appl. Microbiol. 30:873-875.

Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Amer. J. Clin. Path. 45:493-496.

Bauernfeind, A., and R. Jungwirth. 1991. Antibacterial activity of cefpodoxime in comparison with cefixime, cefdinir, cefetamet, ceftibuten, loracarbef, cefprozil, BAY 3522, cefuroxime, cefaclor and cefadroxil. Infection 19:353-362.

Beaman, T., J. Greenamyrem, T. Corner, H. Pankrantz, and P. Gerhardt. 1982. Bacterial spore heat resistance correlated with water content, wet density and protoplast/sporoplast volume ratio. J. Bacteriol. 150:870-877.

Bell, B. P, Goldoft, M., Griffin, P. M., Davis, M. A., Gordon, D. C., Tarr, P. I., Bartleson, C. A., Lewis, J. H., Barrett, T. J., Wells, J. G., Baron, R. and Kobayashi, J. 1994. A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington Experience. JAMA. 272:1349-1353.

Bender, G. R., and R. E. Marquis. 1985. Spore heat resistance and specific mineralization. Appl. Environ. Microbiol. 50:1414-1421.

Bennish, M. L., and M. A. Salam. 1992. Rethinking options for the treatment of shigellosis. J. Antimicrob. Chemother. 30:243-247.

Benveniste, R., and J. Davies. 1973. Mechanisms of antibiotic resistance in bacteria. Annu. Rev. Biochem. 42:471-506.

Beuchat, L. R. 1978. Injury and repair of gram-negative bacteria, with special consideration of the involvement of the cytoplasmic membrane. Adv. Appl. Microbiol. 23:219-243.

Blackburn, C. D., L. M. Curtis, L. Humpheson, C. Billon, and P. J. McClure. 1997. Development of thermal inactivation models for *Salmonella* Enteritidis and *Escherichia coli* O157:H7 with temperature, pH and NaCl as controlling factors. Int. J. Food Microbiol. 38:31-44.

Bloch, A. B., G. M. Cauthen, I. M. Onorato, K. G. Dansbury, G. D. Kelly, C. R. Driver, and D. E. Snider, Jr. 1994. Nationwide survey of drug-resistant tuberculosis in the United States. JAMA 271:665-671.

Bopp, C. A., F. W. Brenner, J. G. Wells, and N. A. Strockbine. 1999. *Escherichia, Shigella,* and *Salmonella,* p. 459-474. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington DC.

Brackett, R. E., Y. –Y. Hao, and M. P. Doyle. 1994. Ineffectiveness of hot acid sprays to decontaminate *Escherichia coli* O157:H7 on beef. J. Food Prot. 57:198-203.

Brackett, R. E., J. D. Schuman, H. R. Ball, and A. J. Scouten. 2001. Thermal inactivation kinetics of *Salmonella* spp. within intact eggs heated using humidity-controlled air. J. Food Prot. 64:934-938.

Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. *Salmonella* nomenclature. J. Clin. Microbiol. 38:2465-2467.

Brey, R. N., B. P. Rosen, and E. N. Sorensen. 1979. Cation/proton antiport systems in *Escherichia coli.* J. Biol. Chem. 255:39-44.

Brown, M. H., and A. C. Baird-Parker. 1982. The microbiological examination of meat, p. 423-520. *In* M. H. Brown (ed.), Meat microbiology. Applied Science Publishers, London, England.

Bryan, F. L. 1983. Epidemiology of milk-borne diseases. J. Food Prot. 46:637-649.

Buchanan, R. L., and S. G. Edelson. 1996. Culturing enterohemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. Appl. Environ. Microbiol. 62:4009-4013.

Buchanan, R. L., and S. G. Edelson. 1999. Effect of pH-dependent, stationary phase acid resistance on the thermal tolerance of *Escherichia coli* O157:H7. Food Microbiol. 16:447-458.

Bunning, V. K., R. G. Crawford, J. T. Tierney, and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella* Typhimurium after sublethal heat shock. Appl. Environ. Microbiol. 56:3216-3219.

Buss, W. C., R. Morgan, J. Guttman, T. Barela, and K. Stalter. 1978. Rifampicin inhibition of protein synthesis in mammalian cells. Science 200:432-434.

Busta, F. F., and D. M. Adams. 1972. Identification of a germination system involved in the heat injury of *Bacillus subtilis* spores. Appl. Microbiol. 24:412-417.

Cabedo, L., J. N. Sofos, and G. C. Smith. 1996. Removal of bacteria from beef tissue by spray washing after different times of exposure to fecal material. J. Food Prot. 59:1284-1287.

Calia, F. M., and D. W. Oldach. 1998. Chloramphenicol, p. 289-298. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Campo, P., A. Gutierrez, and C. L. de Guevara. 1997. Evolution of susceptibility of non-*typhi* *Salmonella* in a Spanish hospital (1992-1994) and report of a *Salmonella* ser. Typhimurium isolate resistant to quinolones. Eur. J. Epidemiol. 13:239-241.

Casolari, A. 1988. Microbial death, p. 1-44. *In* M. J. Bazin, and J. I. Prosser (ed.), Physiological models in microbiololgy. CRC Press, Boca Raton, FL.

Cassens, R. G. 1994. Meat preservation: Preventing losses and assuring safety. Food & Nutrition Press, Inc. Trumbull, CT.

Castanie-Cornet, M. –P., T. A. Penfound, D. Smith, J. F. Ellicott, and J. W. Foster. 1999. Control of acid resistance in *Escherichia coli*. J. Bacteriol. 181:3525-3535.

Castillo, A., J. S. Dickson, R. P. Clayton, L. M. Lucia, and G. R. Acuff. 1998. Chemical dehairing of bovine skin to reduce pathogenic bacteria and bacteria of fecal origin. J. Food Prot. 61:623-625.

Castillo, A., L. M. Lucia, I. Mercado, and G. R. Acuff. 2001a. In-plant evaluation of a lactic acid treatment for reduction of bacteria on chilled beef carcasses. J. Food Prot. 64:738-740.

Castillo, A., L. M. Lucia, D. B. Roberson, T. H. Stevenson, I. Mercado, and G. R. Acuff. 2001b. Lactic acid sprays reduce bacterial pathogens on cold beef carcass surfaces and in subsequently produced ground beef. J. Food Prot. 64:58-62.

CDC. 1996. Surveillance for foodborne-disease outbreaks—United States, 1988-1992. Morb. Mortal. Wkly Rep. 45:1-66.

CDC. 1997. *Salmonella* surveillance, 1996. Centers for Disease Control and Prevention, Atlanta, GA.

Chung, K. –T., J. S. Dickson, and J. D. Crouse. 1989. Attachment and proliferation of bacteria on meat. J. Food Prot. 52:173-177.

Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. 30:175-188.

Cohen, M. L. 1997. Epidemiological factors influencing the emergence of antimicrobial resistance. Ciba Found. Symp. 207:223-231.

Conner, D. E., V. N. Scott, and D. T Bernard. 1990. Growth, inhibition, and survival of *Listeria monocytogenes* as affected by acidic conditions. J. Food Prot. 53:652-655.

Corrier, D. E., C. E. Purdy, and J. R. DeLoach. 1990. Effects of marketing stress on fecal excretion of *Salmonella* spp. in feeder calves. Am. J. Vet. Res. 51:866-869.

