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THERMAL RESISTANCE OF SUBLETHALLY INJURED *SALMONELLA*

By

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ABSTRACT

THERMAL RESISTANCE OF SUBLETHALLY INJURED *SALMONELLA*

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Recovery of stressed cells is often limited by the choice of culture media and environmental conditions, including oxygen exposure. The ability of reducing agents, which can neutralize reactive oxygen species, to enhance recovery of sublethally injured *Salmonella* was studied. Using Trypticase soy agar with 0.6% yeast extract (TSA-YE) supplemented with Oxyrase®, 0.1% sodium pyruvate and 0.6% sodium pyruvate, recovery of heat-shocked (30 min / 54°C), cold-shocked (2 h / 4°C) and starved cells (10 d in phosphate buffer at 4°C) ranged from 89.6 to 90.2%, 94.9 to 95.1% and 99.2 to 101.6%, respectively, and was not significantly different from unsupplemented TSA-YE. Sublethally injured cells may also have heightened sensitivity to thermal processing, due to activated stress response mechanisms. To investigate the effects of sublethal stress on *Salmonella* inactivation kinetics, an 8-strain *Salmonella* cocktail was subjected to heat shock, cold shock, and starvation stress, harvested by centrifugation and inoculated into irradiated comminuted turkey. The $D_{60°C}$-value for heat-shocked *Salmonella* on TSA-YE was 0.64 min, which was significantly higher ($P<0.05$) than that for the unshocked control (0.41 min), whereas those for cold shock (0.38 min) and starvation (0.41 min) were not significantly different from the control. Additionally, more thermal inactivation was observed using selective xylose lysine deoxycholate (XLD) agar than using non-selective TSA-YE, resulting in an underestimation of survivors on the selective medium.

To my Mom and Dad

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KEY TO SYMBOLS OR ABBREVIATIONS

AMI	American Meat Institute
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
ASP	acid shock protein
ATP	adenosine triphosphate
ATR	acid tolerance response
BG	brilliant green
BPB	Butterfield's phosphate buffer
BSA	bismuth sulfite agar
CDC	Centers for Disease Control
CFU	colony forming unit
cm	centimeter(s)
CSP	cold shock protein
d	day(s)
D-value	decimal reduction time
DF	degrees of freedom
DNA	deoxyribonucleic acid
ERS	Economic Research Service
FSIS	Food Safety and Inspection Services
g	gram(s)
GLM	general linear model
HACCP	Hazard Analysis and Critical Control Points
h	hour(s)
HSP	heat shock protein
IFT	Institute of Food Technologists
lb	pound(s)
LPS	lipopolysaccharide layer
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
MPa	megapascal
PFGE	pulsed-field gel electrophoresis
ROS	reactive oxygen species
RTE	ready-to-eat
RNA	ribonucleic acid
s	second(s)
spv	Salmonella Plasmid Virulence
SOD	superoxide dismutase
SSOP	Sanitation Standard Operating Procedure
TCA	tricarboxylic acid cycle
TGE	tryptone-glucose extract
TSA	Trypticase soy agar

TSA-YE	Trypticase soy agar containing 0.6% yeast extract
TSB	Trypticase soy broth
TSB-YE	Trypticase soy broth containing 0.6% yeast extract
U	units
UHT	ultra-high temperature
USDA	United States Department of Agriculture
VRBA	violet red bile agar
WHO	World Health Organization
XLD	xylose lysine deoxycholate

CHAPTER 1

INTRODUCTION

1.1 Foodborne Illness in the United States

Foodborne illness is a considerable public health problem in the United States. An estimated 76 million cases of foodborne disease occur annually, with 325,000 hospitalizations and 5,200 deaths (Mead et al. 1999). Six to 33 million cases of foodborne illness are thought to be bacterial in origin, with *Salmonella* and *Campylobacter* accounting for almost 82% of these cases (CDC 2001a). *Salmonella* and *Listeria,* along with *Toxoplasma*, are responsible for more than 75% of the 5,200 deaths caused by known foodborne pathogens (Mead et al. 1999). The Economic Research Service estimates that the medical costs, productivity losses and lost wages, and value of premature death for the five major pathogens (*Campylobacter, Salmonella, Escherichia coli* O157:H7, *E. coli* non-O157:H7 STEC, and *Listeria monocytogenes*) total at least $6.9 billion annually (Crutchfield 2001). *Salmonella* alone has an estimated annual cost of $2.4 billion (Frenzen 2002). Many sectors of the economy are burdened with the costs of foodborne illness. Those who become ill and their families, as well as other parties, including employers, insurers and taxpayers, generally bear the brunt of these costs (Buzby et al. 2001).

The cost estimate presented for foodborne illness does not include data on product recalls due to bacterial contamination. For instance, from 1998 to 2001, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) recalled over 3,250,000 pounds of meat and poultry due to *Salmonella* contamination (FSIS

2002a). The meat industry is particularly concerned with compromised product safety as a result of *L. monocytogenes*, *E. coli* O157:H7, *Salmonella* and *Campylobacter*, although other microbial hazards (e.g., *Clostridium perfringens*, *Clostridium botulinum*, staphylococcal enterotoxins) are also important.

1.2 The Meat Industry and Relevant Regulations

Microbial contamination of meat and poultry products is paramount when considering the staggering impact of the meat and poultry industry on the United States economy. A U.S. Senate resolution designating the first annual National Meat Week in 1984 described the meat industry, with $70 billion in annual sales, as the "largest single component of U.S. agriculture" (Romans et al. 2001). The impact of the meat and poultry on the agricultural industry has remained virtually unchanged since 1984, although the annual sales are now well over $90 billion (AMI 2000, ERS 2001, Jones 2001, Romans et al. 2001). Red meat and poultry production is continuing along a record setting pace (Southard et al. 2000). Worldwide, the United States leads in beef and veal production and total consumption, placing second only to Argentina in per capita consumption. The poultry record is even more startling, with the United States a clear leader in broiler chicken production, exports, and total and per capita consumption (Romans et al. 2001). Red meat and poultry consumption per capita was 213 and 220 lb in 2001 and 2002, respectively. Consumption of poultry meat alone has soared drastically in the last two decades to 95 and 99 lb per person in 2001 and 2002, respectively (ERS 2002).

In this same timeframe, however, per capita consumption of red meat has actually decreased. Although pork consumption has remained fairly constant, beef consumption

has not (Romans et al. 2001). The marketing of beef has not changed significantly over the last twenty years, compounded by the poultry industry's greater ability to cater to the definitive trend toward convenience foods. It is estimated that three-quarters of all women aged 25-54 in the United States are now in the work force, as opposed to only about one-half twenty years ago. More households have two working adults, allowing for less time to spend preparing meals-a fact that has helped direct the shift toward convenience foods (Carmel 2000).

Pork consumption has remained fairly constant, partly because so many processed meats, including sausage and cold cuts, are made with pork. Likewise, the dramatic increase in poultry consumption can in part be attributed to the use of more chicken and turkey in further processed meat items (Romans et al. 2001). In fact, over sixty percent of all turkey meat produced in the United States is used in the production of convenience items (Romans et al. 2001). The trend towards convenience is perhaps best illustrated by the growth of heat-and-eat meals, such as frozen dinners with meat as the major contributor to the meal. Pre-cooked meat patties and luncheon meats, including bologna, frankfurters and meat loaves, have also benefited from the increased use of convenience foods (Pearson and Gillett 1996).

The United States Department of Agriculture (USDA) defines ready-to-eat (RTE) meat and poultry products as those products that have been appropriately processed so as to be safely consumed without further preparation by the consumer. Such products can be either shelf-stable or non-shelf-stable (i.e., requiring refrigeration to prevent growth of spoilage or pathogenic organisms). USDA specifically divides RTE meat and poultry products into five categories: dried products (e.g., jerky), salt-cured products (e.g.,

prosciutto), fermented products (e.g., pepperoni), cooked and otherwise processed products (e.g., ravioli, turkey franks), and thermally-processed, commercially sterile products (e.g., canned soups with meat or poultry) (FSIS 2001). RTE meat and poultry products are purchased because they offer flavor appeal, nutritional value, convenience and variety to the diet at a price that is competitive with fresh meats and poultry (Tompkin 1986). However, RTE and partially heat-treated meat and poultry products can present a safety problem from a microbiological standpoint. Emphasis is particularly placed on RTE products, as consumers are less likely to reheat these products to sufficiently kill pathogenic microorganisms such as *L. monocytogenes* and *Salmonella* spp. before cooking (Levine et al. 2001). Additionally, while pre-cooked meat patties and other items are widely consumed by the general public, they are particularly popular for school lunch programs, hospitals and other institutional settings where increased numbers of susceptible individuals are present (Romans et al. 2001). However, USDA-FSIS does have certain regulations in place to reduce and eliminate product contamination.

The United States has been inspecting meat and poultry products since 1891, beginning with certain salted pork and bacon items in response to European fears of trichinosis. Federal meat and poultry inspection and regulations expanded throughout the next century. Major milestones included the 1967 Wholesome Meat Act and the 1968 Wholesome Poultry Act requiring that all carcasses and meat products be inspected. By the mid-1990's, FSIS had over 7,400 inspectors in 6,200 slaughter and processing plants. The system these inspectors were operating under, however, did not adequately target and reduce microbial pathogens (Crutchfield et al. 1997). To help remedy this situation, in

1996 FSIS began phasing in sanitation standard operating procedures (SSOPs), Hazard Analysis and Critical Control Points (HACCP) procedures, and microbial testing (Crutchfield et al. 1997).

Most recently, FSIS has finalized new regulations for the production of certain fully and partially cooked meat and poultry products (FSIS 1999). The agency has likewise recently proposed similar regulations for RTE and all partially heat-treated meat and poultry products (FSIS 2001). In both instances, the changes signal a move away from the command-and-control nature of the previous requirements. Rather than dictate step-by-step processing methods with prescribed endpoint temperatures, the regulations are in the form of lethality, stabilization and handling performance standards. In particular, these standards establish lethality levels for pathogen reduction as well as set limits for pathogen growth. Lethality itself is defined as the required reduction in the number of specific pathogenic microorganisms. A 6.5-log reduction of *Salmonella* in cooked beef, roast beef and cooked corn beef and a 7.0-log reduction of *Salmonella* in certain fully and partially cooked poultry products are required. A comparable reduction is proposed for remaining RTE meat and poultry products.

Alternately, processors may choose to achieve an "equivalent probability that no viable *Salmonella* organisms remain in the finished product" (FSIS 1999). Regardless, the new regulations allow processors a certain measure of flexibility accompanied by the opportunity to implement new, cost-effective technology and customized processing procedures. At the same time, however, the importance of demonstrating and validating the lethality of a given process becomes greater, particularly if the process meets alternative lethalities. It is critical to document the relationship between lethality (and

lethality treatments) and product characteristics, in part because processing standards are based largely on thermal inactivation studies performed in a laboratory and not with actual meat products in a processing microenvironment. It also becomes critical to document the relationship between lethality and microorganism characteristics, including strain and sublethal injury. Of particular concern, several antibiotic resistant and heat resistant microorgansims have emerged. Also, several environmental conditions, including starvation and cold shock, have either been proven or are suspected to alter thermal inactivation kinetics of pathogens.

Current regulations have been subject to certain criticisms. Many feel that testing food for pathogens will not necessarily make food safer. However, according to the American Public Health Association (APHA), a group that represents over 50,000 public health professionals in the United States and abroad, "people manage what they measure" (Levinson 2000). Although microbial testing does not suffice as a control for HACCP, microbial testing at appropriate Critical Control Points is imperative to verify the effectiveness of preventative measures and critical limits (Levinson 2000). The APHA and other groups particularly believe that requirements for microbial testing in the form of performance standards accomplish the best balance between public and private intervention and control in food safety. It is not the fundamental role of FSIS to dictate how to exclude pathogens from meat products, but rather to test and affirm that these products meet the established standards. At the same time, enforcing performance standards allows technological innovation, creativity and advancement to stay in the hands of the private sector, where most experts agree it belongs (Levinson 2000).

Proposed and finalized FSIS lethality standards reflect the destruction of specific

"reference" organisms whose elimination or reduction will ordinarily likewise indicate the crucial elimination or reduction of other pathogenic organisms. Except for thermally-processed, commercially sterile products, the lethality performance standards are based on *Salmonella*. *Salmonella* was chosen as the target organism for a number of reasons. It is associated with raw meat (poultry, beef and pork) and is responsible for a high incidence of foodborne illness, which can often be severe. Also, the conditions required for an appropriate reduction in *Salmonella* will simultaneously reduce most other vegetative pathogenic microorganisms. In particular, if the given reduction of *Salmonella* organisms is achieved, a safe reduction of *Trichinella spiralis, Staphylococcus aureus* and *E. coli* O157:H7 should also be met, because these organisms are typically less heat resistant than *Salmonella.* Although more heat resistant than *Salmonella*, *L. monocytogenes*, will most likely enter the product as a post-processing contaminant, due to improper or inadequate sanitation, rather than as a result of insufficient processing. To verify compliance with performance standards (e.g., via challenge studies), FSIS suggests the use of a *Salmonella* cocktail comprised of relatively heat resistant strains or serotypes as well as those implicated in outbreaks (FSIS 2001).

1.3. Justification

The status of the most recent regulation is in limbo. It is not finalized and FSIS is requesting additional scientific information and data. The policy or belief of FSIS is that the regulations must be based on "sound science and common sense measures that involve significant public comment" (FSIS 2001). The research presented here aims to serve as such, helping to answer some of the pressing questions from the regulation for processed products, especially those related to bacterial injury and lethality.

Stressed cells behave differently from cells grown under optimal conditions in the laboratory. Unfortunately, it is typically these latter cells that are used in food safety studies. The possibility that preservation methods based on such studies may not be sufficient to ensure the safety of processed food becomes a major cause for concern (Lou and Yousef 1996).

An additional concern in the design of inactivation studies involving foodborne pathogens is that most studies used various laboratory media rather than food menstra (Farber and Brown 1990). Food components offer a selective advantage to microorganisms for enhanced heat resistance (Van Schothorst and Maggie Duke 1984, Mackey and Derrick 1987b, Farber and Brown 1990, Murphy et al. 1999). Sublethal stress studies must also consider the importance of using actual foods. In fact, initial studies by Mackey and Derrick (1987b) showed that the effect of heat shock was accompanied by increased heat resistance conferred on *Salmonella* ser. Thompson by the composition of the heating medium. Thus, studies designed to compare pathogen stress and inactivation in real foods, specifically meat products, to that in liquid media (Murphy et al. 1999) should be conducted to augment current data with information that may be more directly applicable to real commercial processes.

1.4 Objectives and Hypothesis

After developing sublethal heat, cold and starvation treatments for an 8-strain *Salmonella* cocktail, the objectives of this research were to (1) assess the effect of media supplementation on the recovery of sublethally injured *Salmonella* and (2) investigate the effects of pre-stress treatments on the thermal inactivation kinetics of *Salmonella*. Given the physiological differences between injured and uninjured microorganisms, the

hypothesis is that a prior heat, cold or starvation stress will affect the recovery and thermal resistance profiles of *Salmonella*.

CHAPTER 2

LITERATURE REVIEW

2.1 *Salmonella*

Salmonella spp. are facultatively anaerobic, small, Gram-negative, nonsporing rods belonging to the family *Enterobacteriaceae* (Table 2.1). Currently, there are at least 2,463 serovars (serotypes) of *Salmonella* (Brenner et al. 2000). The major antigens for serotyping are somatic (O lipopolysaccharides of the bacterial outer membrane), flagellar (H antigens) and capsular (Vi antigens occurring in only three *Salmonella* serovars) (Le Minor 1992, D'Aoust 1997). Antigenic structure has applications in diagnostics, identification and confirmation, as well as classification and nomenclature.