Corry, J. E. L. 1972. The water relations and heat resistance of microorganisms. Prog. Ind. Microbiol. 12:73-108.

Craig, W. A. 1998a. Penicillins, p. 173-185. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Craig, W. A. 1998b. Rifampin and related drugs, p. 303-309. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Crosa, J. H. 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. 53:517-530.

Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53:121-147.

Cutter, C. N., and G. R. Siragusa. 1994. Efficacy of organic acids against *Escherichia coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. J. Food Prot. 57:97-103.

CVM-FDA. 1998. Framework document: Proposed framework for evaluating and assuring the human safety of the microbial effects of antimicrobial new animal drugs intended for use in food producing animals. FDA, Washington, DC.

Dabney, P., C. Bopp, F. Tenover, L. Tollefson, F. Angulo, and the NAMS Working Group. 1997. National antimicrobial monitoring system: Antimicrobial resistance in human isolates of *Salmonella* and *Escherichia coli* O157, abstr. C-136, p. 144. *In* Abstracts of the 97th general meeting of the American Society for Microbiology 1997. American Society for Microbiology, Washington, DC.

Dajani, A. S., and R. E. Kauffman. 1981. The renaissance of chloramphenicol. Pediatr. Clin. North Am. 28:195-202.

D'Aoust, J. –Y. 1991. Pathogenicity of foodborne *Salmonella*. Int. J. Food Microbiol. 12:17-40.

D'Aoust, J. -Y. 1997. *Salmonella* species, p. 129-158. *In* L. R. Beuchat, M. P. Doyle, and T. J. Montville (ed.), Food microbiology fundamentals and frontiers. ASM Press, Washington DC.

D'Aoust, J. –Y. 2000. *Salmonella*, p. 1233-1299. *In* B. M. Lund, T. C. Baird-Parker, and G. W. Gould (ed.), The microbiological safety and quality of food, vol. II. Aspen Publishers, Inc., Gaithersburg, MD.

D'Aoust, J. –Y., D. W. Warburton and A. M. Sewell. 1985. *Salmonella typhimurium* phage-type 10 from cheddar cheese implicated in a major Canadian foodborne outbreak. J. Food Prot. 48:1062-1066.

Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, and K. E. Ferris. 2000. Survey of *Salmonella* serotypes shed in feces of beef cows and their antimicrobial susceptibility patterns. J. Food Prot. 63:1648-1653.

Davies, G., W. J. Sojka, C. Wray, and B. M. Williams. 1978. Chloramphenicol-resistant *Salmonella typhimurium*. Vet. Rec. 102:67.

Della Bruna, C., G. Schioppacassi, D. Ungheri, D. Jabes, E. Morvillo, and A. Sanfilippo. 1983. LM 427, a new spiropiperidylrifamycin: *In vitro* and *in vivo* studies. J. Antibiot. (Tokyo) 36:1502-1506.

De Zuniga, A. G., M. E. Anderson, R. T. Marshall, and E. L. Iannotti. 1991. A model system for studying the penetration of microorganisms into meat. J. Food Prot. 54:256-258.

Dibrov, P. A. 1991. The role of sodium ion transport in *Escherichia coli* genetics. Biochim. Biophys. Acta 1056:209-224.

Dickson, J. S. 1992. Acetic acid action on beef tissue surfaces contaminated with *Salmonella typhimurium*. J. Food Sci. 57:297-301.

Dickson, J. S., and M. E. Anderson. 1992. Microbiological decontamination of food animal carcasses by washing and sanitizing systems. A review. J. Food Prot. 55:133-140.

Dickson, J. S., M. D. Hardin, and G. R. Acuff. 1997. Organic acid rinses. Proc. Recip. Meat Conf. American Meat Science Association, Chicago, IL. 49:129-131.

Dixon, Z. R., G. R. Acuff, L. M. Lucia, C. Vanderzant, J. B. Morgan, S. G. May, and J. W. Savell. 1991. Effect of degree of sanitation from slaughter through

fabrication on the microbiological and sensory characteristics of beef. J. Food Prot. 54:200-207.

Dixon, Z. R., C. Vanderzant, G. R. Acuff, J. W. Savell, and D. K. Jones. 1987. Effect of acid treatment of beef strip loin steaks on microbiological and sensory characteristics. Int. J. Food Microbiol. 5:181-186.

Dorsa, W. J. 1997. New and established carcass decontamination procedures commonly used in the beef-processing industry. J. Food Prot. 60:1146-1151.

Dorsa, W. J., C. N. Cutter, and G. R. Siragusa. 1996a. Effectiveness of steam-vacuum sanitizer for reducing *Escherichia coli* O157:H7 inoculated to beef carcass tissue. Letter Appl. Microbiol. 23:61-63.

Dorsa, W. J., C. N. Cutter, and G. R. Siragusa. 1997. Effects of steam-vacuuming and hot water spray-wash on the microflora of refrigerated beef carcass surface tissue inoculated with *Escherichia coli* O157:H7, *Listeria innocua*, and *Clostridium sporogenes*. J. Food Prot. 60:114-119.

Dorsa, W. J., C. N. Cutter, G. R. Siragusa, and M. Koohmaraie. 1996b. Microbial decontamination of beef and sheep carcasses by steam, hot water spray washes, and a steam-vacuum sanitizer. J. Food Prot. 59:127-135.

Doyle, M. E., and A. S. Mazzotta. 2000. Review of studies on the thermal resistance of salmonellae. J. Food Prot. 63:779-795.

Duffy, G., A. Ellison, W. Anderson, M. B. Cole, and G. S. A. B. Stewart. 1995. Use of bioluminescence to model the thermal inactivation of *Salmonella* Typhimurium in the presence of a competitive microflora. Appl. Environ. Microbiol. 61:3463-3465.

Dunne, E. F., P. D. Fey, P. Kludt, R. Reporter, F. Mostashari, P. Shillam, J. Wicklund, C. Miller, B. Holland, K. Stamey, T. J. Barrett, J. K. Rasheed, F. C. Tenover, E. M. Ribot, and F. J. Angulo. 2000. Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with ampC ß-lactamase. JAMA 284:3151-3156.

Dutta, P., M. R. Saha, U. Mitra, R. Rasaily, S. K. Bhattacharya, M. K. Bhattacharya, B. Kundu, and A. Gupta. 1995. Treatment of severe *Salmonella typhimurium* infection with ciprofloxacin. Indian Pediatr. 32:804-807.

Edwards, J. L., Jr., F. F. Busta, and M. L. Speck. 1965. Heat injury of *Bacillus subtilis* spores at ultra-high temperatures. Appl. Microbiol. 13:858-864.

Edwards, J. R., P. J. Turner, C. Wannop, E. S. Withnell, A. J. Grindey, and K. Nairn. 1989. In vitro antibacterial activity of SM-7338, a carbapenem antibiotic with stability to dehydropeptidase I. Antimicrob. Agents Chemother. 33:215-222.

Eliopoulos, G. M. 1998. Mechanisms of bacterial resistance to antimicrobial drugs, p. 319-330. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Ellison, A., W. Anderson, M. B. Cole, and G. S. A. B. Stewart. 1994. Modelling the thermal inactivation of *Salmonella* Typhimurium using bioluminescence data. Int. J. Food Microbiol. 23:467-477.

Ena, J., R. W. Dick, R. N. Jones, and R. P. Wenzel. 1993. The epidemiology of intravenous vancomycin usage in a university hospital: A 10-year study. JAMA 269:598-602.

Esty, J. R., and K. F. Meyer. 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes XI. J. Infect. Dis. 31:650-663.

Ewing, W. H. 1986. Edwards and Ewing's identification of *Enterobacteriaceae*, 4th ed. Elsevier Science Publishing Co., Inc., New York.

Farber, J. M., and F. Pagotto. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. Lett. Appl. Microbiol. 15:197-201.

Feder, H. M., Jr., C. Osier, and E. G. Maderazo. 1981. Chloramphenicol: A review of its use in clinical practice. Rev. Infect. Dis. 3:479-491.

Fedorka-Cray, P. J., D. A. Dargatz, L. A. Thomas, and J. T. Gray. 1998. Survey of *Salmonella* serotypes in feedlot cattle. J. Food Prot. 61:525-530.

Fey, P. D., T. J. Safranek, M. E. Rupp, E. F. Dunne, E. Ribot, P. C. Iwen, P. A. Bradford, F. J. Angulo, and S. H. Hinrichs. 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. N. Engl. J. Med. 342:1242-1249.

Finland, M. 1972. Changing patterns of susceptibility of common bacterial pathogens to antimicrobial agents. Ann. Intern. Med. 76:1009-1036.

Finlayson, M. C. and F. L. Jackson. 1978. Trimethoprim-resistant *Salmonella*. Lancet 2:375.

Firstenberg-Eden, R. 1981. Attachment of bacteria to meat surfaces: A review. J. Food Prot. 44:602-607.

Fleming, A. 1929. On antibacterial action of cultures on *Penicillium*, with special reference to their use in isolation of *B. influenzae*. Br. J. Exp. Pathol. 10:226.

Fliss, I., R. E. Simard, and A. Ettriki. 1991. Comparison of three sampling techniques for microbiological analysis of meat surfaces. J. Food Sci. 56:249-250, 252.

Flowers, R. S., and S. E. Martin. 1980. Ribosome assembly during recovery of heat-injured *Staphylococcus aureus*. J. Bacteriol. 141:645-651.

Foegeding, P. M., and S. B. Leasor. 1990. Heat resistance and growth of *Listeria monocytogenes* in liquid whole egg. J. Food Prot. 53:9-14.

Fontaine, R. E., S. Arnon, W. T. Martin, T. M. Vernon, Jr., E. J. Gangarosa, J. J. Farmer, III, A. B. Moran, J. H. Silliker, and D. L. Decker. 1978. Raw hamburger: an interstate common source of human salmonellosis. Am. J. Epidemiol. 107:36-45.

Foster, J. W. 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. 173:6896-6902.

Foster, J. W. 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. 175:1981-1987.

Foster, J. W. 1995. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Crit. Rev. Microbiol. 21:215-237.

Foster, J. W. 1999. When protons attack: Microbial strategies of acid adaptation. Curr. Opin. Microbiol. 2:170-174.

Foster, J. W. 2000. Microbial responses to acid stress, p. 99-115. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, DC.

Foster, J. W., and H. K. Hall. 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. 172:771-778.

Foster, J. W., and H. K. Hall. 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. 173:5129-5135.

Foster, J. W., and M. P. Spector. 1995. How *Salmonella* survive against the odds. Annu. Rev. Microbiol. 49:145-174.

FSIS-USDA. 1995. Comparison of methods for achieving the zero tolerance standard for fecal, ingesta and milk contamination of beef carcasses: Notice of Conference. Federal Register. 60:49553-49564.

FSIS-USDA. 1996a. Achieving the zero tolerance performance standard for beef carcasses by knife trimming and vacuuming with hot water and steam; Use of acceptable carcass interventions for reducing carcass contamination without prior agency approval: Notice of Policy Change. Federal Register. 61:15024-15027.

FSIS-USDA. 1996b. Pathogen Reduction: Hazard Analysis and Critical Control Point (HACCP) Systems; Final Rule. Federal Register. 61 (No. 144): 38806-38989.

Galan, J. E. 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. 20:263-271.

Galan, J. E., and C. Ginocchio. 1994. The molecular genetic bases of *Salmonella* entry into mammalian cells. Biochem. Soc. Trans. 22:301-306.

Gangar, V., and M. S. Curiale. 1999. Dry rehydratable film method for enumerating confirmed *Escherichia coli* in poultry, meats, and seafood: Collaborative study. J. AOAC Int. 82:73-78.

Garcia-del Portillo. 2000. Molecular and cellular biology of *Salmonella* pathogenesis, p. 3-49. *In* J. W. Cary, J. E. Linz, and D. Bhatnagar (ed.), Microbial foodborne diseases: Mechanisms of pathogenesis and toxin synthesis. Technomic Publishing Co, Inc., Lancaster, PA.

Garcia-del Portillo, F., and B. B. Finlay. 1994. Invasion and intracellular proliferation of *Salmonella* within non-phagocytic cells. Microbiologia SEM 10:229-238.

Garcia-del Portillo, F., M. G. Pucciarelli, W. A. Jeffries, and B. B. Finlay. 1994. *Salmonella typhimurium* induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells. J. Cell Sci. 107:2005-2020.

Gill, C. O., M. Badoni, and T. Jones. 1996. Hygienic effects of trimming and washing operations in a beef-carcass-dressing process. J. Food Prot. 59:666-669.

Gill, C. O., M. Badoni, and J. C. McGinnis. 1999. Assessment of the adequacy of cleaning of equipment used for breaking beef carcasses. Int. J. Food Microbiol. 46:1-8.

Gill, C. O., M. Badoni, and J. C. McGinnis. 2001a. Microbiological sampling of meat cuts and manufacturing beef by excision or swabbing. J. Food Prot. 64:325-334.

Gill, C. O. and J. Bryant. 1997. Decontamination of carcasses by vacuum-hot water cleaning and steam pasteurizing during routine operations at a beef packing plant. Meat Sci. 47:267-276.

Gill, C. O., J. C. McGinnis, and J. Bryant. 2001b. Contamination of beef chucks with *Escherichia coli* during carcass breaking. J. Food Prot. 64:1824-1827.

Ginocchio, C. C., S. B. Olmsted, C. L. Wells, and J. E. Galan. 1994. Contact with epithelial cells induces the formation of surface appendages on *Salmonella typhimurium*. Cell 76:717-724.

Glew, R. H., and M. A. Keroack. 1998. Vancomycin and teicoplanin, p. 260-269. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Glynn, M. K., C. Bopp, W. Dewitt, P. Dabney, M. Mokhtar, and F. J. Angulo. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N. Engl. J. Med. 338:1333-1338.

Goldstein, F. W., J. C. Chumpitaz, J. M. Guevara, B. Papadopoulou, J. F. Acar, and J. F. Vieu. 1986. Plasmid-mediated resistance to multiple antibiotics in *Salmonella typhi*. J. Infect. Dis. 153:261-266.

Goodson, M., and R. J. Rowbury. 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sublethal acid pH value. Lett. Appl. Microbiol. 8:77-79.

Gordon, D. 2000. Ceftriaxone-resistant *Salmonella* infection from antibiotic-treated cattle. Gastroenterology 119:3.