Salmonella taxonomy and nomenclature are complex and have undergone several significant changes in recent years. Most state and local public health agencies follow the nomenclatural system used by the Centers for Disease Control and Prevention (CDC). This system, based on recommendations from the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* (Institut Pasteur, Paris), divides the genus *Salmonella* into two species, *S. enterica* and *S. bongori*, each containing multiple serotypes (see Table 2.2). *S. enterica*, the so-called type species, is divided into six subspecies differentiated by biochemical reactions, by genomic relatedness and by the Kauffman-White serotyping scheme. Although the subspecies are differentiated, they do form a single DNA hybridization group. The majority of all serotypes fall within *S. enterica* subsp. I (*S. enterica* subsp. *enterica*). Within this group, CDC uses names for serotypes (e.g., Enteritidis, Typhimurium, Heidelberg) and follows

Table 2.1. Biochemical characteristics of a typical *Salmonella* isolate (D'Aoust 1997, Madigan et al. 1997, Bell and Kyriakides 2002).

Biochemical Characteristic	Reaction
H_2S	+
Catalase	+
Citrate	+
Indole	-
Lactose	-
Lysine Decarboxylase	+
Ornithine Decarboxylase	+
Oxidase	-
Urease	-

+ = positive reaction; - = negative reaction

Table 2.2. Species, subspecies and serovars of the genus *Salmonella* (adapted from Brenner et al. 2000).

Species	No. of serovars	Natural habitat
Salmonella enterica		
I. subsp. *enterica*	1454	warm-blooded animals
II. subsp. *salamae*	489	
IIIa. subsp. *arizonae*	94	
IIIb. subsp. *diarizonae*	324	
IV. subsp. *houtenae*	70	cold-blooded animals and the environment[a]
VI. subsp. *indica*	12	
Salmonella bongori	20	
Total	2463	

[a]Isolates from all subspecies have been recovered from human sources.

the naming format of the WHO Collaborating Centre (see Table 2.3) (Brenner et al. 2000, D'Aoust 1997, Le Minor 1992).

For purposes related to epidemiological investigation, salmonellae can be divided into three groups (Jay 1998, Poppe 1999):

1. Serovars infecting humans only. These include S. ser. Typhi, the causative agent of typhoid fever, and the paratyphoid salmonellae. Typhoid and paratyphoid fevers are the most severe diseases caused by salmonellae (Jay 1998).

2. Host adapted serovars including S. ser. Abortusequi (horses), S. ser. Abortusovis (sheep), S. ser. Choleraesuis (swine), S. ser. Dublin (cattle) and S. ser. Gallinarum and S. ser. Pullorum (poultry). Several organisms in this group are human pathogens and potentially can be contracted from foods (Jay1998, Poppe 1999).

3. Unadapted serovars with no host preference. The largest group, these serovars may be pathogenic for both humans and animals. This group includes most foodborne salmonellae and is most commonly responsible for foodborne infections in human adults (Jay 1998, Le Minor 1992).

Many different serotypes are responsible for gastroenteritis. Table 2.4 gives the serotypes most commonly isolated from human sources between 1996 and 2000. In 2000, the top three *Salmonella* serotypes (Typhimurium, Enteritidis and Newport) represented 51% of all isolates. As in other years, most isolates (25% in 2000) were from children under 5 years of age (CDC 2001b). In 1999, 31% of human *S.* Typhimurium isolates received by the National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline, the multi-drug resistance pattern associated with isolates of *S.* ser. Typhimurium belonging to phage type DT104 (Ribot et al. 2002).

Symptoms resulting from infection with nontyphoid salmonellae generally appear 8 to 72 hours after exposure and include diarrhea, nausea, fever and abdominal cramps.

Table 2.3. *Salmonella* nomenclature recommended by the WHO Collaborating Centre for Reference and Research on *Salmonella* and used at the CDC (adapted from Brenner et al. 2000).

Nomenclature	
Genus (italicized)	*Salmonella*
Species (italicized)	• *enterica* • *bongori*
Serotype (capitalized, not italicized)	(The serotype name should be preceded by the word "serotype" or "ser." the first time it is mentioned in the text.)
Examples	
S. enterica[a] subsp. *enterica* ser. Typhi	S. ser. Typhi (*S. typhi* also used)
S. enterica[a] subsp. *enterica* ser. Typhimurium	S. ser. Typhimurium (*S. typhimurium* also used)
S. enterica[a] subsp. *enterica* ser. Hadar	S. ser. Hadar

[a]*S. Choleraesuis* or *S. enteritidis* may also be used

Table 2.4. Predominant clinical serovars of salmonellae in the United States between 1996 and 2000[a].

Year	Total	Rank	Serotype (%)
1996	39,035	1	Enteritidis (24.5)
		2	Typhimurium* (24.3)
		3	Heidelberg (5.1)
			Newport (5.1)
		4	Montevideo (3.1)
		5	Javiana (1.9)
1997	34,608	1	Typhimurium* (26.3)
		2	Enteritidis (22.9)
		3	Heidelberg (6.1)
		4	Newport (4.6)
		5	Agona (2.1)
			Montevideo (2.1)
1998	33,971	1	Typhimurium* (26.0)
		2	Enteritidis (17.7)
		3	Newport (6.7)
		4	Heidelberg (5.6)
		5	Javiana (3.4)
1999	32,782	1	Typhimurium* (24.6)
		2	Enteritidis (16.3)
		3	Newport (8.0)
		4	Heidelberg (5.5)
		5	Muenchen (4.1)
2000	32,022	1	Typhimurium* (22.1)
		2	Enteritidis (19.4)
		3	Newport (9.3)
		4	Heidelberg (5.2)
		5	Javiana (3.6)

*Typhimurium includes var. Copenhagen

[a]Data compiled by the Public Health Laboratory Information System (CDC 2001b, 2000, 1999, 1998, 1997a).

Although acute symptoms can last for weeks, the self-limiting condition is most typically resolved within 4 to 7 days. Successful treatment of patients without complications may only require fluid and electrolyte replacement. However, septicemia and other secondary complications such as reactive arthritis have been reported, particularly among highly susceptible individuals (D'Aoust 1997, Frenzen et al. 1999).

Salmonellosis consistently ranks at or near the top of the list of foodborne diseases. The CDC estimates 1.4 million cases of salmonellosis, 95% of which are foodborne, occur annually in the United States (Frenzen et al. 1999). The overall incidence of diagnosed *Salmonella* infections per 100,000 people was 15.1 in 2001 (CDC 2002a) and 14.4 in 2000 (CDC 2001c). The Healthy People 2010 national health objective for *Salmonella* is 6.8 cases per 100,000 people (CDC 2002a). The estimated annual economic cost (medical costs, productivity losses, lost wages, and value of premature death) of *Salmonella* is $2.4 billion (Frenzen 2002).

Outbreaks associated with *Salmonella* have been reported in a variety of foods, including meat, poultry, cantaloupe, cheese, ice cream and chocolate (D'Aoust 1997). Specific examples of salmonellosis outbreaks are numerous. For instance, *S.* ser. Hadar was identified as the causative agent in a 1999 Georgia outbreak that sickened at least 58 people who ate barbecued pork at a local restaurant. Stool specimens and barbecue samples from the restaurant and patient's homes had identical pulsed-field gel electrophoresis (PFGE) patterns (Toomey et al. 2001). Other examples include a 1998 outbreak of *S.* ser. Heidelberg in turkey that sickened at least 25 people at a New York church luncheon, a 1997 outbreak of *S.* ser. Reading in corned beef that sickened 130 New Jersey restaurant-goers (CDC 1997b), three outbreaks between 2000 and 2002 of *S.*

ser. Poona in cataloupe that sickened 155 people in the United States and Canada (CDC 2002b) and 241 confirmed egg-associated *S.* ser. Enteritidis outbreaks between 1999 and 2001 (CDC 2003). Outbreaks caused by different *Salmonella* serotypes occur in a wide variety of establishments, including private homes, hospitals, and restaurants.

The primary habitat of *Salmonella* is the intestinal tract of humans and animals (Le Minor 1992). The ubiquity of *Salmonella* within the natural environment, along with intensive husbandry practices employed in the meat, fish and shellfish industries has helped foster the continued importance of this human pathogen in the global food chain (D'Aoust 1997). Poultry and pigs are well established as major reservoirs for salmonellae, with cattle being a primary source of S. Typhimurium (Gilbert and Humphrey 1998).

According to Gilbert and Humphrey (1998), meat may come in contact with salmonellae through an infected animal, an animal slaughtered near an infected animal, other meat on a contaminated work surface, or from processors/employees with salmonellosis. Since most *Salmonella* infections are asymptomatic in animals, one way their prevalence may be reflected is in the frequency with which the micoorganism can be detected in products of animal origin (Gilbert and Humphrey 1998).

The presence of salmonellae in the poultry, pork, beef and sheep industries stems from the exposure of animals to environmental sources of contamination, contaminated feeds, and parental transmission of infection. However, the frequency with which salmonellae are detected in meat varies with location, season, and species of animal (D'Aoust, 1997, Gilbert and Humphrey 1998). Of the various sectors within the meat industry, poultry products remain the foremost reservoirs of salmonellae in many

countries, largely dominating other meat products as possible vehicles of infection (D'Aoust 1997). In 2001, the prevalence of *Salmonella* in ground turkey was 26.2% and 19.5% in ground chicken, compared to 2.8% in ground beef (FSIS 2002b).

Salmonella spp. can be contracted from foods of animal origin and foods contaminated with animal feces (Shallow et al. 2000). Although contaminated poultry meat and poultry products (e.g., eggs) may dominate this group, any foodstuff can conceivably be a vehicle for human salmonellosis. This is often a result of cross-contamination, as has been documented with fruits and vegetables (D'Aoust 1997).

2.2 Thermal Inactivation of *Salmonella*

The decimal reduction time (D-value) is the time required to reduce a microbial population by 90%. Historically, it has been used in the literature to assess the death rate or heat resistance of microorganisms, with many intrinsic and extrinsic factors influencing heat resistance.

Serotypes and strains of *Salmonella* are known to exhibit different degrees of heat resistance. In egg products, for example, certain strains of *S.* Enteritidis are often more heat resistant than *S.* Typhimurium (Doyle and Mazzotta 2000). A D-value of 4.09 min for *S.* Enteritidis ME-14 in liquid whole egg at 56.7°C was significantly greater than a D-value of 3.13 min for *S.* Typhimurium DT104 (Brackett et al. 2001). However, while the D-value of *S.* Enteritidis PT34 in liquid egg yolk at 56°C ranged from 5.14 to 7.39 min, *S.* ser. Seftenberg had a D-value of 19.96 min (Chantarapanont et al. 2000).

S. Seftenberg is well known for its remarkable heat resistance in culture media and various food products (Doyle and Mazzotta 2000). In ground chicken breast meat

heated to 67.5°C, a 1.10-log reduction in *S.* Seftenberg was observed as opposed to a >6-log reduction in *S.* Heidelberg, *S.* ser. Mission, *S.* ser. Montevideo and *S.* ser. California (Murphy et al. 1999). After heating for five minutes at 60°C, no viable cells of *S.* Typhimurium Tm-1 remained in ground chicken pectoral muscle initially inoculated at 10^8 CFU/g. However, an exposure of 10 to 15 min at 65°C was required to kill an equal number of cells of *S.* Seftenberg 775W (Bayne et al. 1965). *S.* Seftenberg 775W is often used as a test organism in validation studies. If a thermal process effectively reduces *S.* Seftenberg 775W, it is expected to be as effective or more so against salmonellae that are more commonly encountered in foods (Doyle and Mazzotta 2000).

Bacteria attached to meat tissue or surfaces are generally more heat resistant than those dispersed throughout a food or broth or those suspended in liquid media (Murphy et al. 2002, Doyle and Mazzotta 2000). Humphrey et al. (1997) reported that D-values at 58°C for *S.* Typhimurium DT104 in pork muscle tissue were >10 min for attached cells versus only 2 min for free cells. Additionaly, growth and inactivation of microorganisms in food and model systems may be quite different than in liquid media. For example, Murphy et al (1999) found that a six strain *Salmonella* cocktail was more heat resistant in ground chicken breast patties than in an agar-water solution. A 7-log (CFU/g) reduction of *Salmonella* in agar-peptone occurred 19% faster than in chicken meat. Additionally, Quintavalla et al. (2001) found that the heat resistance of eight strains of *Salmonella* (*S.* Typhimurium strains ATCC 14028, 133 and 1116, *S.* ser. Derby B4373, *S.* ser. Potsdam 1133, *S.* ser. Menston 179. *S.* ser. Eppendorf 166, and *S.* Kingston I124) was 1.5-4 times higher in pork meat as opposed to Trypticase soy broth. While published data comparing the heat resistance of *Salmonella* in actual meat products versus liquid media is limited,

variations in food composition and culture media contribute to differences in thermal inactivation.

Certain food components such as fat offer protection against heat treatments (Jay 1998, Hansen and Riemann 1963, Line at al 1991, Ahmed et al. 1995). Maurer (2001) found that increasing the level of fat in turkey increased the D-value of *S.* Seftenberg. Juneja and Eblen (2000) observed significantly higher D-values at 58°C for an eight strain *S.* Typhimurium DT104 cocktail in ground beef containing 7, 12, 18 and 24% fat than in chicken broth containing 3% fat. They suggested that these differences could be due to poor heat transfer through the heating matrix as a result of the high fat levels. Thermal inactivation in ground beef was non-linear, and although the absolute D-values of the *S.* Typhimurium DT104 cocktail at 58°C, 60°C, 62.5°C and 65°C in ground beef decreased as the fat content increased, lag times increased substantially. Considering the combination of lag time and D- value, 7.07 min at 65°C was required to reduce *S.* Typhimurium DT104 by 7 logs in ground beef containing 7% fat, as opposed to 20.16 min in ground beef containing 24% fat (Juneja and Eblen 2000). The thermal protection afforded by increased fat levels may be due to a localized absence of moisture (reduced water activity) within bacterial cells (Hansen and Riemann 1963, Jay 1998, Juneja and Eblen 2000).

Water activity (a_w) is defined as the ratio of the water vapor pressure of a food substrate to the vapor pressure of pure water at the same temperature (Jay 1998). In practice, it can be interpreted as the moisture available in foods for microbial growth. It is believed that proteins are more stable in a drier state; therefore, a greater input of energy is required to denature proteins when the moisture content is decreased (Hansen

and Riemann 1963). As a result, a decrease in water activity is generally associated with a corresponding increase in the thermal resistance of microorganisms (Doyle and Mazzotta 2000, Carlson 2002, Jay 1998, Hansen and Riemann 1963). Archer et al. (1998) heated *S.* ser. Weltevreden in flour with hot air at temperatures ranging from 57°C to 77°C. As the initial a_w increased over a range of values from 0.2 to 0.6, D-values decreased for all temperatures tested. The effects of a_w on heat resistance may depend on the solute used (Mattick et al. 2001, Moats et al. 1971). Mattick et al. (2000) found that reducing the a_w of Trypticase soy broth with glucose-fructose more effectively increased the thermal resistance of *S.* Typhimurium DT104 than did glycerol or NaCl.

Acid adaptation not withstanding, microorganisms tend to be most heat resistant at their optimum pH for growth (Jay 1998). Casadei et al. (2001) found that a drop in pH from 7 to 3 caused a 100-fold reduction in *S.* Typhimurium D-values at temperatures between 48°C and 54°C, and Juneja and Eblen (1999) reported an increase in the heat sensitivity of *L. monocytogenes* at 55°C and 60°C with increasing acidity. A theoretical explanation for this effect of pH is that the logarithm of the rate of heat-destruction increases linearly in the acidic and alkaline ranges but has a minimum at the optimum growth pH (Juneja and Eblen 1999, Reichart 1994). However, Leyer and Johnson (1993) found that acid-adapted (pH 5.8) cells of *S.* Typhimurium were more thermally tolerant than nonadapted cells, exhibiting 10-fold more survivors after 20 min at 50°C, 10 min at 55°C and 2.5 min at 57.5°C. Acid adaptation includes two phases, pre-shock and acid shock, that alter the expression of at least 70 proteins and are required for protection against extreme acid stress (Leyer and Johnson 1993). Acid shock and adaptation may fall under the phenomenon of sublethal injury and cross-protection.

2.3 Sublethal Injury and Stress

2.3.1 Definitions

The simplest definition of injury is the effect sublethal treatments have on microorganisms (Hurst 1984). By extension, sublethal injury is a consequence of exposure to a chemical or physical process that damages but does not kill a microorganism (Russell 1984, Hurst 1977). Yousef and Courtney (2003) include damage to cellular components in their description of injury and according to Gilbert (1984), "Sublethal injury of microorganisms implies damage to structures within the cells, the expression of which entails some loss of cell function that may be transient or permanent." Rather than produce only viable or dead cells, pathogen and spoilage controls produce a continuum of effects, and a considerable proportion of microorganisms in foods likely harbors sublethal lesions (IFT 2002, Mossel and van Netten 1984, Zhao and Doyle 2001).

The term stress has also been used to illustrate the effect of sublethal treatments. However, Hurst (1984) considers injury to be the preferred term as, by analogy with higher organisms, its description evokes an image of "temporary and repairable physical damage." By similar analogy, the term stress apparently carries a more subtle meaning, not necessarily causing physical damage but able to alter the behavior of an individual. Current literature pertaining to microbial injury typically does not maintain this distinction, and the terms are used interchangeably.

The term stress, however, is universally used in reference to the agents or treatments causing injury. There is a tendency to perceive food as a friendly environment for bacteria. More than likely, the food itself is an unfriendly environment compromised

by competition, lack of moisture, acids and other by-products (Archer 1996), and almost all microorganisms in the food have been subject to certain environmental and processing stresses (Tables 2.5 and 2.6). The stresses organisms in a food environment can be subjected to vary broadly from acid shock, oxidants, salt and starvation to heat shock, freezing and thawing (Miller et al. 2000). In addition, certain emerging technologies (e.g., high hydrostatic pressure) cause sublethal injury, although others (e.g., pulsed electric field) do not (Wuytack et al. 2003, Yousef and Courtney 2003). Potential stresses generally fit into three categories (physical, chemical or nutritional), can occur at several stages of the food chain (pre-harvest, processing, distribution, storage) and cause a wide range of damage to the bacterial cell.

Storz and Hengge-Aronis (2000) also include as a definition of stress any departure from optimal conditions that has the potential to decrease growth rate. Any situation that induces the expression of genes recognized to respond to specific environmental conditions may also define a stressful situation and may be important in the effect of bacterial stress responses on host-pathogen interactions.

Given that the perception of a stressful situation is coordinately linked to characteristics of the individual cell, merging the above definitions of stress into one universal definition is difficult. However, the working definition from the standpoint of food microbiology is typically taken to be a physical or chemical (or nutritional) condition insufficiently severe to kill, resulting in sublethally injured microbes (Hurst 1977, Murano and Pierson 1993). The physical and chemical conditions can be processing treatments, but they may also be environmental conditions, such as limiting nutrients in water.

Table 2.5. Physical and chemical treatments (stresses) with the ability to reversibly injure microorganisms (Ray 1989, Ray 2001, Wuytack et al. 2003, Thanomsub et al. 2002).

Physical:
Drying-including air drying and freeze-drying
Heat-particularly sublethal heating processes
High hydrostatic pressure
Low temperatures-including both refrigeration and freezing
Pulsed white light
Radiation-gamma, ultraviolet and X-ray
Solids-concentrations of sugars, salts
Chemical:
Chemical sanitizers-chlorine, iodine, quaternary ammonium compounds
Oxidative treatments-including ozone
pH-alkali and acids (organic and inorganic)
Preservatives-sorbate, benzoate, nitrate, nisin, etc.

Table 2.6. Microorganisms known to experience reversible injury from sublethal stresses that are of importance in food systems (Ray 1989, Ray 2001).

Microorganism	**Main Importance in Food**
Escherichia coli	Indicator, Pathogenic
Enterobacter aerogenes	Indicator
Klebsiella sp.	Indicator, Pathogenic
Streptococcus faecalis	Indicator
Salmonella sp.	Pathogenic
Listeria monocytogenes	Pathogenic
Shigella sp.	Pathogenic
Vibrio parahaemolyticus	Pathogenic
Yersinia enterocolitica	Pathogenic
Campylobacter jejuni	Pathogenic
Staphylococcus aureus	Pathogenic
Clostridium perfringens	Pathogenic
Clostridium botulinum	Pathogenic
Pseudomonas sp.	Spoilage
Bacillus sp.	Spoilage, Pathogenic
Lactococcus lactis	Bioprocessing (Fermentation)
Lactobacillus bulgaricus	Bioprocessing (Fermentation)
Lactobacillus acidophilus	Dietary adjunct (Probiotic)
Saccharomyces cerevisiae	Bioprocessing (Fermentation)
Candida sp.	Spoilage
Aspergillus flavus	Spoilage

Storz and Hengge-Aronis (2000) believe there are different levels of stress severity, from minor to severe, extreme and eventually lethal. With minor stress, bacterial cells adapt completely to the changed conditions, and growth rate is not affected. Low levels of stress may cause a transient adaptation (adaptive response) accompanied by a temporary change in physiology that often results in increased stress tolerance (Yousef and Courtney 2003). At the other extreme, lethal stress can cause the death of some but not necessarily all bacterial cells. The occurrence of death in only a certain fraction of the population indicates that lethal stress may induce responses or even adaptive mutations that may actually improve survival of the overall population (Storz and Hengge-Aronis 2000, Archer 1996). Moderate stress may result in injury, and there is also thought to be a continuum of injury, ranging from mild to severe along with healthy and dead cells (Hurst et al. 1976, Hurst 1984, Mackey 2000, Stephens et al. 1997). The relationship between the different stress levels and degrees of injury, as well as adaptation, is not well defined. For practical purposes, sublethal injury is essentially anything less than death and, at some point, injured cells have likely undergone some type of stress adaptation.

The expression of sublethal injury can be manifested in several ways in the laboratory (Hurst 1977). Metabolic injury is described by the inability of bacterial cells to grow on defined minimal media (Gilbert 1984). This inability is often temporary, indicating repair of injury can occur (Hurst 1984). Structural injury is viewed as the inability to proliferate or survive in media containing selective agents (e.g., bile salts, tellurite, sodium desoxycholate, bismuth sulfite, sodium chloride, crystal violet, brilliant green) with no obvious inhibitory effects on unstressed cells (Hurst 1977, Gilbert 1984,

Hurst 1984, Semanchek and Golden 1998, Ray 2001). Depending on other conditions (e.g., enrichment, incubation conditions, etc.), the inability to grow on selective media is also temporary, which once again indicates that repair of injury is imminent.

Some authors consider metabolic and structural to be different degrees of the same injury (Hurst 1984, Kang and Siragusa 1999). Both are discernable by the inability to form visible colonies under given conditions, making injury recognizable by the loss of characteristic growth capabilities regardless of the method of evaluation (Hurst 1977, Busta 1976). However, it seems that while all injured cells suffer structural damage, which upsets the permeability barrier in the cell wall and membrane, metabolic injury is further damage to various functional components of the cell (Brashears et al. 2001). Consequently, growth on selective media, or the lack thereof, is the primary method of assessing sublethal injury (Hurst 1984, Gilbert 1984, Czechowicz et al. 1996, Semanchek and Golden 1998, Brashears et al. 2001). The difference in plate counts between selective and non-selective media is used to quantify sublethal injury as a proportion or percentage of the entire population (Kang and Siragusa 1999, Semanchek and Golden 1998, Baylis et al. 2000a, Dickson and Frank 1993, Restaino et al. 1980, Brashears et al. 2001).

2.3.2 Specific Stresses

Abrupt acid shock or gradual acid stress can occur in low pH conditions when H^+ ions cross the bacterial cell membrane and create an acidic intracellular pH. Likewise, unprotonated organic acids can diffuse across the bacterial cell membrane and lower the internal pH upon dissociation (Foster 2000, Abee and Wouters 1999). While acid injury is thought to be the result of an easily reversible pH equilibrium shift, it is more complex

than simple neutralization of the acidic conditions (Przybylski and Witter 1979).

Acid stress can occur with fermentation (e.g., summer sausage) or the addition of preservatives such as acetic and propionic acids (Smith and Palumbo 1978, Abee and Wouters 1999). Current recommendations for the use of acid washes to decontaminate beef carcasses include concentrations of lactic acid at 1%-2.5% (Gerris 2000). Dickson and Siragusa (1994) found that washing beef tissue with 1% lactic or acetic acid sublethally injured *S.* Typhimurium. Also, an alkaline stress can occur under high pH conditions. Many detergents and chemical sanitizers, such as caustic soda (NaOH) and ammonium compounds, used to clean food processing facilities, including food contact surfaces, are alkaline in nature (Taormina and Beuchat 2001).

Starvation stress can occur on animal carcasses, in food, on equipment surfaces, on walls and floors, and in water (Lou and Yousef 1996, Dickson and Frank 1993). Dickson and Frank (1993) define starvation stress as the survival of bacteria in oligotrophic environments with low or no available nutrients to provide for growth and/or reproduction. Typically, natural environments are places with limiting amounts of nutrients and rapidly changing nutrient availability (Hengge-Aronis 1993, Givskov et al. 1994). Bacteria are present in different physiological states, including exponential growth, slow growth or stationary phase and death. Of the stages or types of growth, cryptic must also be considered. Cryptic growth is the phenomenon where dead organisms provide nutrients for the multiplication of survivors (Postgate and Hunter 1963). Cryptic growth may contribute to the survival of populations well after growth has ceased.

Salmonella has been reported to survive in cold storage at 5°C for up to 8 months

(Jeffreys et al. 1998, D'Aoust et al. 1985). This is likely due to cold shock and subsequent cold adaptation. The cold shock phenomenon occurs when growing bacteria are suddenly chilled, for instance from 37°C to 15°C, 10°C or lower, and it has been established that rather small temperature drops (e.g., from 37°C to room temperature) can also generate cold shock in susceptible organisms (Mackey 1984, Jones et al. 1996). The associated cold shock response is divided into stages of initial cessation of growth, resumption of growth after an adaptive period and changes in protein synthesis (Miller et al. 2000). Microorganisms inhabiting foods that must be refrigerated for pre and/or post-processing storage are subject to cold shock. Additionally, injury due to cold shock may occur if dilutions are placed in the refrigerator when subsequent laboratory tests cannot be immediately completed (Van Schothorst and Maggie Duke 1984).

Sensitivity to cold varies broadly. No single property explains this sensitivity, but population density, growth temperature, cooling rate and the temperature range over which cooling occurs all impact the survival of cold-shocked cells (Postgate and Hunter 1963, Mackey 1984). The effect of food components on the degree of injury and survival of cold-stressed microorganisms has not been studied in great detail. Preliminary work has shown that water or diluents are more stressful environments than broth or food (Mackey 1984). The time to decrease salmonellae levels 90% in water at 0 to 5°C was between 2 and 16 d, while in vacuum-packaged beef in the same temperature range, only a 50% reduction was observed after 28 d (Mackey 1984, Mitchell and Starzyk 1975, Kennedy et al. 1980). However, chilling in oyster homogenate at 4°C for 24 h decreased populations of *Vibrio vulnificus* nearly 7 logs (lethal cold stress), while cold storage in a salt-based culture medium had no effect (sublethal cold stress) (Oliver 1981).

Although it is not known to be an effective method for destroying bacteria, freezing can be an injurious treatment. Injury results from continued exposure to concentrated solutes and physical damage caused by ice crystal formation. Many constituents of food and culture media are protective against freeze damage. These cryoprotectants include glycerol, sodium glutamate, certain sugars, peptides and proteins (Mackey 1984). The presence of such protectants may be why Semanchek and Golden (1998) found freezing to have a minimal impact on the development of injury in *E. coli* O157:H7.

Osmotic stress seems to be associated with both freezing and freeze-drying. Freeze-dried microorganisms are subject to freezing, drying, storage and rehydration stresses (Mackey 1984). Rapid rehydration of foods and freeze-dried cultures can cause osmotic injury (Van Schothorst and Maggie Duke 1984). Osmotic stress can occur when shifts in external osmolarity cause water to flow either into or out of the bacterial cell. In extreme conditions, this type of stress can cause physical damage to the cell. Less severe osmotic changes affect water availability and can be caused by sodium or other salt challenges (Bremer and Krämer 2000). Water availability is intimately linked to water activity. In food processing, microorganisms respond to both a "storage" a_w and a "treatment" a_w (Quesnel 1984).

In contrast to other stresses, heat shock is perhaps the most studied and best understood. Heat shock occurs when organisms are shifted for short periods of time from lower to higher temperatures within or above their normal growth range (Bunning et al. 1990, Mackey and Derrick 1986, Pagán et al. 1997, Farber and Brown 1990). The shifted temperatures are not yet lethal, particularly given that the bacterial population is

heterogeneous with respect to growth phase and heat sensitivity. Even temperatures and treatments meant to be lethal will not completely destroy all organisms, as a potentially significant number of cells actually survive a given heat process and are merely injured (Murano and Pierson 1993).

Both pre-processing and processing environments present conditions that mimic or cause heat shock. Guidelines for the use of acid washes to decontaminate carcasses include a spray temperature of 20°C to 60°C (Gerris 2000). Heat shock was reportedly induced in *Escherichia coli* O157:H7 at 42°C (Murano and Pierson 1993), *Campylobacter jejuni* at 46°C (Palumbo 1984), *S.* Typhimurium at 48°C (Mackey and Derrick 1986, Bunning et al. 1990), and *Listeria monocytogenes* in a fermented beef/pork sausage homogenate at 48°C and 52°C (Farber and Brown 1990). Hot acid sprays may elevate the superficial temperature of the carcass, altering the growth and resistance profile of indigenous flora (Castillo et al. 2001).

Thermal processing can cause injury to microorgansims. Food products requiring long heating lag phases, including products pasteurized at low temperatures for long times and egg products, are subject to heat shock conditions. (Mackey and Derrick 1987b, Murano and Pierson 1993, Bunning et al. 1990). The behavior of microorganisms in slowly heated foods will presumably mimic the behavior of microorganisms subject to isothermal heat shock (Mackey and Derrick 1987a). Thus, meat products and bulk products that are slow cooked to a final internal temperature and sous vide foods (Pagán et al. 1997) are also subject to heat shock conditions (Mackey and Derrick 1987a, Farber and Brown 1990, Quintavalla and Campanini 1991). Microorganisms present in meat products left on warming trays before receiving a final re-heating could also experience a

heat shock (Farber and Brown 1990).

2.3.3. Cellular Modification and Damage

Environmental stresses and stresses caused by processing are thought to cause loss of some cell structure functions and/or protein denaturation (Lou and Yousef 1996). Table 2.7 outlines the types of cellular damage caused by various treatments. Dead, injured and otherwise normal cells likely differ in the degree of injury to structural and functional components (Ray 1984).

Exposure to low pH environments may remove or sequester cations from key sites in a microorganism (Gould 1984). Damage to ribonucleic acid (RNA) is linked to low pH, and could be due to removal of magnesium (Mg^{2+}) (Przybylski and Witter 1979, Hurst 1984). Mg^{2+} is necessary for ribosomal integrity with acetic acid reportedly interfering with the ribosome and/or protein synthesis (Hurst 1984, Mossel and van Netten 1984). Shifts in pH can disrupt the proton motive force (Russell 1984, Rowbury 2003) and also alter the protein profile of the outer membrane in Gram-negative cells, the cytoplasmic membrane in Gram-positive cells, or the protein coat of spores (Leyer and Johnson 1993, Gould 1984). Low cytoplasmic pH can also damage deoxyribonucleic acid (DNA) (Yousef and Courtney 2003, Rowbury 2003).