Gorman, B. M., J. N. Sofos, J. B. Morgan, G. R. Schmidt, and G. C. Smith. 1995. Evaluation of hand-trimming, various sanitizing agents, and hot water spray-washing as decontamination interventions for beef brisket adipose tissue. J. Food Prot. 58:899-907.

Gould, G. W. 1989. Heat-induced injury and inactivation, p. 11-42. *In* G. W. Gould (ed.), Mechanisms of action of food preservation procedures. Elsevier Science Publishers Ltd., New York, NY.

Graves Delmore, L. R., Sofos, J. N., Schmidt, G. R. and Smith, G. C. 1997. Inactivation of pathogenic bacteria by the chemical dehairing process proposed for use on beef carcasses during slaughter. Proc. Recip. Meat Conf. American Meat Science Association, Chicago, IL. 50:163-164.

Graves Delmore, L. R., Sofos, J. N., Schmidt, G. R. and Smith, G. C. 1998. Decontamination of inoculated beef with sequential spraying treatments. J. Food Sci. 63:890-893.

Grecz, N., and G. Bruszer. 1981. Lethal heat induces single strand breaks in the DNA of bacterial spores. Biochem. Biophys. Res. Commun. 98:191-196.

Griffith, R. S. 1981. Introduction to vancomycin. Rev. Infect. Dis. 3:S200-S204.

Gurney, T. R., and L. B. Quesnel. 1980. Thermal activation and dry-heat inactivation of spores of *Bacillus subtilis* var. *niger*. J. Appl. Bacteriol. 48:231-247.

Gurney, T. R., and L. B. Quesnel. 1981. Amino acid enhancement of recovery in dry-heat damaged spores of *Bacillus subtilis*. J. Appl. Bacteriol. 51:67-80.

Hall, R. L. 1997. Foodborne illness: Implications for the future. Emerg. Infect. Dis. 3:555-559.

Hall, H. K., and J. W. Foster. 1996. The role of Fur in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J. Bacteriol. 178:5683-5691.

Hall, H. K., K. L. Karem, and J. W. Foster. 1995. Molecular responses of microbes to environmental pH stress. Adv. Microb. Phys. 37:229-272.

Hamilton, I. R., and N. D. Buckley. 1991. Adaptation by *Streptococcus mutants* to acid tolerance. Oral Microbiol. Immunol. 6:65-71.

Hardin, M. D., G. R. Acuff, L. M. Lucia, J. S. Oman, and J. W. Savell. 1995. Comparison of methods for decontamination from beef carcass surfaces. J. Food Prot. 58:368-374.

Hartke, A., S. Bouche, J. C. Giard, A. Benachour, P. Boutibonnes, and Y. Auffray. 1996. The lactic acid stress response of *Lactococcus lactis* subsp. *lactis*. Curr. Microbiol. 33:194-199.

Helmuth, R. and D. Protz. 1997. How to modify conditions limiting resistance in bacteria in animals and other reservoirs. Clin. Infect. Dis. 24:S136-S138.

Herikstad, H., P. S. Hayes, J. Hogan, P. Floyd, L. Snyder, and F. J. Angulo. 1997a. Ceftriaxone-resistant *Salmonella* in the United States. Pediatr. Infect. Dis. J. 16:904-905.

Herikstad, H., P. Hayes, M. Mokhtar, M. L. Fracaro, E. J. Threlfall, and F. J. Angulo. 1997b. Emerging quinolone-resistant *Salmonella* in the United States. Emerg. Infect. Dis. 3:371-372.

Hicks, E. W. 1961. Uncertainties in canning process calculations. J. Food Sci. 26:218-226.

Hill, C., B. O'Driscoll, and I. Booth. 1995. Acid adaptation and food poisoning microorganisms. Int. J. Food Microbiol. 28:245-254.

Hof, H., I. Ehrhard, and H. Tschape. 1991. Presence of quinolone resistance in a strain of *Salmonella typhimurium*. Eur. J. Clin. Microbiol. Infect. Dis. 10:747-749.

Huis in't Veld, J. H. J. 1996. Microbial and biochemical spoilage of foods: An overview. Int. J. Food Microbiol. 33:1-18.

Humphrey, T. J., P. A Chapman, B. Rowe, and R. J. Gilbert. 1990. A comparative study of the heat resistance of salmonellas in homogenized whole egg, egg yolk or albumen. Epidemiol. Infect. 104:237-241.

Humphrey, T. J., E. Slater, K. McAlpine, R. J. Rowbury, and R. J. Gilbert. 1995. *Salmonella* Enteritidis phage type 4 isolates more tolerant of heat, acid, or hydrogen peroxide also survive longer on surfaces. Appl. Environ. Microbiol. 61:3161-3164.

Hurst, A., and A. Hughes. 1978. Stability of ribosomes of *Staphylococcus aureus* S6 sublethally heated in different buffers. J. Bacteriol. 91:134-142.

Iandolo, J. J., and Z. J. Ordal. 1966. Repair of thermal injury of *Staphylococcus aureus.* J. Bacteriol. 91:134-142.

ICMSF. 1996. Microorganisms in foods 5: Characteristics of microbial pathogens. Blackie Academic & Professional, London, England.

Idziak, E. S., and P. Suvanmongkol. 1971. Effect of pH on the pathogenic function of *Salmonella typhimurium.* Can. J. Microbiol. 18:9-12.

Jay, J. M. 2000. Modern food microbiology, 6th ed. Aspen Publishers, Inc., Gaithersburg, MD.

Jayasheela, M., U. K. Sharma, A. K. Mondal, and S. N. Saxena. 1987. Plasmid mediated enterotoxigenicity in *Salmonella* strains isolated from patients with gastroenteritis. Indian J. Med. Res. 85:496-499.

Jenkins, D. E., J. E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli.* J. Bacteriol. 170:3910-3914.

Jensen, M. J., C. Genigeorgis, and S. Lindroth. 1987. Probability of growth of *Clostridium botulinum* as affected by strain, cell and serological type, inoculum size and temperature and time of incubation in a model broth system. J. Food Safety 8:109-126.

Johnstone, K., G. S. Stewart, I. R. Scott, and D. J. Ellar. 1982. Zinc release and the sequence of biochemical events during triggering of *Bacillus megaterium* KM spore germination. Biochem. J. 208:407-411.

Judge, M. D., E. D. Aberle, J. C. Forrest, H. B. Hedrick, and R. A. Merkel. 1989. Principles of meat science, 2nd ed. Kendall/Hunt Publishing Co., Dubuque, IA.

Juneja, V. K., and B. S. Eblen. 2000. Heat inactivation of *Salmonella typhimurium* DT104 in beef as affected by fat content. Lett. Appl. Microbiol. 30:461-467.

Juneja, V. K., B. S. Eblen, and G. M. Ransom. 2001. Thermal inactivation of *Salmonella* spp. in chicken broth, beef, pork, turkey, and chicken: Determination of *D*- and z-values. J. Food Sci. 66:146-152.

Juneja, V. K., P. G. Klein and B. S. Marmer. 1998. Heat shock and thermotolerance of *Escherichia coli* O157:H7 in a model beef gravy system and ground beef. J. Appl. Microbiol. 84:677-684.

Jung, Y. S., and L. R. Beuchat. 2000. Sensitivity of multidrug-resistant *Salmonella typhimuirium* DT104 to organic acids and thermal inactivation in liquid egg products. Food Microbiol. 17:63-71.

Kadota, H., A. Uchida, Y. Sako, and K. Harada. 1978. Heat-induced DNA injury in spores and vegetative cells of *Bacillus subtilis*, p. 27-30. *In* G. Chambliss, and J. C. Vary (ed.), Spores, vol. VII. American Society for Microbiology, Washington DC.

Kapperud, G., S. Gustavsen, I. Hellesnes, A. H. Hansen, J. Lassen, J. Hirn, M. Jahkola, M. A. Montenegro, and R. Helmuth. 1990. Outbreak of *Salmonella typhimurium* infection traced to contaminated chocolate and caused by a strain lacking the 60-megadalton virulence plasmid. J. Clin. Microbiol. 28:2597-2601.

Karem, K., J. W. Foster, and A. K. Bej. 1994. Adaptive acid tolerance response (ATR) in *Aeromonas hydrophila*. Microbiology 140:341-352.

Karita, M., and M. J. Blaser. 1998. Acid-tolerance response in *Helicobacter pylori* and differences between *Cag*A$^+$ and *Cag*A$^-$ strains. J. Infect. Dis. 178:213-219.

Katsui, N., T. Tsuchido, M. Takano, and I. Shibasaki. 1981. The effect of preincubation temperature on the heat resistance of *Escherichia coli* having different fatty acid compositions. J. Gen. Microbiol. 122:357-361.

Katsui, N., T. Tsuchido, M. Takano, and I. Shibasaki. 1982. Viability of heat-stressed cells of micro-organisms as influenced by pre-incubation and post-incubation temperatures. J. Appl. Bacteriol. 53:103-108.

Kiessling, C. R., J. H. Cutting, M. Loftis, W. M. Kiessling, A. R. Datta, and J. N. Sofos. 2002. Antimicrobial resistance of food-related *Salmonella* isolates, 1999-2000. J. Food Prot. 65:603-608.

Kilsby, D. C., and M. E. Pugh. 1981. The relevance of the distribution of microorganisms within batches of food to the control of microbiological hazards from foods. J. Appl. Bacteriol. 51:345-354.

Klein, J. O., T. C. Eickhoff, J. G. Tilles, and M. Finland. 1964. Cephalothin: Activity *in vitro*, absorption and excretion in normal subjects and clinical observation in 40 patients. Am. J. Med. Sci. 248:640-656.

Kluytmans, J., A. Van Belkum, and H. Verbrugh. 1997. Nasal carriage of *Staphylococcus aureus*: Epidemiology, underlying mechanisms, and associated risks. Clin. Microbiol. Rev. 10:505-520.

Kochevar, S. L., J. N. Sofos, R. R. Bolin, J. O. Reagan, and G. C. Smith. 1997. Steam-vacuuming as a pre-evisceration intervention to decontaminate beef carcasses. J. Food Prot. 60:107-113.

Koo, F. C. W., J. W. Peterson, C. W. Houston, and N. C. Molina. 1984. Pathogenesis of experimental salmonellosis: Inhibition of protein synthesis by cytotoxin. Infect. Immun. 43:93-100.

Kormendy, I., and L. Kormendy. 1997. Considerations for calculating heat inactivation processes when semilogarithmic thermal inactiation models are non-linear. J. Food Eng. 34:33-40.

Kotula, A. W., Lusby, W. R., Crouse, J. D. and de Vries, B. 1974. Beef carcass washing to reduce bacterial contamination. J. Ani. Sci. 39:674-679.

Kroll, R. G., and I. R. Booth. 1983. The relationship between intracellular pH, the pH gradient and potassium transport in *Escherichia coli*. J. Biochem. 216:706-719.

Kroll, R. G., and R. A. Patchett. 1992. Induced acid tolerance in *Listeria monocytogenes*. Lett. Appl. Microbiol. 14:224-227.

Kropp, H., J. G. Sundelof, J. S. Kahan, F. M. Kahan, and J. Birnbaum. 1980. MK0787 (*N*-formimidoyl thienamycin): Evaluation of in vitro and in vivo activities. Antimicrob. Agents Chemother. 17:993-1000.

Kucers, A. 1980. Current position of chloramphenicol chemotherapy. J. Antimicrob. Chemother. 6:1-4.

Lee, I. S., J. Lin, H. K. Hall, B. Bearson, and J. W. Foster. 1995. The stationary-phase sigma factor σ^S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol. Microbiol. 17:155-167.

Lee, L. A., N. D. Puhr, E. K. Mahoney, N. H. Bean, and R. V. Tauxe. 1994a. Increase in antimicrobial-resistant *Salmonella* infections in the United States, 1989-1990. J. Infect. Dis. 170:128-134.

Lee, I. S., J. L. Slonczewski, and J. W. Foster. 1994b. A low-pH-inducible, stationary-phase acid tolerance response in *Salmonella typhimurium*. J. Bacteriol. 176:1422-1426.

Lelievre, H., G. Lina, M. E. Jones, C. Olive, F. Forey, M. Roussel-Delvallez, M. –H. Nicolas-Chanoine, C. M. Bebear, V. Jarlier, A. Andremont, F. Vandenesch, and J. Etienne. 1999. Emergence and spread in French hospitals of methicillin-resistant *Staphylococcus aureus* with increasing susceptibility to gentamicin and other antibiotics. J. Clin. Microbiol. 37:3452-3457.

Le Minor, L. 1981. The genus *Salmonella*, p. 1148-1159. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes. Springer-Verlag, New York, NY.

Lerner, S. A., R. P. Gaynes, and L. N. -Lerner. 1998. Aminoglycosides, p. 204-227. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Leyer, G. J., and E. A. Johnson. 1992. Acid adaptation promotes survival of *Salmonella* spp. in cheese. Appl. Environ. Microbiol. 58:2075-2080.

Leyer, G. J., and E. A. Johnson. 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. Appl. Environ. Microbiol. 59:1842-1847.

Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster. 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. J. Bacteriol. 177:4097-4104.

Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. 62:3094-3100.

Lindsay, J. A. 1997. Chronic sequelae of foodborne disease. Emerg. Infect. Dis. 3:443-451.

Livermore, D. M. 1993. Determinants of the activity of ß–lactamase inhibitor combinations. J. Antimicrob. Chemother. 31(Suppl A):9-21.

Livermore, D. M., and J. E. Corkill. 1992. Effects of CO_2 and pH on inhibition of TEM-1 and other ß–lactamases by penicillanic acid sulfones. Antimicrob. Agents Chemother. 36:1870-1876.

Livermore, D. M., and J. D. Williams. 1996. ß-Lactams: Mode of action and mechanisms of bacterial resistance, p. 502-578. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, MD.

Lorca, G., R. Raya, M. Taranto, and G. de Valdez. 1998. Adaptive acid tolerance response in *Lactobacillus acidophilus*. Biotechnol. Lett. 20:239-241.

Lou, Y., and A. E. Yousef. 1996. Resistance of *Listeria monocytogenes* to heat after adaptation to environmental stresses. J. Food Prot. 59:465-471.

Lou, Y., and A. E. Yousef. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. 63:1252-1255.

Lund, B. M., A. F. Graham, and J. G. Franklin. 1987. The effect of pH on the probability of growth of proteolytic strains of *Clostridium botulinum*. Int. J. Food Microbiol. 4:215-226.