Starvation conditions can lead to an increase or decrease in exopolysaccharide levels (Dickson and Frank 1993), as well as entry into stationary phase, a mode characterized by drastic changes in the cell envelope, membrane composition, and DNA structure (Hengge-Aronis 1993, Rowbury 2003). Cells under starvation experience physiological changes that include decreased cell size and membrane fluidity and increased protein turnover (Lou and Yousef 1996). Microorganisms in low nutrient

Table 2.7. Types of cell damage resulting from different sublethal treatments (adapted from Mossel and van Netten 1984, Ray 1986, Wuytack et al. 2003, Rowbury 2003).

	damage to:				
observed in cells treated by:	cell wall or cell wall synthesis	membrane (leakage)	proteins	RNA (ribosomes) or RNA synthesis	DNA or DNA synthesis
freezing		x		x	x
drying	x	x		x	x
freeze-drying	x	x		x	x
heating		x	x	x	x
gamma-irradiation	x	x	x	?	x
change in oxygen potential		x			
osmotic shock	x	x			
starvation			?	x	
high hydrostatic pressure		x	x		
pulsed white light		x	x		x
salt shock			x		

environments likely integrate cell density and starvation stress signals to allow for cell surface modifications and utilization of alternate energy sources (Lazazzera 2000, Postgate and Hunter 1963). Alterations in cellular morphology and modifications in cell surface components enhance adherence and may contribute to biofilm formation.

Damage and modification of the cell membrane are associated with almost all forms of physical stress (Mackey 2000). The membrane is a common site of injury by chilling, freezing and heating (Boziaris and Adams 2001). Damage to the outer membrane of Gram-negative cells includes release of lipopolysaccharides (LPS), lipids, phospholipids, divalent cations necessary for LPS stability and periplasmic enzymes, all of which disrupt the permeability barrier (Boziaris and Adams 2001, Ray 2001, Hurst 1977). Permeability control is also lost at low temperatures because of decreased membrane fluidity (Graumann and Marahiel 1996, Mackey 1984). Changes in permeability have led to the suggestion that certain treatments (heating, freezing, drying, radiation) create small pores in the outer membrane (Mackey 2000). Repair of sublethal injury involves repair of these pores, but conditions for repair of the outer membrane does not necessarily facilitate repair of the cytoplasmic membrane (Hurst 1977).

While Gram-positive organisms do not have an outer membrane, they do have a surface protein layer outside the cell wall. Both the surface protein layer and cell wall are potential sites of injury. Freeze injury to *Lactobacillus bulgaricus* has been shown to damage the protein layer (Mackey 2000, Wright and Klaenhammer 1981). Mg^{2+} and D-alanine were lost from cell wall teichoic acid polymers of *Staphylococcus aureus* heated in phosphate buffer (Hurst and Hughes 1981).

VanBogelen and Neidhardt (1990) theorize that ribosomes may function as

sensors of conditions or temperatures that elicit a heat or cold shock. Ribosome and ribosomal RNA (rRNA) degradation are often observed as heat-induced lesions (Genthner and Martin 1990, Tolker-Nielsen and Molin 1996). Due to destruction of 16S rRNA, the 30S ribosomal subunit is damaged or destroyed with thermal stress in *S. aureus*, *S.* Typhimurium B and *E. coli* (Hurst 1977). Destruction of the 30S subunit is prohibited when Mg^{2+} (as $MgCl_2$) is added to the heating menstruum. Loss of ribosomes also occurs with starvation, particularly when conditions lead to intracellular Mg^{2+} depletion (Mackey 2000). As Mg^{2+} is necessary for ribosomal integrity and inhibition of ribonuclease, it appears ribosomal damage is largely a consequence of Mg^{2+} loss (Hurst 1977).

While DNA damage (Figure 2.1) has been observed in heated, freeze-thawed, dried, and acid-treated cells, it may be an indirect result of these treatments (Mackey 2000, Russell 1984). For instance, strand breakage during or after heat treatment is likely due to the stimulation of endonuclease activity (Russell 1984, Hurst 1977). Death in cold-shocked *E. coli* is connected to the loss of Mg^{2+}, which is required by DNA ligase for DNA synthesis and repair (Sato and Takahashi 1970).

Certain treatments, particularly heating, can denature proteins, leading to the inactivation of enzymes and disruption of the active transport of cations, sugars and amino acids (Hurst 1977). Dehydrogenases are especially heat sensitive and glycosylases and endonucleases, enzymes involved in DNA repair, are heat-labile at mild temperatures of 40°C-50°C (Pellon and Sinskey 1984). Sublethally heat-injured *S. aureus* have reduced catabolic capabilities, and glucose-transport is disrupted in heat damaged *S.* Typhimurium (Busta 1976). Heating has been shown to inactivate catalase and

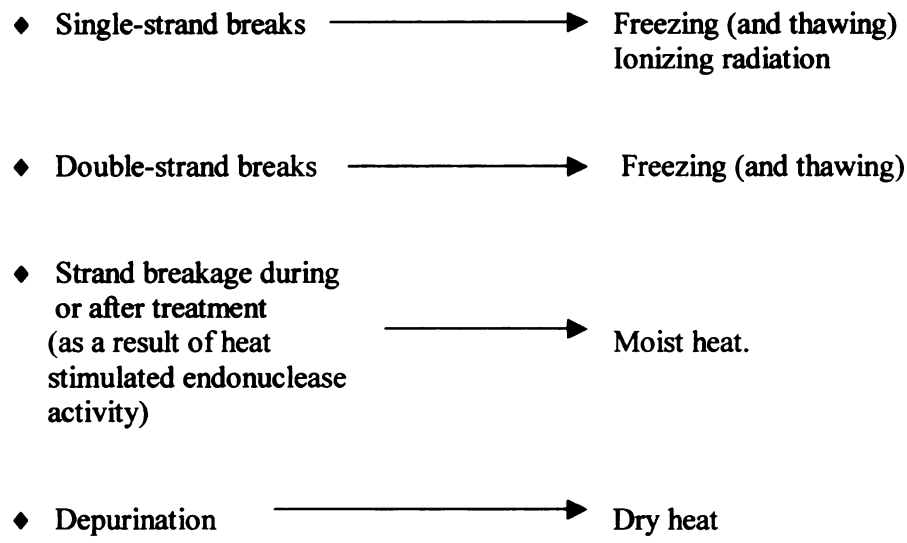

Figure 2.1. Specific types of damage to bacterial DNA (Russell 1984).

superoxide dismutase in several microorganisms, including *L. monocytogenes* and *S. aureus* (Andrews and Martin 1979, Dallmier and Martin 1988). The loss of this activity may contribute to the oxygen sensitivity of injured microorganisms.

2.3.4 Oxygen Sensitivity of Injured Cells

Oxygen species lethal to injured microorganisms may be rapidly formed in both selective and non-selective culture media. Reactive oxygen species (ROS) include the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$), singlet oxygen, hypochlorous acid, lipid peroxides, protein hydroperoxides and ozone (Valentine et al. 1995, Simpson et al. 1992). Autooxidation of reducing sugars in the presence of phosphate during autoclaving has been implicated in the generation of ROS in culture media (Stephens et al. 2000, Baylis et al. 2000a). Media containing manganese and/or citrate can also autooxidize to form peroxides. If peroxides accumulate in media in the presence of superoxide radicals, hydroxyl radicals will form. Hydroxyl radicals can also form when H_2O_2 reacts with Fe^{2+} (McDonald et al. 1983, Suh and Knabel 2000). ROS can also be formed endogenously as a consequence of the normal metabolic reduction of molecular oxygen to water. Reactive intermediates include $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ (Stephens et al. 2000).

Oxidative stress caused by elevated levels of $O_2^{\cdot-}$, H_2O_2 or $^{\cdot}OH$ can lead to damage of cellular components (Storz and Zheng 2000). Although the superoxide radical is believed to be only moderately reactive, it is still capable of exerting detrimental effects, including oxidation of membrane phospholipids, proteins and DNA, inhibition of catalase and peroxidases, and reduction of metal ions (Stephens et al. 2000, Schellhorn and Hassan 1988, Takeda and Avila 1986). Metal ions in a reduced state are unstable and

have the potential to reduce H_2O_2, producing the extremely toxic hydroxyl radical. H_2O_2 is more reactive than the superoxide radical, and H_2O_2 produced in the media can readily diffuse across the cell membrane and disrupt membrane transport systems, as well as enzyme and nucleic acid synthesis. Of the reduced forms of oxygen, the hydroxyl radical is the most reactive, being able to immediately oxidize cellular components, including proteins, nucleic acids and lipids (Stephens et al. 2000).

Two antioxidant enzymes, superoxide dismutase (SOD) and catalase, work together to protect bacteria from the harmful effects of oxygen radicals produced during the course of normal respiration. SOD converts superoxides to O_2 or H_2O_2 and catalase converts H_2O_2 to H_2O and O_2 (Schellhorn and Hassan 1988, Takeda and Avila 1986).

As early as 1925, it was suggested that survival of heated microorganisms is affected by the presence or absence of air, and that this factor should be included in studies of thermal death time (Jackson and Woodbine 1963, Oerskov 1925). $D_{62.8°C}$-values were six-fold higher when *L. monocytogenes* was enumerated with strictly anaerobic techniques than with aerobic recovery (Knabel et al. 1990). Equivalent heat treatments at 55°C for 100 min, 59°C for 5 min and 61°C for 1 min effected a 6-log reduction in *E. coli* O157:H7 (Bromberg et al. 1998). Only half of this 6-log reduction was due to thermal inactivation (as determined by anaerobic enumeration), with the remainder due to the inability of sublethally injured cells to grow in the presence of oxygen. Gôrt and Imlay (1998) suggest *E. coli* produce only enough SOD to protect intracellular components from endogenously generated superoxide. Hence, even unstressed microorganisms face the challenge of neutralizing ROS produced in culture media. The challenge is even greater for stressed cells, which typically exhibit

heightened sensitivity to peroxides and superoxide radicals due to diminshed SOD and catalase activity and the inability of injured organisms to synthesize new enzymes (Suh and Knabel 2000, Patel et al. 1995, McDonald et al.1983, Czechowicz et al. 1996).

Research on the stability of catalase and SOD in a wide variety of food-related microorganisms to different environmental stresses is limited. Heating *S. aureus* in phosphate buffer at 52°C for 20 min reduced catalase activity by nearly 40%, with catalase loss increasing to about 90% during the first 2-4 h of repair in Trypticase soy broth (TSB) (Andrews and Martin 1979). Amin and Olson (1968) saw a 10- to 20-fold reduction in staphylococcal catalase activity at 54.4°C compared to 37.8°C. Dallmier and Martin (1988) and Zemser and Martin (1998) reported a sharp decrease in catalase activity when *L. monocytogenes* was heated between 55°C and 60°C. Shifting the temperature of *E. coli* from 30°C to 45°C inhibited catalase (Smirnova et al. 2001a), and Mackey and Seymour (1987) attributed the increased peroxide sensitivity of heat-injured *E. coli* K12 to at least partial inactivation of inducible catalase/peroxidase. Murano and Pierson (1993) found that heat shock at 42°C lowered the activity of catalase and SOD in *E. coli* O157:H7, and that even without the heat shock, heating at 55°C could eliminate the activity of both enzymes. While Vasconcelos and Deneer (1994) saw no change in SOD levels in *L. monocytogenes* subjected to a heat shock at 42°C, Dallmier and Martin (1988) demonstrated a decrease in SOD activity after heating at 45°C.

Smirnova et al. (2001b) showed that the responses of *E. coli* to cold shock (from 37°C to 20°C) and long-term cultivation at 20°C were similar to the typical oxidative stress response, with an enhanced expression of SOD and catalase. Stead and Park (2000) identified superoxide radicals, and H_2O_2 in the event that these radicals are not

detoxified, as the toxic species produced during freeze-thaw treatment of *Campylobacter coli*. By showing that SOD-deficient mutants are sensitive to freeze-thaw, their work demonstrated that SOD is important in the resistance of *C. coli* to this stress. Catalase-deficient mutants also had an increased sensitivity to freezing and thawing. However, the increased sensitivity to H_2O_2 of *C. jejuni* injured by freezing and mild heating observed by Humphrey (1988) was not associated with changes in total catalase activity. However, these cells required the addition of catalase for growth on nutrient agar.

Efforts have been made to increase the recovery of injured microorganisms by eliminating oxidative stress by incorporating reducing agents or other agents that act to scavenge O_2 or degrade H_2O_2 in recovery media (Suh and Knabel 2000, Patel et al. 1995, McDonald et al. 1983). Overall effectiveness of the various supplements is dependent on the organism and variations in the type, degree and site of injury.

When sodium pyruvate or α-ketoglutaric acid, both nonenzymatic H_2O_2-degrading compounds, were added to Luria-Bertani agar, recovery of starvation (sterilized distilled water) and low-temperature (4°C) stressed *E. coli* O157:H-strain E32511/HSC increased from less than 0.1 CFU/mL to 10^4-10^5 CFU/mL within 48 h (Mizunoe et al. 1999). Calabrese and Bissonnette (1990) observed a significant increase in the recovery of chlorine-stressed coliforms when standard recovery media was supplemented with catalase, pyruvate or a combination of the two. Rayman et al. (1978) found that the addition of pyruvate or catalase to Trypticase soy agar (TSA) stimulated the growth of heat-injured *S.* Seftenberg but not of *Streptococcus faecium*, an organism not possessing catalase. TSA supplemented with 0.1% sodium pyruvate was shown to recover 10 times as many heat-damaged *E. coli* O157:H7 as unsupplemented TSA

(Czechowicz et al. 1996). Pyruvate has also been shown to enhance the recovery of heat-stressed *L. monocytogenes* (Busch and Donnelly 1992, Patel et al. 1995), *Shigella flexneri* (Smith and Dell 1990), *S. aureus* (Hurst et al. 1976, Martin et al. 1976), and heat- and freeze-injured *E. coli* (McDonald et al. 1983).

The addition of catalase to media has also been shown to increase the recovery of injured cells, up to 1100-fold in the case of heated and freeze-dried *S. aureus*, as well as *S. aureus* subjected to low a_w (Flowers et al. 1977). Martin et al. (1976) reported that the addition of catalase to TSA containing 7.0% NaCl increased the enumeration of heat-injured *S. aureus* 10^4-fold. Although Stephens et al. (2000) determined catalase did not have a significant effect on the recovery of heat-injured *S.* Typhimurium, Rayman et al. (1978) reported a 151-fold increase in heat-injured *S.* Seftenberg counts when catalase was added to TSA. Catalase has also been shown to increase the recovery of acid-injured *E. coli* (Martin et al. 1976) and heat-injured *L. monocytogenes* (Patel et al. 1995) and *E. coli* (Mackey and Seymour 1987).

The metabolic reduction of O_2 to H_2O is achieved by the action of electron transport enzymes located in the cytoplasmic membrane. Oxygen-reducing membrane fractions have been derived from several bacterial species (Adler 1990). Oxyrase®, the partially purified, oxygen-reducing membrane fragment of *E. coli*, can remove O_2 from culture media and destroy oxygen radicals. The latter is probably due to the presence of catalase. Oxyrase® has been shown to enhance recovery of *L. monocytogenes* following heat injury (Yu and Fung 1991, Suh and Knabel 2000). Additionally, Patel and Beuchat (1995) and Patel et al. (1995) showed that Oxyrase® increased recovery *L. monocytogenes* from heat-pasteurized whole milk and filtrates of homogenized beef, but

not from skim milk or cabbage filtrates. Media containing Oxyrase® recovered up to 1.5-log CFU/mL more heat-stressed *S.* Typhimurium and up to 2.0-log CFU/mL more acid/salt-stressed *S.* Typhimurium DT104 than media without Oxyrase® (Stephens et al. 2000). Oxyrase® also can reportedly enhance recovery of radiation-damaged *E. coli* (Adler et al. 1981), as well as heat-injured *E. coli* O157:H7 and *Yersinia enterocolitica* (Thippareddi et al. 1995).

2.3.5 Repair and Stress Responses

Several key factors define the concept of injured cells. The injured population may contain bacterial cells that are slightly to severely injured, with viable nonculturable and dead cells also likely to be present. If appropriate intrinsic (nutrients, a_w) and extrinsic (temperature, relative humidity) parameters exist during the farm-to-fork continuum, most injured cells will repair and regain the characteristics of normal cells (Ray 1986, Busta 1994), as well as the additional characteristic of increased stress tolerance associated with stress adaptation. The repair process is reflected by delayed germination of spores, a prolonged lag phase for vegetative organisms and the inability to multiply until repair has taken place (Busta 1994). Following repair, resistance to selective agents and the ability to proliferate in their presence is regained (Brashears et al. 2001).

Repair times can be estimated from the length of the extended lag phase or by the differential plating method. Using non-selective media, the estimated lag phase is the time to repair injury and begin multiplication. The differential plating method uses a selective medium containing a substance inhibitory to injured microorganisms (i.e., due to membrane damage) and a non-selective medium with no obvious inhibitory effects on

unstressed cells. The non-selective (reference) medium enumerates the entire population and the selective medium only the healthy fraction. With differential plating, repair time is the time necessary to regain resistance to the selective medium. The time needed for repair depends on the bacterial species and strain, the type and severity of injury and the inhibitory nature of the selective media (Mackey 2000).

Measurements with differential plating suggest that, under optimal conditions, injury caused by treatment with acid, chilling, freezing, freeze-drying, gamma-radiation or mild heating can be repaired within 4-5 h, with severe heat injury sometimes requiring much longer repair times (Mackey 2000). Repair of *C. jejuni* subject to heat shock in potassium phosphate buffer at 46°C for 45 min was complete within 4 h of incubation at 37°C. No repair occurred at 5°C (Palumbo 1984). Damage to *E. coli* caused by sublethal acidfication (sodium acetate buffer, pH 4.2, 60 min) was completely repaired after 1-2 h at 32°C in TSB, with 95% repair reported after 120 min in potassium phosphate buffer (pH 8.0) (Przybylski and Witter 1979). Freeze- and alkali-injured *E. coli* cells can also recover when incubated in phosphate buffer (Musarrat and Ahmad 1988).

Repair times determined by estimating lag time are not always in agreement with those determined by differential plating. Mackey and Derrick (1982) determined that gamma-irradiated and dried *S.* Typhimurium sometimes recovered full resistance to the selective medium before the lag phase was complete. In some instances, the time individual cells of heat-injured *S.* Typhimurium required to regain tolerance to salt (up to 14 h) was longer than the lag time (9 h). Mathew and Ryser (2002) reported growth of heat-injured *L. monocytogenes* within the first two hours of incubation, although percent injury was not fully repaired when assessed by the differential plating method. Meyer

and Donnelly (1992) also reported growth of heat-injured *L. monocytogenes* before resistance to the selective medium was fully regained.

Lag and repair times for injured bacteria reported in the literature must be viewed with caution. Cell-to-cell variability (including growth phase and cell-cycle stage) during stress exposure typically leads to variations in injury within a population (Stephens et al. 1997), and repair time as determined by differential plating or estimation of lag phase can be biased due to this heterogeneity. In general, less severely injured cells repair quicker and have a shorter lag period than more severely injured cells. When the cells with the shorter lag period begin multiplying, they can mask the presence, on non-selective and selective media, of cells requiring longer repair times. Consequently, a single or average repair time does not reflect the distribution within a population and may well be an underestimation (Mackey and Derrick 1982, Mackey 2000). This may help explain the growth observed by Mackey and Derrick (1982), Meyer and Donnelly (1992) and Mathew and Ryser (2002) before all cells regained resistance to selective agents.

Stephens et al. (1997) examined the distribution of lag times of heat-injured *S.* Typhimurium by serially diluting the culture, inoculating the dilutions across many microtitre plates, monitoring growth by turbidity and calculating lag times from a model that extrapolated the growth curve back to initial inoculum levels. While the lag times for individual healthy cells were narrowly distributed, the lag times for injured cells were widely distributed, from <12 h to >20 h, with some >30 h. These results highlight the heterogeneous nature of bacterial populations and have apparent implications for traditional and rapid methodology involving pre-enrichment and repair steps, particularly when the overgrowth of competing bacteria is considered.

Repair requires that specific biochemical events occur, the nature of which differ with the type of stress. Metabolic processes that occur during repair can include the synthesis of adenosine triphosphate (ATP), DNA, RNA, proteins and the reorganization of existing macromolecules, including LPS in Gram-negative organisms and teichoic acid in Gram-positive organisms (Ray 2001, Hurst 1984, Ray 1986). Repair of the cell membrane through lipid synthesis must occur relatively early so that cells can fully repair other stress-induced lesions (Beuchat 1978).

Normal cellular functions may be re-established by the transient synthesis of general or specific stress proteins (Lou and Yousef 1996). Since the confirmation of heat shock proteins in *Drosophila* sp. in 1974, virtually every organism subsequently studied has been shown to respond to a moderate temperature shock with the increased production of specific proteins (Mackey and Derrick 1986, Bunning et al. 1990, Farber and Brown 1990, Watson 1990, Snyder and Champness 1997). Bacterial responses to stress can be general or specific. In some instances, a component of a given stress response may be part of both general and specific response pathways (IFT 2002).

Characteristics of stress proteins include increased production under conditions that repress the synthesis of most other cellular proteins (Lindquist 1992), a functional role in adaption of bacteria to growth- and survival-limiting conditions (Völker et al. 1992), and the overall mediation of recovery of stress-induced damage (Bunning et al. 1990). Stress proteins in prokaryotic and eukaryotic organisms are among the most evolutionarily conserved proteins (Watson 1990, Snyder and Champness 1997), and the occurrence of the heat shock response in prokaryotes, eukaryotes and archaebacteria indicate it must have developed in the earliest common form of cellular life on Earth,

possibly 2 to 2.5 billion years ago (Neidhardt and VanBogelen 1987).

The general stress response in most Gram-negative bacteria, including the enteric bacteria *E. coli*, *Shigella flexneri* and *S.* Typhimurium, is regulated by RpoS, the alternative sigma-subunit of RNA polymerase (σ^S) (Abee and Wouters 1999). In *E. coli*, RpoS controls the expression of more than 50 genes involved in the general stress response (Loewen et al. 1998). RpoS can be induced by a number of stresses, including nutrient starvation, osmotic shock, high and low temperatures, pH stress and oxidative stress (Abee and Wouters 1999, Hengge-Aronis 2000, Rowbury 2003). Dodd and Aldsworth (2002) demonstrated induction of RpoS in *Salmonella* with changes in a_w produced using different humectants and food preservatives. Bacteria defective in the gene for RpoS experience greater sensitivity to food processing conditions, such as heat shock, starvation, acid and ethanol (Abee and Wouters 1999, Rees et al. 1995).

In *L. monocytogenes*, *S. aureus*, *Bacillus subtilis* and other Gram-positive bacteria, the alternative sigma-subunit σ^B regulates the general stress response. In *B. subtilis*, σ^B controls the expression of over 40 genes involved in the general stress response (Abee and Wouters 1999). Like RpoS, σ^B can be induced by a number of environmental stresses, including ethanol, nutrient starvation, oxygen limitation, low and high temperatures, high salt concentrations and hydrogen peroxide (Abee and Wouters 1999, Völker et al. 1992). Disruption of σ^B in *B. subtilis* can increase sensitivity to oxidative stress, and disruption in *L. monocytogenes* reportedly decreases resistance to acid and osmotic stress (Abee and Wouters 1999).

Several specific stress responses have been identified, and like the general stress response, these are adaptive responses that allow bacteria to survive and, in some cases,

multiply under stressful conditions (Abee and Wouters 1999, Ray 2001). The heat shock response and associated heat shock proteins (HSPs) are the most studied. Several HSPs act as molecular chaperones or chaperonins. These chaperones help cells survive by re-folding or targeting for destruction proteins that are improperly assembled or denatured by the abrupt rise in temperature (Snyder and Champness 1997, Jones et al. 1996, Graumann et al. 1996, Lindquist 1992). Some HSPs possess proteases or peptidases activity for degrading irreparably denatured proteins (Snyder and Champness 1997, Jones et al. 1996, Lindquist 1992, Yousef and Courtney 2003). HSPs may also play a role in DNA repair and replication, cell division and modification of cellular morphology (*E. coli* exhibits a transient tendency to elongate), and the accumulation of osmolytes, which may maintain or enhance protein stability (Abee and Wouters 1999, Tsuchido et al. 1986, Neidhardt and VanBogelen 1987).

One theory of the heat shock response is that it is responsible for the synthesis of proteins that replace thermolabile components of macromolecule-synthesizing systems, such as ribosomes. Presence of an inducible rather than constitutive heat shock system in thermophilic bacteria suggests involvement as a mediator of temperature shifts rather than high temperatures (Neidhardt and VanBogelen 1987). HSPs have been identified following several other stresses, including starvation and anaerobiosis, as well as exposure to ethanol, other organic solvents, oxidative agents and high salt concentration (Jenkins et al. 1988, Watson 1990, Lindquist 1992, Rowbury 2003, Girgis et al. 2003). Accumulation of damaged or denatured proteins is thought to trigger the synthesis of HSPs following environmental stress (Watson 1990).

Many bacteria respond to abrupt decreases in temperature by transiently

synthesizing a number of protective proteins (Graumann and Marahiel 1996). Cold shock proteins (CSPs) have not been studied in prokaryotes and eukaryotes to the same degree as HSPs, but they have been demonstrated in *Bacillus cereus* (Mayr et al. 1996), *B. subtilis* (Lottering and Streips 1995, Graumann et al. 1996), *E. coli* (Jones et al. 1987, Goldstein et al. 1990), *Lactococcus lactis* (Wouters et al. 1998), *L. monocytogenes* (Bayles et al. 1996), *V. vulnificus* (McGovern and Oliver 1995), *S.* Enteritidis (Jeffreys et al. 1998) and *S.* Typhimurium (Craig et al. 1998). These CSPs are thought to play a critical role in various cellular and physiological functions, including DNA recombination, transcription, translation, messenger RNA and protein folding, sugar uptake, chemotaxis and general metabolism efficiency (Jones et al. 1996, Graumann and Marahiel 1996, Wouters et al. 2000). The maintenance of membrane fluidity at low temperatures is necessary for cold adaptation and involves at least one constitutively expressed enzyme that is active only at low temperatures (Abee and Wouters 1999).

S. Typhimurium can withstand potentially lethal acid shock conditions (pH<4) if preceded by adaptation to milder conditions (Bearson et al. 1996, Foster 1995). This acid adaptation, stemming from a gradual decrease in pH to non-lethal levels (pH>4), induces the acid tolerance response (ATR) (Leyer and Johnson 1993, Abee and Wouters 1999, Bang et al. 2002). Acid responses, also described for *L. monocytogenes*, *E. coli* and *S. flexneri* (Abee and Wouters 1999), can be induced by organic acids commonly used in the food industry (O'Driscoll et al. 1996). The more effective acid resistance systems for *E. coli* and *S. flexneri* allow survival at pH 2, while the ATR of *S.* Typhimurium is a pH 3 tolerance system (Audia et al. 2001, Abee and Wouters 1999, Foster and Moreno 1999). During acid adaptation and shock, *S.* Typhimurium induces the expression of over 50

acid shock proteins (ASPs) in the exponential phase and 15 ASPs in the stationary phase (Foster 1991, Foster and Moreno 1999, Foster 1995). These proteins are under the control of multiple, overlapping regulatory systems and protect cells against acid and perhaps other environmental stresses (Audia et al. 2001).

The oxidative response typically involves proteins for preventing (e.g., catalase and superoxide dismutase) and repairing (e.g., exonucleases and glycosylases) oxidative damage (Farr and Kogoma 1991). Starvation proteins also have been demonstrated, independent of the nutrient for which a cell is starved. Some of these proteins are unique to starvation while others are common to other stresses (Hengge-Aronis 1993, Matin 1991, Jenkins et al. 1988, Völker et al. 1992). Starvation and stationary phase proteins are likely responsible for maintaining long-term survival and perhaps enhancing general cellular resistance by stabilizing the ribosome against degradation (Tolker-Nielsen and Molin1996), morphing cells into a more spherical shape (Givskov et al. 1994) and improving metabolic potential through the utilization of alternative growth substrates (Matin 1991).

Their overlapping nature and the wide variety of stresses capable of inducing their expression complicates the classification of stress proteins as general or specific (Watson 1990). The universal induction of many of the same stress proteins after exposure to different stresses has been reported in *B. subtilis*, *E. coli*, *E. faecalis* and *L. lactis* (Girgis et al. 2003). Jenkins et al. (1988) studied starvation followed by heat and hydrogen peroxide stress in *E. coli*. While each stress produced its own distinct protein pattern, eleven heat shock and six oxidative stress proteins were common to starvation proteins, with three proteins common to all three stresses. Völker et al. (1992) determined that the

proteins induced by *B. subtilis* following heat stress were both general stress proteins and heat shock specific proteins, indicating that the heat shock and general stress responses are related. Some genes associated with the general stress response have clear functions for managing specific stresses, such as oxidative and osmotic stress (Yousef and Courntey 2003) while others play a role in general protection under multiple stress conditions (IFT 2002).

2.3.6 Cross-Protection

Sykes (1963) attributed survival in adverse environments to sublethal treatments insufficient to cause death and/or to protection from the unfavorable conditions. It was inferred from the latter that bacterial cells could adapt or acquire resistance to different conditions by modifying metabolic activities, adjusting nutrient utilization or making use of enzymes previously present in a recessive role. Although the exact mode of action is not fully understood, a role in stress protection has been proposed for stress proteins, especially HSPs and starvation proteins (Pagán et al. 1997, Hengge-Aronis 1993, Abee and Wouters 1999, Hecker at al. 1996). The long-term survival of *E. coli* is dependent on the synthesis of starvation proteins (Matin 1991), and Givskov et al. (1994) concluded that protein synthesis induced by starvation was necessary for *Pseudomonas putida* to develop a general stress-resistant state. Jenkins et al. (1988) correlated the induction of starvation proteins with protection of *E. coli* against heat and H_2O_2.

That the induction of certain stress proteins is coincident with the development of acquired resistance to heat (acquired thermotolerance) and other stresses (cross-protection) does not establish a direct cause-and-effect relationship (Jenkins et al. 1988, Juneja and Novak 2003). However, the heat shock and general stress responses may

induce changes that protect cells from the effects of heat and other stress conditions (IFT 2002). The phenomenon that one stress offers protection when subsequently stressed at higher levels (especially for heat) or by other types of stresses is widely documented. Lou and Yousef (1996, 1997) describe this as "stress-hardening", suggesting "if the first encountered stress is not lethal to a microorganism, the adaptation of the microorganism to this sublethal stress may 'harden' the microorganism and allow its survival at subsequent stresses."

Watson (1990) believes that acquired thermotolerance is the most characteristic physiological response microorganisms have to mild temperature shock. Novak et al. (2001) reported that vegetative cells of *Clostridium perfringens* incubated at 46°C for 60 min had significantly higher $D_{60°C}$-values than those incubated at 28°C. Exponential phase cells of *Vibrio parahaemolyticus* heat shocked at 42°C for 30 min had a $D_{47°C}$-value of 3.33 min and were significantly more heat resistant than unshocked cells having a $D_{47°C}$-value of just 2.03 min (Wong et al. 2002). Heat shocking *E. coli* O157:H7 at 42°C for 5 min before thermal inactivation at 55°C also increased the D-value >2-fold (Murano and Pierson 1992, 1993). Juneja et al. (1998) sublethally heated samples of beef gravy and ground beef previously inoculated with a four-strain cocktail of *E. coli* O157:H7 at 46°C for 15 to 30 min. The heat-shocked cells were more thermotolerant than unshocked cells, with a 1.56- and 1.50-fold increase in the time to achieve a 10,000-fold reduction at 60°C in beef gravy and ground beef, respectively. When treated at 62°C, cells of *L. monocytogenes* heat-shocked in Trypticase soy broth containing 0.6% yeast extract (TSB-YE) at 45°C for 180 min were six-fold more heat resistant than unshocked cells (Pagán et al. 1999), and a heat shock in TSB-YE at 48°C for 10 min

increased the $D_{55°C}$-value of *L. monocytogenes* more than two-fold (Linton et al. 1992). Mackey and Derrick (1987b) determined that *S.* Thompson inoculated in TSB, liquid whole egg, and 10% or 40% reconstituted dried milk was more thermal tolerant at 54°C or 60°C if held at 48°C for 30 min prior to treatment.