MacDonald, K. L., M. L. Cohen, N. T. Hargrett-Bean, J. G. Wells, N. D. Puhr, S. F. Collin, and P. A. Blake. 1987. Changes in antimicrobial resistance of *Salmonella* isolated from humans in the United States. JAMA 258:1496-1499.

Maggi, N., C. R. Pasqualucci, R. Ballotta, and P. Sensi. 1966. Rifampicin: A new orally active rifamycin. Chemotherapy 11:285-292.

Malik, V. S. 1972. Chloramphenicol. Adv. Appl. Microbiol. 15:297-336.

Marshall, K. C., R. Stout, and R. Mitchell. 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. 68:337-348.

McAlister, L., and D. B. Finkelstein. 1980. Heat shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. 93:819-824.

McDonald, L. C., H. P. Fleming, and H. M. Hassan. 1990. Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Appl. Environ. Microbiol. 56:2120-2124.

McDonald, L. C., C. R. Hackney, and B. Ray. 1983. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. Appl. Environ. Microbiol. 45:360-365.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5:607-625.

Mermin, J. H., J. M. Townes, M. Gerber, N. Dolan, E. D. Mintz, and R. V. Tauxe. 1998. Typhoid fever in the United States, 1985-1994: Changing risks of international travel and increasing antimicrobial resistance. Arch. Intern. Med. 158:633-638.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Miller, S. I., E. L. Hohmann, and D. A . Pegues. 1995. *Salmonella* (including *Salmonella* Typhi), p. 2013-2033. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, NY.

Moellering, R. C., Jr., and D. E. Sentochnik. 1998. Cephalosporins, p. 185-197. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Morse, S. S. 1995. Factors in the emergence of infectious diseases. Emerg. Infect. Dis. 1:7-15.

Murray, B. E. 1986. Resistance of *Shigella*, *Salmonella* and other selected enteric pathogens to antimicrobial agents. Rev. Infect. Dis. 8:S172-S181.

NCCLS. 1999a. Development of *in vitro* susceptibility testing criteria and quality control parameters for veterinary antimicrobial agents; approved guideline. NCCLS document M37-A. NCCLS, Wayne, PA.

NCCLS. 1999b. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. NCCLS document M31-A. NCCLS, Wayne, PA.

Neidhardt, F. C. 1987. Multigene systems and regulons, p. 1313-1317. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM Press, Washington, DC.

Ng, H., H. G. Bayne, and J. A. Garibaldi. 1969. Heat resistance of *Salmonella*: The uniqueness of *Salmonella* Senftenberg 775W. Appl. Microbiol. 17:78-82.

Nojoumi, S. A., D. G. Smith, and R. J. Rowbury. 1995. Tolerance to acid in pH 5.0-grown organisms of potentially pathogenic gram-negative bacteria. Lett. Appl. Microbiol. 21:359-363.

NRC. 1999. The use of drugs in food animals: Benefits and risks. National Academy Press, Washington, DC.

Nutsch, A. L., R. K. Phebus, M. J. Riemann, D. E. Schafer, J. E. Boyer, Jr., R. C. Wilson, J. D. Leising, and C. L. Kastner. 1997. Evaluation of a steam pasteurization process in a commercial beef processing facility. J. Food Prot. 60:485-492.

Nutsch, A. L., R. K. Phebus, M. J. Riemann, J. S. Kotrola, R. C. Wilson, J. E. Boyer, Jr., and T. L. Brown. 1998. Steam pasteurization of commercially slaughtered beef carcasses: Evaluation of bacterial poulations at five anatomical locations. J. Food Prot. 61:571-577.

O'Brien, L. M., S. V. Gordon, I. S. Roberts, and P. W. Andrew. 1996. Response of *Mycobacterium smegmatis* to acid stress. FEMS Microbiol. Lett. 139:11-17.

O'Driscoll, B., C. G. M. Gahan, and C. Hill. 1996. Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl. Environ. Microbiol. 62:1693-1698.

Palumbo, S. A. 1986. Is refrigeration enough to restrain foodborne pathogens? J. Food Prot. 49:1003-1009.

Palumbo, M. S., S. M. Beers, S. Bhaduri, and S. A. Palumbo. 1995. Thermal resistance of *Salmonella* spp. and *Listeria monocytogenes* in liquid egg yolk and egg yolk products. J. Food Prot. 58:960-966.

Peleg, M. 1999. On calculating sterility in thermal and non-thermal preservation methods. Food Res. Int. 32:271-278.

Peleg, M. 2000. Microbial survival curves—the reality of flat "shoulders" and absolute thermal death times. Food Res. Int. 33:531-538.

Peleg, M., and M. B. Cole. 1998. Reinterpretation of microbial survival curves. Crit. Rev. Food Sci. 38:353-380.

Pestka, S. 1971. Inhibitors of ribosomal functions. Annu. Rev. Microbiol. 25:487-562.

Pflug, I. J., and R. G. Holcomb. 1977. Principles of thermal destruction of microorganisms, p. 933-994. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation. Lea & Febiger. Philadelphia, PA.

Pflug, I. J., and R. G. Holcomb. 1991. Principles of the thermal destruction of microorganisms, p. 85-128. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation. Lea & Febiger. Philadelphia, PA.

Phebus, R. K., A. L. Nutsch, and D. E. Schafer. 1996. Laboratory and commercial evaluation of a steam pasteurization process for reduction of bacterial populations on beef carcass surfaces. Proc. Recip. Meat Conf. 49:121-124. American Meat Science Association, Chicago, IL.

Phebus, R. K., A. L. Nutsch, D. E. Schafer, R. C. Wilson, M. J. Riemann, J. D. Leising, C. L. Kastner, J. R. Wolf, and R. K. Prasai. 1997. Comparison of steam pasteurization and other methods for reduction of pathogens on surfaces of freshly slaughtered beef. J. Food Prot. 60:476-484.

Piddock, L. J. V., K. Whale, and R. Wise. 1990. Quinolone resistance in *Salmonella*: Clinical experience. Lancet 335:1459.

Popoff, M. Y., and L. Le Minor. 1997. Antigenic formulas of the *Salmonella* serovars, 7th revision. WHO Collaborating Centre for Reference and Research on *Salmonella*, Pasteur Institute, Paris, France.

Poppe, C., N. Smart, R. Khakhria, W. Johnson, J. Spika, and J. Prescott. 1998. *Salmonella typhimurium* DT104: A virulent and drug-resistant pathogen. Can. Vet. J. 39:559-565.

Prasai, R. K., G. R. Acuff, L. M. Lucia, D. S. Hale, J. W. Savell, and J. B. Morgan. 1991. Microbiological effects of acid decontamination of beef carcasses at various locations in processing. J. Food Prot. 54:868-872.

Prescott, J. F., and J. D. Baggot (ed.). 1993. Antimicrobial therapy in veterinary medicine, 2nd ed. Iowa State University Press, Ames, IA.

Prescott, L. M., J. P. Harley, and D. A. Klein. 1993. Microbiology, 2nd ed. Wm. C. Brown Publishers, Dubuque, IA.

Rahal, J. J., Jr., and M. S. Simberkoff. 1979. Bactericidal and bacteriostatic action of chloramphenicol against meningeal pathogens. Antimicrob. Agents Chemother. 16:13-18.