The response of microorganisms in foods heated slowly is thought to be the same as the response to an instantaneous heat shock, namely acquired thermotolerance. There is evidence to indicate that heating rate should be included when determining inactivation kinetics. Quintavalla and Campanini (1991) found that *L. monocytogenes* was more heat resistant at 60°C, 63°C and 66°C when heated slowly (0.5°C/min) in a pork emulsion than if heated virtually instantaneously to these temperatures. Mackey and Derrick (1987a) examined the heat resistance of *S.* Typhimurium as affected by rising temperatures and concluded that slowly increasing the temperature (2°C/min, 1°C/min and 0.6°C/min) from 20°C up to 55°C, versus instantaneous heating at 55°C, increased thermal resistance. The resistance of cells increased with decreasing heating rates, with the most resistance afforded by a heat shocking at 48°C for 30 min before heating. The observation of increasing thermal resistance with decreasing heating rates may be a general phenomenon, as it was also reported by Stephens et al. (1994) for *L. monocytogenes*.

Thermotolerance can also be induced by stresses other than heat. Table 2.8 summarizes some of the available data for different microorganisms. In addition, many sublethal treatments can cross-protect against stresses other than heat. Jenkins et al. (1988) reported that starvation or adaptive treatments with heat, H_2O_2 or ethanol protects *E. coli* against further oxidative (H_2O_2) challenges. According to Zook et al. (2001),

Table 2.8. Treatments known to enhance thermotolerance for microorganisms.

Microorganism	Stress Treatment	Heat Challenge	Reference
E. coli O157:H7	Starvation in distilled water for 24 h at 37°C	56°C for up to 90 min	Rowe and Kirk 2000
	Starvation in 0.85% NaCl (pH 6.6) for 48 h at 37°C	56°C for 50 min	Leenanon and Drake 2001
	Acid adaptation (pH 4.8-4.9) in TSB for 18 h at 37°C	56°C for 50 min	Leenanon and Drake 2001
Non-pathogenic *E.coli*	Starvation in 0.85% NaCl (pH 6.6) for 48 h at 37°C	56°C for 50 min	Leenanon and Drake 2001
	Acid adaptation (pH 4.8-4.9) in TSB for 18 h at 37°C	56°C for 50 min	Leenanon and Drake 2001
	Glucose starvation in M9 minimal media for 4 h at 29°C	57°C	Jenkins et al. 1988
S. Typhimurium	Starvation in a minimal medium with 0.02% glucose for 10 h at 37°C	52°C	Tolker-Nielsen and Molin 1996
	Acid adaptation (pH 5.8) in medium E at 37°C	50°C for up to 60 min	Leyer and Johnson 1993
L. monocytogenes	Adaptation in Tryptose phosphate broth (pH 12.0) at 37°C for 45 min	56°C or 59°C	Taormina and Beuchat 2001

Table 2.8 (cont'd).

Microorganism	Stress Treatment	Heat Challenge	Reference
L. monocytogenes	Exposure to 4-8% (vol/vol) ethanol at 35°C for 1 h	56°C	Lou and Yousef 1996
	Starvation in 0.1M phosphate buffer (pH 7) at 30°C for up to 163 h	56°C	Lou and Yousef 1996
	Exposure to 500 ppm H_2O_2 at 35°C for 1 h	56°C	Lou and Yousef 1996
	Acid adaptation in TSB-YE (pH 4.5) at 35°C for 1 h	56°C	Lou and Yousef 1996
	Acid shock (pH 4) in TSB-YE at room temperature for 1 h	58°C	Farber and Pagotto 1992
P. putida	Starvation at 30°C by exhaustion of carbon, nitrogen, phosphate or sulfate	47°C	Givskov et al. 1994

treatment with a commercial sanitizer (0.1% peroxyacetic acid) also increased oxidative tolerance of *E. coli* O157:H7. Starvation of *P. putida* resulted in enhanced resistance to ethanol, heat shock and osmolarity (Givskov et al. 1994). Bang and Drake (2002) observed that starvation increased freeze-thaw resistance of *V. vulnificus*, while Leenanon and Drake (2001) observed an increase in freeze-thaw resistance of *E. coli* O157:H7 following both starvation and acid adaptation. Bollman et al. (2001) observed that cold shocking *E. coli* O157:H7 in milk, whole egg or sausage, but not beef or pork, enhanced survival during frozen storage. Adaptation of *S.* Typhimurium to pH 5.8 reportedly increases tolerance to osmotic (salt) stress, crystal violet and an activated lactoperoxidase system (Leyer and Johnson 1993). Lou and Yousef (1997) found that adaptation of *L. monocytogenes* to 5% (vol/vol) ethanol significantly increased resistance to lethal doses of acid, ethanol and NaCl. Similarly, adaptation to acid (pH 4.5-5), ethanol, 500 ppm H_2O_2, 7% (wt/vol) NaCl and heat (1 h at 45°C) increased resistance to H_2O_2.

The response to a given stress differs among bacterial species. While Lou and Yousef (1997) showed that both acid and ethanol adaptation increased the resistance of *L. monocytogenes* to lethal doses of acid, adaptation of *S.* Typhimurium to stresses other than acid did not protect against lethal acid treatment (Lee et al. 1995). Bunning et al. (1990) found that a 48°C heat shock significantly increased thermotolerance at 57.8°C for *S.* Typhimurium but not for *L. monocytogenes*. Mazzotta (2001) determined that while acid adaptation (pH 5, 18-24h) increased the heat resistance at 56°C, 58°C and 60°C for *L. monocytogenes*, *E. coli* O157:H7 and two *Salmonella* composites (*S.* Gaminara, Rubislaw and Hartford, and *S.* Typhimurium and Enteritidis) in apple, orange and white grape juices adjusted to pH 3.9, the increase in heat resistance was greater for *L.*

monocytogenes and *E. coli* O157:H7 than for *Salmonella*.

The effect of adaptive pre-treatments can also vary between different bacterial strains. Cheng et al. (2002) found that acid adaptation at pH 5 for 4 h increased the thermal tolerance at 52°C of two strains of *E. coli* O157:H7, but not of a third strain. Similarly, starvation was shown to increase the heat resistance of only two of three *E. coli* O157:H7 strains (Rowe and Kirk 2000). These observed differences for species and strains may be due to inherent degrees of heat or other stress resistance.

The magnitude and nature of a protective stress response also varies with the treatment. Völker et al. (1992) found that treatment of *B. subtilis* with low salt was less effective at inducing thermotolerance than a preceding heat shock. Jenkins et al. (1988) reported that the oxidative and thermal resistance of starved *E. coli* cells was greater than for heat-adapted cells, with resistance to H_2O_2 and heat increasing as the starvation period increased. Looking at *L. monocytogenes*, Lou and Yousef (1996) also found that the magnitude of the increase in thermotolerance provided by starvation, acid, ethanol or H_2O_2 depended on the duration of starvation (i.e., the longer the starvation period, the greater the increase in heat resistance), the pH level and the concentration of ethanol or H_2O_2. The type of acid can also affect the outcome, as Farber and Pagotto (1992) observed that, while hydrochloric was effective at inducing thermotolerance in *L. monocytogenes*, acetic and lactic acids were not. For heat-shocked *S.* Typhimurium, the degree of thermal resistance and rapidity of its onset was shown to increase with increasing heat shock temperatures (Mackey and Derrick 1986). While cells of *L. monocytogenes* incubated at pH 12 for 45 min were significantly more heat resistant at 56°C than those incubated at pH 7.3, cells incubated at pH 9, 10 or 11 did not exhibit this

enhanced thermotolerance (Taormina and Beuchat 2001). The strength of the agent used to adjust the pH to12 also had an effect on thermotolerance.

2.3.7 Virulence

The presence of injured microorganisms in food gives rise to significant public health concerns. Injury may initially leave cells undetected during quality control checks and at critical control points during manufacture. However, subsequent repair in the food may allow for growth and its consequences, including spoilage and the production of toxins and other virulence factors (Johnson and Busta 1984). As an example, three virulence factors of *E. coli* O157:H7, verotoxin (VT)1, VT2 and the attaching and effacing gene, *eae*, were shown to be retained following starvation and heat stress (Rowe and Kirk 2000). According to Singh and McFeters (1987), virulence of *Y. enterocolitica* in orally inoculated mice was also unaffected by chlorine stress. A bacterium's pathogenicity or virulence may be considered the ultimate expression of its ability to repair injury (Dillon and Bezanson 1984).

Mekalanos (1992) defines virulence determinants as those factors contributing to infection and disease, but not to general "housekeeping" functions. There is not always a clear line of distinction between the two, but virulence genes, to some degree, are part of an adaptive response to stresses encountered in a host (IFT 2002). Many of the stresses that are intrinsically part of a host's defense system are similar to those encountered in the natural environment (see Figure 2.2). Exposure to stresses in both natural environments and food processing facilities may be seen by pathogenic microorganisms as a signal for the expression of virulence factors (Lou and Yousef 1997). A strain of *S.*

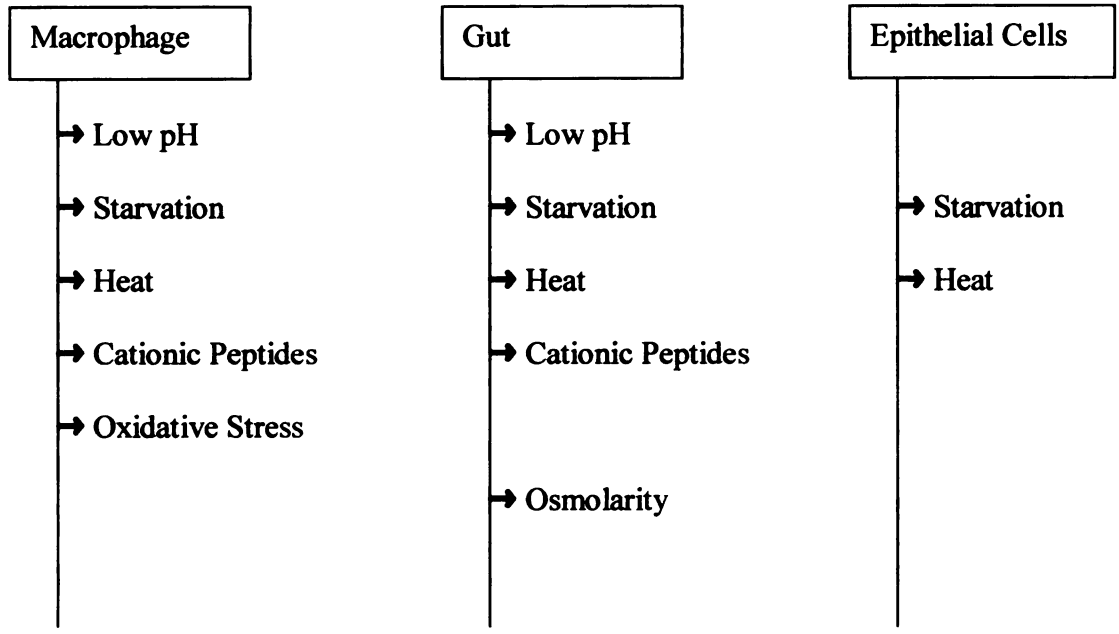

Figure 2.2. Certain stresses encountered by microorganisms in the environment are also imposed by a host's defense mechanisms (Foster and Spector 1995).

Enteritidis possessing enhanced acid and heat tolerance was shown to be more virulent for mice and more invasive for chickens than a non-resistant reference strain (Humphrey et al. 1996).

Expression of many virulence factors is dependent on environmental cues (Mekalanos 1992, Knøchel and Gould 1995). Several environmental conditions have been identified that induce expression of Spv (*Salmonella* plasmid virulence) proteins, including glucose starvation, low pH, elevated temperature and iron limitation (D'Aoust 1997, Valone et al. 1993, Foster and Spector 1995). The *spv* genes are thought to facilitate rapid multiplication in host cells and systemic spread and infection of extraintestinal tissues by several serovars of *Salmonella* (Typhimurium, Dublin, Enteritidis) (D'Aoust 1997). An invasion gene of *S.* Typhimurium, *invA*, is reportedly induced by high osmolarity (Mekalanos 1992, Archer 1996) and expression of listeriolysin, a major virulence factor of *L. monocytogenes*, by heat shock, oxidative stress and transition to the stationary phase (Sokolovic et al. 1990, Mekalanos 1992, O'Driscoll et al. 1996, Datta and Kothary 1993, Sokolovic et al. 1993). Production of thermostable direct hemolysin, a major virulence factor of *V. parahaemolyticus*, is enhanced by heat shock at 42°C (Wong et al. 2002).

Temperature-regulated virulence factors have been identified in enteroinvasive *E. coli* (Berlutti et al. 1998), *S. flexneri* (Maurelli 1989, Mekalanos 1992, Dorman et al.1990), *L. monocytogenes* (Gahan and Hill 1999, Leimeister-Wachter et al. 1992) and *Y. enterocolitica* (Skurnik and Toivanen 1992), and heat shock has been linked to virulence in *L. monocytogenes* (Archer 1996, Mekalanos 1992), *S.* Typhimurium (Archer 1996, Foster and Spector 1995) and *Shigella* species (Sunil 2000). As pathogens traverse from

the natural environment, through contaminated food, water or insect vectors, into mammalian hosts, the sudden increase in body temperature triggers a strong heat shock-like response, which is intensified when host defense mechanisms (including fever) are encountered (Lindquist 1992).

The facultative intracellular pathogen *Y. enterocolitica* experiences a global stress response to the hostile environment of macrophages, including the induction of heat shock proteins (Yamamoto et al. 1994). While no set of specific stess-induced proteins is induced in its entirety within the macrophage environment, *S.* Typhimurium also expresses a number of stress proteins, including HSPs (Foster and Spector 1995, Abshire and Neidhardt 1993, Buchmeier and Heffron 1990). To a certain degree, HSP synthesis appears to protect pathogens from host defense mechanisms and HSPs themselves may well be ubiquitous evasion factors of pathogens (Kaufmann 1989). Due perhaps to their wide distribution and homology among different species, HSPs are often dominant antigens of the immune response (Zugel and Kaufmann 1999, Buchmeier and Heffron 1990, Lindquist 1992). Host cells can also synthesize HSPs, and an extended assault on the immune system with HSP antigens that are similar in the host and infecting pathogens may promote autoimmune disease (Zugel and Kaufmann 1999, Kaufmann 1989).

Acid tolerance is thought to enhance virulence in one of two ways. Resistance to strong acid conditions facilitates survival in the stomach, thereby decreasing a pathogen's necessary infective dose. Resistance to moderate acid conditions improves pathogen survival in foods dependent on low pH for microbial inactivation (Leenanon and Drake 2001). Acid tolerance of *E. coli* O157:H7 likely contributes to its low infective dose. Acid sensitive strains of *S.* Typhimurium exhibit reduced virulence (Foster 1995),

whereas acid tolerant mutants of *L. monocytogenes* exhibit increased virulence in the mouse model (O'Driscoll et al. 1996). For many pathogens, acid tolerance seems to enhance survival in the host macrophage (Archer 1996, Gahan and Hill 1999).

Due to the limited oxygen availability in the small intestine, anaerobic stress is hypothesized to enhance virulence of pathogens invading the gastrointestinal tract (Kapoor et al. 2002). Anaerobiosis can reportedly induce the invasion phenotype in *S.* Typhimurium, confirmed by the restricted invasion of aerobically grown cells (Mekalanos 1992, Foster and Spector 1995). Exposure of *S.* Typhi to anaerobic conditions also enhances virulence (Kapoor et al. 2002). Osmotic stress may prepare microorganisms for survival within a host, as the expression of some virulence factors may be enhanced in the osmolarity range of host tissues. Osmoregulation of virulence is noted for several microorganisms, including *S.* Typhimurium, *S. flexneri*, and *Vibrio cholerae* (Mekalanos 1992, Sleator and Hill 2002).

Preceding examples indicate alterations in cellular physiology, including stress protein synthesis, in response to environmental stresses may strongly impact virulence. A bacterium's capability of successfully handling environmental stresses in part defines its virulence, as the response to such stresses often includes the expression and control of various virulence factors (Archer 1996). The consequences of this has led Archer (1996) to question if a "reduction in preservation might not in fact lead to a reduction in the immediate virulence of certain pathogens, and, additionally, to a lowering of the rate of emergence of new or better host-adapted pathogens."

2.3.8 Recovery of Injured Microorganisms

Recovery is the "getting back" of injured or otherwise treated microorganisms

from their environment (Harris 1963). Some of these organisms will be repaired and others will not. Damage to microorganisms cannot be understood and characterized unless the injured population has been recovered. The return of colony-forming ability, particularly on selective media, is recognized as recovery from initial injury and indicates repair of the structural and metabolic functions damaged by stress (Przybylski and Witter 1979). It is very important that appropriate techniques and media are used to quantitatively recover all cells, regardless of injury status, following exposure to stressful treatments, since any of these cells could potentially induce illness (Brashears et al. 2001). Historically, the term "resuscitation" has described treatments or processes used for complete recovery (Mossel and van Netten 1984, Allen et al. 1952).

Resuscitation requires incubation under optimal conditions in order to facilitate the repair of injured bacteria (Hurst 1984). Any generalizations regarding the recovery of injured microorganisms must be made with caution, as optimal conditions vary widely between different species, strains and stress treatments. Incubation at temperatures well above or below the optimum can significantly reduce recovery of injured cells (Mackey et al. 1994). For example, better recovery of heat-injured *L. monocytogenes* occurred at temperatures between 20°C and 25°C than at 5°C (Mackey et al. 1994) or >30°C (Teo and Knabel 2000). Although in many instances the optimum repair temperature has been reported to be below the optimum growth temperature (Mackey 2000), the effect of incubation temperature varies with the organism and the type of stress (Mossel and van Netten 1984). Although the recovery of certain strains of irradiated *E. coli* was improved at temperatures below 37°C, temperatures above 37°C are also reportedly favorable for recovery of irradiated *E. coli* (Harris 1963). For microorganisms for which cold

enrichment has been recommended (e.g., *L. monocytogenes*, *Y. enterocolitica*), it has been suggested that incubation at 25°C for 2 to 3 h before incubation at 4°C could produce a favorable environment for the repair and recovery of injured cells (Restaino et al. 1980).

Stressed cells are affected by the levels of several nutrients, including Ca^{2+}, Mg^{2+}, K^{+}, carbohydrates and amino acids (Mossel and van Netten 1984). Mg^{2+} is important in stabilization of the ribosome, which can be compromised by many environmental stresses. Although injured cells typically repair faster in rich as opposed to minimal media (Mackey 2000), the effect of nutrients in the recovery medium is likely dependent on a number of additional factors, including the phase of growth when the cells are stressed and the medium used to grow the cells before stressing (Mossel and van Netten 1984, Harris 1963, Mackey 2000). As a result, the effect of minimal medium recovery must not be overlooked. In some instances, recovery of stressed cells is dramatically increased on minimal salts media. Gomez and Sinskey (1975) determined that minimal medium recovery of heated *S.* Typhimurium depended on the presence of air, and several authors (Mackey and Derrick 1986, Mackey 2000, Stephens et al. 2000) suggested that minimal medium recovery is an effect of oxidative stress, including sensitivity to peroxides in rich media.

By eliminating the challenges of oxidative stress, some authors have improved the recovery of sublethally injured microorganisms with anaerobic incubation. Bromberg et al. (1998) recovered 10^3 CFU/mL when heat-treated cells of *E. coli* O157:H7 were recovered aerobically, but 10^6 CFU/mL when they were recovered anaerobically. Anaerobically held cells gradually regained their ability to grow in the presence of

oxygen, perhaps due to the synthesis of enzymes that detoxify oxygen radicals. Xavier and Ingham (1993) reported that anaerobic incubation recovered significantly more heat-treated cells of *S.* Enteritidis than did aerobic incubation, and strictly anaerobic conditions recovered significantly more heat-injured cells of *L. monocytogenes* compared to aerobically incubated controls (Knabel et al. 1990).

Other factors must also be considered when optimizing the recovery of injured microorganisms, including pH, presence of reducing agents or H_2O_2-degrading compounds and salt concentration, among others. Although often included in culture media formulations, sodium chloride can impair repair and recovery of injured microorganisms (Harris 1963). However, inhibition from the recovery medium may be partially overcome by the protective effect from the heating medium (Mañas et al. 2001). Other salts, such as ferric chloride, can enhance recovery of phenol-treated *E. coli* (Harris 1963). The method of inoculating media must also be considered, because if not carefully done, pour plating can result in additional thermal injury and a reduction in the recovery of stressed cells (Mossel and van Netten 1984, Czechowicz et al. 1996).

Even if optimum repair/recovery conditions are used, the extended lag period characteristic of injury will still be observed. The length of the required repair or lag time depends on the bacterial species, strain and the type/severity of injury. For this reason, any attempt to shorten or eliminate pre-enrichment periods, which are designed to allow for resuscitation of injured cells or to enable target organisms to reach a detectable level, are often met with false-negatives (Andrews 1986, Palumbo 1984, Flowers et al. 1992, Mackey 2000). Rapid methods employing a non-selective enrichment step might be affected by this, particularly in food matrices where the overgrowth of competing

bacteria must be considered; however, more research is needed, since studies on the impact of injury on incubation conditions required to reach detectable microbial levels are limited (Mackey 2000). Direct selective enrichment of highly contaminated products is not uncommon. This method seems to be inappropriate for detecting injured microorganisms, particularly in the presence of large numbers of competitive flora, and the incorporation of a non-selective pre-enrichment can significantly increase recovery (D'Aoust 1981, Blackburn and McCarthy 2000).

CHAPTER 3

EFFICACY OF SODIUM PYRUVATE AND OXYRASE® FOR RECOVERING HEAT-, COLD-, AND STARVATION-INJURED *SALMONELLA*

3.1 Abstract

Recovery of heat-injured, cold-shocked or otherwise stressed cells is often limited by choice of media and environmental conditions, including oxygen exposure. Reliable enumeration of these injured and potentially more resistant cells is particularly important in thermal death-time studies so as to not underestimate D-values, which can lead to a false sense of security in cooked products. This study assessed the efficacy of adding two reducing agents, Oxyrase® and sodium pyruvate, to a non-selective culture medium for recovery of heat-, cold- and starvation- stressed *Salmonella.* Using TSA-YE supplemented with Oxyrase®, 0.1% sodium pyruvate and 0.6% sodium pyruvate, recovery of heat-shocked, cold-shocked and starved cells ranged from 89.6 to 90.2%, 94.9 to 95.1% and 99.2 to 101.6%, respectively, and was not significantly different from unsupplemented TSA-YE. From these findings, it is apparent that, under the specified conditions, supplementation of TSA-YE with Oxyrase® or sodium pyruvate is not required to enhance the recovery of heat-shocked, cold-shocked or starved salmonellae.

3.2 Introduction

Salmonella is a major cause of gastroenteritis in humans when contaminated food is undercooked or eaten raw. In the United States, an estimated 1.4 million cases of salmonellosis occur each year (Frenzen et al. 1999) at a cost of $2.4 billion (Frenzen

2002). Outbreaks associated with *Salmonella* have been reported in a wide range of foods, including meat, poultry, eggs, fruits, vegetables, dairy products and chocolate (D'Aoust 1997).

Physical and chemical treatments intended to inactivate *Salmonella* can lead to injury, with such cells harboring various sublethal lesions that adversely affect the permeability barrier and other physiological properties. As a result, injured cells typically are unable to undergo repair and proliferation on solid media containing selective components (Hurst 1977, Gilbert 1984, Hurst 1984, Semanchek and Golden 1998, Ray 2001). Repair and subsequent growth is also inhibited by selective agents in enrichment broth (Patel and Beuchat 1995). Consequently, the potential for obtaining false-negative results or underestimation of injured target cells is increased (Blackburn and McCarthy 2000). Even if undetected in food products, injured cells are still virulent if ingested by susceptible individuals (Reissbrodt et al. 2002).

Methods developed to allow injured organisms to repair before exposure to a selective medium do not always take into account potential inhibition from the non-selective medium (McDonald et al. 1983). Recovery of injured organisms is often highly sensitive to factors that do not affect, to the same degree, the recovery of uninjured organisms, with non-selective media differing in their ability to resuscitate injured cells (Stephens et al. 2000). Enhanced recovery in simple media, a phenomenon known as minimal medium recovery, was suggested to result from sensitivity to peroxides present in rich media (Stephens et al. 2000, Mackey and Derrick 1986). Autooxidation of reducing sugars in the presence of phosphate during autoclaving can result in the production of toxic oxygen species, particularly hydrogen peroxide (H_2O_2) (Stephens et

al. 2000, Baylis et al. 2000a). Media containing manganese or citrate can also autooxidize to form peroxides. If peroxides accumulate in media in the presence of superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) will form. Alternatively, hydroxyl radicals can form when H_2O_2 reacts with Fe^{2+} (McDonald et al. 1983, Suh and Knabel 2000). Reactive oxygen species (ROS), including $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$, can be produced endogenously as a consequence of the normal metabolic reduction of molecular oxygen to water (Stephens et al. 2000).

Oxidative stress caused by elevated levels of ROS can damage cellular components (Storz and Zheng 2000) and possibly inhibit repair and recovery of injured microorganisms in culture media. Injured cells, particularly those that are heat-injured, often have diminished activity of superoxide dismutase (SOD) and catalase, the enzymes instrumental in neutralizing the harmful effects of oxygen radicals (Suh and Knabel 2000, McDonald et al.1983). Research on the stability, in response to different environmental stresses, of catalase and SOD in *Salmonella* is limited. However, a number of studies have attempted to enhance recovery of sublethally injured microorganisms by supplementing various media with reducing agents, O_2 scavengers and/or H_2O_2-degrading agents (Suh and Knabel 2000, Patel et al. 1995, McDonald et al. 1983).

Incorporating sodium pyruvate in culture media reportedly improved recovery of heat-injured *Escherichia coli* O157:H7 (Czechowicz et al. 1996), *Listeria monocytogenes* (Patel et al. 1995, Busch and Donnelly 1992), *Salmonella* Seftenberg (Rayman et al. 1978), *Shigella flexneri* (Smith and Dell 1990) and *Staphylococcus aureus* (Hurst et al. 1976, Martin et al. 1976), as well as heat- and freeze-injured *E. coli* (McDonald et al. 1983). Addition of catalase also enhanced recovery of heat-injured and freeze-dried *S.*

aureus (Martin et al. 1976, Flowers et al. 1977), acid-injured *E. coli* (Martin et al. 1976), and heat-injured *S.* Seftenberg (Rayman et al. 1978), *L. monocytogenes* (Patel et al. 1995) and *E. coli* (Mackey and Seymour 1987). The benefits of other agents, including α-ketoglutaric acid (degrades H_2O_2) (Mizunoe et al. 1999), sodium thioglycolate (blocks formation of H_2O_2) (McDonald et al. 1983, Xavier and Ingham 1993, Yu and Fung 1991), L-cysteine (reducing agent) (Knabel and Thielen 1995) and Oxyrase® (oxygen-reducing membrane fraction from *E. coli*) (Stephens et al. 2000, Yu and Fung 1991, Reissbrodt et al. 2002, Patel and Beuchat 1995, Patel et al. 1995) have also been reported.

Research on the elimination of oxidative stress for the enhanced recovery of injured *Salmonella* is limited, particularly when non-thermal injury is considered. The aim of the present study was to assess the impact of sodium pyruvate and Oxyrase® on the recovery of heat-, cold- and starvation-stressed *Salmonella*.

3.3 Materials and Methods

3.3.1 Bacterial Strains

The following eight strains of *Salmonella* were used: *S.* Thompson FSIS 120 (chicken isolate), *S.* Enteritidis H3527 (clinical isolate phage type 13A), *S.* Enteritidis H3502 (clinical isolate phage type 4), *S.* Typhimurium H3380 (DT104 clinical isolate), *S.* Hadar MF60404 (turkey isolate), *S.* Copenhagen 8457 (pork isolate) and *S.* Heidelberg F5038BG1 (clinical isolate), all obtained from V.K. Juneja (Agricultural Research Service, Eastern Regional Research Center, USDA-ARS, Philadelphia, PA), and *S.* Typhimurium 420, a DT104 strain isolated from a pork processing facility. All strains were maintained at –70°C in Trypticase soy broth (TSB) (Difco Laboratories, Detroit,

MI) containing 10% (v/v) glycerol (J.T. Baker, Mallinckbrodt Baker, Inc., Phillipsburg, NJ).

3.3.2 Culture Preparation

Each culture was propagated by transferring one loopful of the frozen cell suspension into 9 mL of TSB containing 0.6% yeast extract (TSB-YE) (Difco). Cultures were subjected to a minimum of two consecutive overnight transfers (37°C, 18-24 h) in TSB-YE before use.

3.3.3 Heat Shock Treatment

An 8-strain *Salmonella* cocktail was prepared by combining 3 mL of the individual overnight cultures in a sterile 50 mL centrifuge tube (Clear Propylene, Plug Seal Cap, Corning Inc., Corning, NY), centrifuging at 10,000 rpm for 15 min at 4°C (Super T21, Sorvall® Products, L.P., Newtown, CT) and re-suspending the pellet in 3 mL 0.1% peptone (Difco) to obtain a culture containing approximately 10^{10} CFU/mL. Heat-injured cells were obtained using the methods of Mathew and Ryser (2002) and Busch and Donnelly (1992), with minor modifications. In this procedure, 200 mL of TSB-YE (in a 2800 mL wide-mouth Fernbach flask) was tempered to 54°C in a shaking (30 rpm) water bath (Reciprocal Shaking Bath, Precision Scientific, Winchester, VA), inoculated with 2 mL (~10^{10} CFU/mL) of the *Salmonella* cocktail and heated at 54°C for 30 min. After 30 min, heat-shocked cells were immediately cooled in an ice-water bath for 3 to 5 min.

The initial temperature for heat shock was 48°C, which was also used by Mackey and Derrick (1986, 1987a, 1987b) and Bunning et al. (1990) for *Salmonella*. However, for the individual *Salmonella* strains in the cocktail, the extent of injury observed at 48°C

(6-54%), as well as at 52°C (23-68%), was decided to be insufficient for the current study (see Appendix B, Table B.1). At 56°C, a 30 min heat shock was lethal for 4 of the 8 strains. As 54°C resulted in approximately 90% injury in preliminary studies, it was this temperature that was incorporated into the heat shock procedure.

3.3.4 Cold Shock Treatment

The 8-strain *Salmonella* cocktail was prepared as previously described for heat shock, except the pellet was re-suspended in 4 mL 0.1% peptone to obtain a culture containing approximately 10^{10} CFU/mL. Cold-stressed cells were obtained by inoculating 200 mL TSB-YE (in a 500 mL Erlenmeyer flask) previously tempered to 4°C with 3 mL (~10^{10} CFU/mL) of the *Salmonella* cocktail. This cell suspension was then gently agitated for 1 min and held quiescently at 4°C for 2 h.

The 2 h time period for the cold shock treatment was chosen because preliminary work showed, that by 4 h, injury had decreased for the individual *Salmonella* strains in the cocktail (see Appendix B, Table B.2).

3.3.5 Starvation Treatment

The method for starvation was similar to that of Dickson and Frank (1993). The 8-strain *Salmonella* cocktail was prepared as previously described for heat shock, except the pellet was re-suspended in 24 mL Butterfield's phosphate buffer (BPB), pH 7.18 (prepared according to the Bacteriological Analytical Manual (FDA 1998)). To prevent carry-over of nutrients, the culture was centrifuged again at 10,000 rpm for 15 min at 4°C and the pellet re-suspended in 3 mL BPB to obtain a cell suspension containing approximately 10^{10} CFU/mL. Cells were starved by transferring 1 mL of the *Salmonella*

cocktail into 99 mL BPB in a 160-mL screw-capped glass bottle, gently agitating the cell suspension for 1 min and holding it quiescently at 4°C for 10 d.