Raja, N., W. C. Goodson, C. Chui, D. G. Smith, and R. J. Rowbury. 1991. Habitation to acid in *Escherichia coli*: Conditions for habitation and its effects on plasmid transfer. J. Appl. Bacteriol. 70:59-65.

Rathman, M., M. D. Sjaastad, and S. Falkow. 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. 64:2765-2773.

Rees, C. E., C. E. Dodd, P. T. Gibson, I. R. Booth, and G. S. Stewart. 1995. The significance of bacteria in stationary phase to food microbiology. Int. J. Food Microbiol. 28:263-275.

Renberg, I., T. Korsman, and H. J. B. Birks. 1993. Prehistoric increases in the pH of acid-sensitive Swedish lakes caused by land-use changes. Nature 362:824-827.

Rice, L. B., and R. A. Bonomo. 1996. Genetic and biochemical mechanisms of bacterial resistance to antimicrobial agents, p. 453-501. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, MD.

Riley, L. W., M. L. Cohen, J. E. Seals, M. J. Blaser, K. A. Birkness, N. T. Hargrett, S. M. Martin, and R. A. Feldman. 1984. Importance of host factors in human salmonellosis caused by multiresistant strains of *Salmonella*. J. Infect. Dis. 149:878-883.

Roberts, T. A., and A. D. Hitchins. 1969. Resistance of spores, p. 611-670. *In* G. W. Gould, and A. Hurst (ed.), The bacterial spore. Academic Press, London.

Rowbury, R. J., and M. Goodson. 1993. PhoE porin of *Escherichia coli* and phosphate reversal of acid damage and killing and of acid induction of the CadA gene product. J. Appl. Bacteriol. 74:652-661.

Rowbury, R. J., and M. Goodson. 1998. Glucose-induced acid tolerance appearing at neutral pH in log-phase *Escherichia coli* and its reversal by cyclic AMP. J. Appl. Microbiol. 85:615-620.

Rowe, M. T., and R. Kirk. 1999. An investigation into the phenomenon of cross-protection in *Escherichia coli* O157:H7. Food Microbiol. 16:157-164.

Russell, N. J. 1988. Environmentally-induced changes in lipid composition and membrane function, p. 130-145. *In* R. Whittenbury, G. W. Gould, J. G. Bands, and R. G. Board (ed.), Homeostatic mechanisms in microorganisms. Bath University Press, Bath.

Ryu, J-. H., and L. R. Beuchat. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. Int. J. Food Microbiol. 45:185-193.

Ryu, J-. H., and L. R. Beuchat. 1999. Changes in heat tolerance of *Escherichia coli* O157:H7 after exposure to acidic environments. Food Microbiol. 16:317-324.

Saito, H., H. Tomioka, M. Emori, T. Yamane, K. Yamashita, K. Hosoe, and T. Hidaka. 1991. In vitro antimycobacterial activities of newly synthesized benzoxazinorifamycins. Antimicrob. Agents Chemother. 35:542-547.

Salyers, A. A., and D. D. Whitt. 1994. Bacterial pathogenesis: A molecular approach. ASM Press, Washington, DC.

Samelis, J., J. N. Sofos, P. A. Kendall, and G. C. Smith. 2001a. Fate of *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104, and *Listeria monocytogenes* in fresh meat decontamination fluids at 4 and 10°C. J. Food Prot. 64:950-957.

Samelis, J., J. N. Sofos, P. A. Kendall, and G. C. Smith. 2001b. Influence of the natural microbial flora on the acid tolerance response of *Listeria monocytogenes* in a model system of fresh meat decontamination fluids. Appl. Environ. Microbiol. 67:2410-2420.

Sanders, C. C., and K. S. Thomson. 1998. Other ß-lactam antibiotics, p. 197-203. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

SAS. 1995. SAS® System under Microsoft Windows, Release 6.12. SAS Institute Inc., Cary, NC.

SAS. 1999. SAS® System under Microsoft Windows, Release 8e. SAS Institute Inc., Cary, NC.

SAS. 2001. SAS® System under Microsoft Windows, Release 8.2. SAS Institute Inc., Cary, NC.

Schafer, V., P. M. Shah, H. W. Doerr, M. Ziemen, S. Hellwich, and G. Seibert. 1992. In-vitro activity of cefpirome against isolates from patients with urinary tract, lower respiratory tract and wound infections. J. Antimicrob. Chemother. 29(Suppl A):7-12.

Schnell, T. D., J. N. Sofos, V. G. Littlefield, J. B. Morgan, B. M. Gorman, R. P. Clayton, and G. C. Smith. 1995. Effects of postexsanguination dehairing on the microbial load and visual cleanliness of beef carcasses. J. Food Prot. 58:1297-1302.

Schuman, J. D., B. W. Sheldon, and P. M. Foegeding. 1997. Thermal resistance of *Aeromonas hydrophila* in liquid whole egg. J. Food Prot. 60:231-236.

Schwach, T. S., and E. A. Zottola. 1982. Use of scanning electron microscopy to demonstrate microbial attachment to beef and beef contact surfaces. J. Food Sci. 47:1401-1405.

Scott, V. N., and D. T. Bernard. 1985. The effect of lysozyme on the apparent heat resistance of non-proteolytic type B *Clostridium botulinum*. J. Food Safety 7:145-154.

Scroggins, C. D. 1988. Stepping stone or stumbling blocks: A consumer perspective of animal drug use, p. 53-58. *In* Animal drug use: Dollars and sense. Department of Health and Human Services Publication # 88.6045, Center for Veterinary Medicine, Rockville, MD.

Sensi, P. 1983. History of the development of rifampin. Rev. Infect. Dis. 5: S402-S406.

Setlow, B., and P. Setlow. 1979. Localization of low-molecular weight basic proteins in *Bacillus megaterium* spores by cross-linking with ultraviolet light. J. Bacteriol. 139:486-494.

Shah, D. B., J. G. Bradshaw, and J. T. Peeler. 1991. Thermal resistance of egg-associated epidemic strains of *Salmonella* Enteritidis. J. Food Sci. 56:391-393.

Shannon, K. and G. French. 1998. Multiple-antibiotic-resistant *Salmonella*. Lancet 352:490.

Sheridan, J. J., and D. A. McDowell. 1998. Factors affecting the emergence of pathogens on foods. Meat Sci. 49:S151-S167.

Small, P. L. C. 1998. *Shigella* and *Escherichia coli* strategies for survival at low pH. Jpn. J. Med. Sci. Biol. 51:S81-S89.

Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski. 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: Role of *rpoS* and growth pH. J. Bacteriol. 176:1729-1737.

Smith, S. M., P. E. Palumbo, and P. J. Edelson. 1984. *Salmonella* strains resistant to multiple antibiotics: Therapeutic implications. Pediatr. Infect. Dis. 3:455-460.

Smulders, F. J. M., and G. G. Greer. 1998. Integrating microbial decontamination with organic acids in HACCP programs for muscle foods: Prospects and controversies. Int. J. Food Microbiol. 44:149-169.

Snijders, J. M. A., M. H. W. Janssen, G. E. Gerats, and G. P. Corstiaensen. 1984. A comparative study of sampling techniques for monitoring carcass contamination. Int. J. Food Microbiol. 1:229-236.

Sofos, J. N., S. L. Kochevar, G. R. Bellinger, D. R. Buege, D. D. Hancock, S. C. Ingham, J. B. Morgan, J. O. Reagan, and G. C. Smith. 1999a. Sources and extent of microbiological contamination of beef carcasses in seven United States slaughtering plants. J. Food Prot. 62:140-145.

Sofos, J. N., S. L. Kochevar, J. O. Reagan, and G. C. Smith. 1999b. Extent of beef carcass contamination with *Escherichia coli* and probabilities of passing U.S. regulatory criteria. J. Food Prot. 62:234-238.

Sofos, J. N., S. L. Kochevar, J. O. Reagan, and G. C. Smith. 1999c. Incidence of *Salmonella* on beef carcasses relating to the U.S. meat and poultry inspection regulations. J. Food Prot. 62:467-473.

Sofos, J. N., and G. C. Smith. 1993. The new headache of the U.S. meat industry: *E. coli* 0157:H7. Meat Focus Int. 2:317-325.

Sofos, J. N., and G. C. Smith. 1998. Nonacid meat decontamination technologies: Model studies and commercial applications. Int. J. Food Microbiol. 44:171-188.

Spika, J. S., S. H. Waterman, G. W. Soo Hoo, M. E. St Louis, R. E. Pacer, S. M. James, M. L. Bissett, L. W. Mayer, J. Y. Chiu, B. Hall, K. Greene, M. E. Potter, M. L. Cohen, and P. A. Blake. 1987. Chloramphenicol resistant *Salmonella newport* traced through hamburger to dairy farms. A major persisting source of human salmonellosis in California. N. Engl. J. Med. 316:565-570.

Stratton, C. W. 1996. Mechanisms of action for antimicrobial agents: General principles and mechanisms for selected classes of antibiotics, p. 579-603. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 4th ed. Williams & Wilkins, Baltimore, MD.

Stumbo, C. R. 1973. Thermobacteriololgy in food processing, 2nd ed. Academic Press, New York, NY.

Sykes, R. B., and D. P. Bonner. 1985. Discovery and development of the monobactams. Rev. Infect. Dis. 7(Suppl 4):S579-S593.

Tauxe, R. V. 1991. *Salmonella*: A postmodern pathogen. J. Food Prot. 54:563-568.

Tauxe, R. V. 1996. An update on *Salmonella*. Health Environ. Digest 10:1-4.

Thornsberry, C., B. C. Hill, J. M. Swenson, and L. K. McDougal. 1983. Rifampin: Spectrum of antibacterial activity. Rev. Infect. Dis. 5:S412-S417.

Threlfall, E. J., F. J. Angulo, and P. G. Wall. 1998. Ciprofloxacin-resistant *Salmonella typhimurium* DT104. Vet. Rec. 142:255.

Todd, E. C. D. 1989. Preliminary estimates of costs of foodborne disease in the United States. J. Food Prot. 52:595-601.

Tollefson, L., F. J. Angulo, and P. J. Fedorka-Cray. 1998. National surveillance for antibiotic resistance in zoonotic enteric pathogens. Vet. Clin. North Am. Food Anim. Pract. 14:141-151.

Tollefson, L, P. J. Fedorka-Cray, and F. J. Angulo. 1999. Public health aspects of antibiotic resistance monitoring in the USA. Acta Vet. Scand. Suppl. 92, 67-75.

Upmann, M., P. Jakob, and G. Reuter. 2000. Microbial transfer during cutting and deboning of pork in a small-scale meat processing plant. Dairy Food Environ. Sanit. 20:14-23.

Van Gerwen, S. J. C., and M. N. Zwietering. 1998. Growth and inactivation models to be used in quantitative risk assessment. J. Food Prot. 61:1541-1549.

Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli*: Partial purification and some properties. J. Biol. Chem. 218:97-106.

Walker, R. A., A. J. Lawson, E. A. Lindsay, L. R. Ward, P. A. Wright, F. J. Bolton, D. R. A. Wareing, J. D. Corkish, R. H. Davies, and E. J. Threlfall. 2000. Decreased susceptibility to ciprofloxacin in outbreak-associated multiresistant *Salmonella typhimurium* DT104. Vet. Rec. 147:395-396.

Wang, G., and M. P. Doyle. 1998. Heat shock response enhances acid tolerance of *Escherichia coli* O157:H7. Lett. Appl. Microbiol. 26:31-34.

Ware, L. M., M. L. Kain, J. N. Sofos, K. E. Belk, and G. C. Smith. 1999. Comparison of sponging and excising as sampling procedures for microbiological analysis of fresh beef-carcass tissue. J. Food Prot. 62:1255-1259.

Warth, A. D. 1978. Relationship between the heat resistance of spores and the optimum and maximum growth temperatures of *Bacillus* species. J. Bacteriol. 134:699-705.

Weitzel, G., U. Pilatus, and L. Rensing. 1987. The cytoplasmic pH, ATP content and total protein synthesis rate during heat-shock protein inducing treatments in yeast. Exp. Cell Res. 170:64-79.

White, D. G., S. Zhao, R. Sudler, S. Ayers, S. Friedman, S. Chen, P. F. McDermott, S. McDermott, D. D. Wagner, and J. Meng. 2001. The isolation of antibiotic resistant *Salmonella* from retail ground meats. New Engl. J. Med. 345:1147-1166.

Whiting, R. C. 1995. Microbial modeling of foods. Crit. Rev. Food Sci. Nutr. 35:467-494.

Wierup, M. 2000. The control of microbial diseases in animals: Alternatives to the use of antibiotics. Int. J. Antimicrob. Agents 14:315-319.

Wilde, S., F. Jorgensen, A. Campbell, R. Rowbury, and T. Humphrey. 2000. Growth of *Salmonella enterica* Serovar Enteritidis PT4 in media containing glucose results in enhanced RpoS-independent heat and acid tolerance but does not affect the ability to survive air-drying on surfaces. Food Microbiol. 17:679-686.

Williams, B. M. 1975. Environmental considerations in salmonellosis. Vet. Rec. 96:318-321.

Williams, D. N. 1998. Tetracyclines, p. 227-231. *In* S. L. Gorbach, J. G. Bartlett, and N. R. Blacklow (ed.), Infectious diseases, 2nd ed. The W. B. Saunders Co., Philadelphia, PA.

Williams, J. D., and F. Moosdeen. 1987. In vitro antibacterial effects of cephalosporins. Drugs 34(Suppl 2):1-14.

Wilmes-Riesenberg, M. R., B. Bearson, J. W. Foster, and R. Curtiss, III. 1996. Role of the acid tolerance response in virulence of *Salmonella typhimurium*. Infect. Immun. 64:1085-1092.

Wise, R. 1992. Oral cephalosporins. J. Antimicrob. Chemother. 29:91-92.

Xavier, I. J., and S. C. Ingham. 1997. Increased *D*-values for *Salmonella* Enteritidis following heat shock. J. Food Prot. 60:181-184.

Yamamori, T., and T. Yura. 1982. Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in *Escherichia coli* K12. Proc. Natl. Acad. Sci. 79:860-864.

Zilberstein, D., V. Agmon, S. Schuldiner, and E. Padan. 1984. *Escherichia coli* intracellular pH, membrane potential and cell growth. J. Bacteriol. 158:246-252.