Preliminary work showed that 5 d of starvation resulted in insufficient injury for the individual *Salmonella* strains in the cocktail and by 14 d, injury had begun to decrease from those levels observed at 10 d (see Appendix B, Table B.3). As a result, the 10 d period was incorporated into the starvation procedure. To verify that the injury observed at 10 d was due to starvation in BPB and not to the effects of low temperature, injury was also determined after 10 d at 4°C for cultures of the *Salmonella* strains grown and held in TSB-YE (see Appendix B, Table B.4).

3.3.6 Enumeration of Cells

Four different non-selective recovery media were used: Trypticase soy agar (Difco) containing 0.6% yeast extract (TSA-YE), TSA-YE containing 0.1% (w/v) sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO) (TSA-YE + 0.1% NaPyr), TSA-YE containing 0.6% (w/v) sodium pyruvate (TSA-YE + 0.6% NaPyr) and TSA-YE containing Oxyrase® (Oxyrase, Inc., Mansfield, OH). Oxyrase® was added after sterilizing TSA-YE at a final concentration of 10% (v/v) as recommended by the manufacturer, whereas sodium pyruvate was added to TSA-YE before autoclaving. Bismuth sulfite agar (Difco) (BSA) was used as the selective medium to assess sublethal injury. Before and after stressing, the cells were enumerated by serial dilution in 0.1% peptone followed by spiral plating (Autoplate® 4000, Spiral Biotech, Inc., Norwood, MA) in duplicate on the five different recovery media. All plates were counted after 18 to 24 h of incubation at 37°C.

Percent injury following the individual treatments was determined according to the formula: % injury = [(count on non-selective agar – count on selective agar)/count on non-selective agar] x 100. The unsupplemented, non-selective medium, TSA-YE, and the selective BSA were used to calculate injury. Percent injury was calculated for each replication of each treatment, with the results reported as averages.

3.3.7 Data Analysis

All experiments with Oxyrase® were repeated three times, whereas experiments with TSA-YE + 0.1% NaPyr and TSA-YE + 0.6% NaPyr were repeated nine times for heat shock and six times for cold shock and starvation. Results are reported as averages. The significance of bacterial recovery was assessed with a mixed model analysis of variance (ANOVA) (Proc Mixed, SAS® Version 8.02, SAS Institute, Inc., Cary, NC).

3.4 Results

3.4.1 Sublethal Injury

The individual stress treatments produced *Salmonella* cocktails containing 86.83 ± 8.09% (mean injury ± standard deviation) heat-injured, 68.25 ± 8.94% cold-injured, and 85.58 ± 8.23% starvation-injured cells, respectively.

3.4.2 Recovery of Injured Cells

Following heat shock, TSA-YE recovered 89.63 ± 2.25% of the original cells present, while TSA-YE + 0.1% NaPyr, TSA-YE + 0.6% NaPyr and TSA-YE + Oxyrase® recovered 90.18 ± 1.67%, 90.01 ± 2.32% and 89.56 ± 0.41%, respectively (Table 3.1). The application of heat significantly decreased ($P<0.05$) the populations on each individual medium, indicating that recovery was affected by the presence of dead and/or

injured cells. After heat shock, differences between the populations recovered on the different non-selective media were not statistically significant ($P>0.05$). The same was also true before heat shock. The ANOVA results for heat shock, as well as cold shock and starvation, can be found in Appendix B (Table B.6).

After cold shock, TSA-YE recovered 94.94 ± 1.85% of the original population, while TSA-YE + 0.1% NaPyr, TSA-YE + 0.6% NaPyr and TSA-YE + Oxyrase® recovered 95.10 ± 3.10, 94.93 ± 2.39% and 95.06 ± 2.39%, respectively (Table 3.2). Cold shock significantly decreased ($P<0.05$) the numbers recovered on each medium tested; however, before and after cold shock, the differences between the populations recovered on the four non-selective media were not statistically significant ($P>0.05$).

Following starvation, TSA-YE recovered 101.64 ± 5.92% of the original *Salmonella* population, while TSA-YE + 0.1% NaPyr, TSA-YE + 0.6% NaPyr and TSA-YE + Oxyrase® recovered 99.22 ± 5.68%, 100.00 ± 8.20% and 100.25 ± 8.03%, respectively (Table 3.3). There were no significant differences ($P>0.05$) in the initial and post-starvation populations, indicating no treatment effect. As with heat shock and cold shock, the differences between the populations recovered on the four non-selective media were not statistically significant ($P>0.05$). Recovery following starvation, as well as heat and cold shock, is shown in Figure 3.1.

Table 3.1 The effect of sodium pyruvate and Oxyrase® on recovery of heat-injured *Salmonella.*

	Initial	Post-Heat Shock	
Medium	Log (CFU/mL)	Log (CFU/mL)	% Recovery[a]
TSA-YE	10.40 ± 0.31	9.32 ± 0.34	89.63 ± 2.25
TSA-YE + 0.1% NaPyr	10.29 ± 0.32	9.38 ± 0.28	90.18 ± 1.67
TSA-YE + 0.6% NaPyr	10.37 ± 0.33	9.36 ± 0.29	90.01 ± 2.32
TSA-YE + Oxyrase®	10.02 ± 0.02	8.97 ± 0.03	89.56 ± 0.41

Arithmetic Means ± Standard Deviations
[a] Percent recovery was calculated with initial counts on TSA-YE as 100%.

Table 3.2 The effect of sodium pyruvate and Oxyrase® on recovery of cold-stressed *Salmonella.*

	Initial	Post-Cold Shock	
Medium	Log (CFU/mL)	Log (CFU/mL)	% Recovery[a]
TSA-YE	10.66 ± 0.44	10.11 ± 0.38	94.94 ± 1.85
TSA-YE + 0.1% NaPyr	10.60 ± 0.41	10.13 ± 0.25	95.10 ± 3.10
TSA-YE + 0.6% NaPyr	10.66 ± 0.45	10.11 ± 0.29	94.93 ± 2.39
TSA-YE + Oxyrase®	10.22 ± 0.21	9.78 ± 0.01	95.06 ± 2.39

Arithmetic Means ± Standard Deviations
[a] Percent recovery was calculated with initial counts on TSA-YE as 100%.

Table 3.3 The effect of sodium pyruvate and Oxyrase® on recovery of starved *Salmonella*.

Medium	Initial Log (CFU/mL)	Post-Starvation Log (CFU/mL)	Post-Starvation % Recovery[a]
TSA-YE	10.59 ± 0.04	10.76 ± 0.62	101.64 ± 5.92
TSA-YE + 0.1% NaPyr	10.57 ± 0.03	10.50 ± 0.56	99.22 ± 5.68
TSA-YE + 0.6% NaPyr	10.56 ± 0.03	10.58 ± 0.86	100.00 ± 8.20
TSA-YE + Oxyrase®	10.55 ± 0.02	10.60 ± 0.85	100.25 ± 8.03

Arithmetic Means ± Standard Deviations
[a] Percent recovery was calculated with initial counts on TSA-YE as 100%.

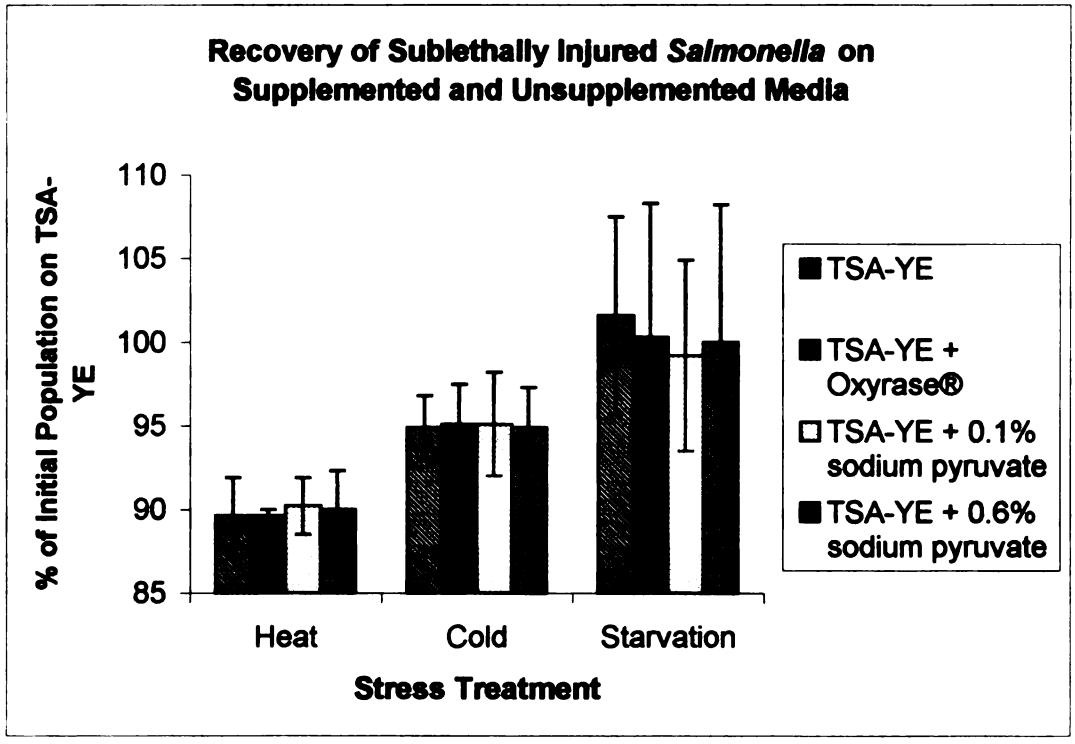

Figure 3.1 Recovery of sublethally injured *Salmonella* on unsupplemented and supplemented TSA-YE. Values are plotted as averages, with standard deviations shown as error bars.

3.5 Discussion

While it is common for authors to describe how they calculated the proportion of injured cells in a culture following a stress treatment, many fail to report actual numerical values. Although this limits the comparisons of our data to earlier findings, several observations can be made. In the current work, between 68% and 87% of the *Salmonella* cocktail was injured by heat shock, cold shock or starvation. With the exception of *S.* Montevideo FSIS 051 in place of the DT104 laboratory strain *S.* Typhimurium 420, Smith (2000) used the same 8-strain *Salmonella* cocktail and found that 90% of the population was injured following heat shock in TSB at 56°C for 30 min. While this is in close agreement with our findings, Baylis et al. (2000a) observed a much greater range of injury (59% and 98%) in *S.* Typhimurium CRA 8378 heat-treated in nutrient broth for 25 min at 51.5°C. For the same heat shock conditions and strain of *S.* Typhimurium, Baylis et al. (2000b) calculated 86% and 95% injury in two cultures, but observed little or no injury in a third culture. Using other test organisms, >99% of *Listeria monocytogenes* was injured by heating in tryptose phosphate broth at 56°C for 30 min (Mathew and Ryser 2002), 90% by heating in TSB at 54°C for 30 min (Pascual et al. 2001) and 73% by heating in tryptose phosphate broth at 52°C for 18 min (Patel et al. 1995). Percent injury following starvation in BPB for 5 d at 10°C was 2.1% for *L. monocytogenes*, 3.55% for *Escherichia coli*, and non-detectable for *S.* Typhimurium (Dickson and Frank 1993). This latter finding may differ from the high percent injury by starvation reported in the present study due to the shorter starvation period used (see Appendix B, Table B.3). Also, for *S.* Enteritidis, Boziaris and Adams (2001) observed a lesser degree of

injury (<50%) following cold shock in nutrient broth or phosphate-buffered saline for 10 min at 0.5°C.

The extent of injury following exposure to sublethal stress is dictated by differences between bacterial species and strains, treatment or stress conditions, the type, degree and site of injury, the medium in which the test organisms is grown and stressed, the selective and non-selective media used to assess injury and the growth phase of the culture (Ray 1989, Ray 2001). Heat, for instance, simultaneously affects the cell membrane, proteins and nucleic acids (Wuytack et al. 2003), and the degree of membrane damage and protein denaturation associated with heat could be more extensive than that associated with other treatments. Also, exponentially growing cells are more susceptible to injury than are stationary phase cells (Pascual et al. 2001, McMahon et al. 2000), and at least in the case of heat, injury may be difficult to manipulate and control in the stationary phase (Stephens et al. 1997). In addition, injury of planktonic cells (i.e., free, single, grown in broth, etc.) is probably quite different from injury of cells grown on an agar plate. Growth on an agar plate is perhaps the simplest expression of a biofilm, and bacterial cells in biofilms express enhanced resistance to heat, chemicals and sanitizers (Montville 1997, Costerton et al. 1995).

Standard deviations associated with average injury for each treatment were relatively large, indicating that some degree of variability exists. Since preliminary work (see Appendix B, Table B.5) indicated that all 8 strains in the cocktail were equally susceptible to injury by the three treatments, these variations are more likely due to the inherent heterogeneity present in every bacterial population and the choice of selective media.

All three supplemented media (TSA-YE containing 0.1% sodium pyruvate, 0.6% sodium pyruvate and Oxyrase®) were as equally effective as unsupplemented TSA-YE for recovering heat-, cold- and starve-injured *Salmonella*. McDonald et al. (1983) reported that tryptone-glucose extract (TGE) agar and violet red bile agar (VRBA) supplemented with 0.33% or 0.66% sodium pyruvate recovered significantly more freeze- and heat-stressed *E. coli* than did their unsupplemented counterparts. TGE and VRBA with sodium pyruvate at a concentration of 1.2% recovered more freeze-stressed cells, but 1.2% sodium pyruvate was only effective in TGE for heat-injured cells. In their study, McDonald et al. (1983) used pour plating, a method with the potential to impart an additional thermal stress on the test organism.

Czechowicz et al. (1996) found that recovery of heat-stressed *E. coli* O157:H7 increased 10- to 1000-fold using Trypticase soy agar (TSA) and plate count agar (PCA) containing 1% sodium pyruvate, as compared to unsupplemented TSA. Since the supplemented media were plated by the spread plate method and unsupplemented TSA by the pour plate method, part of the increased recovery with sodium pyruvate was perhaps due to the lack of exposure to additional thermal stress during spread plating. Czechowicz et al. (1996) also noted that spread plating on TSA containing 1% sodium pyruvate did not enhance recovery of heat-stressed cells over that seen using unsupplemented TSA. In the current study, the impact of the different supplements could have been outweighed by the impact of surface inoculation of media by spiral plating.

The increased recovery reported by Czechowicz et al. (1996) on PCA with 1% sodium pyruvate was not due to spread plating alone. The authors suggested that the presence of glucose and yeast extract in PCA may be as important as the addition of

sodium pyruvate. Growth factors available in yeast extract could contribute to the repair of injured organisms. Although the potential exists for pyruvate to be utilized as an energy-generating compound by injured organisms, most authors believe its main function is to degrade H_2O_2, and it is unlikely to be used as a nutrient (Mizunoe et al. 1999, Baird-Parker and Davenport 1965). However, if the use of pyruvate (and other TCA cycle intermediates) were to promote growth, the benefits of the supplement could be masked by the presence of yeast extract in our non-selective media.

The concentration of the chosen supplement may also affect the recovery of stressed cells. Nebra et al. (2002) found that sodium pyruvate used at a concentration of 0.66% in selective media enhanced recovery of metabolically injured (3 days of storage at 11°C) *Bifidobacterium* cells, while a concentration of 0.33% was not effective. Mizunoe et al. (1999) tested different concentrations of sodium pyruvate, catalase and α-ketoglucaric acid in Luria-Bertani agar for improving the recovery of starvation- and low-temperature-stressed *E. coli* O157:H-strain E32511/HSC. Each supplement had a particular concentration that afforded the highest recovery. In the case of sodium pyruvate, 0.1% recovered more cells than did 0.01%, 0.02%, 0.05%, 0.5%, 1%, 1.5% or 2%. Suh and Knabel (2000) also observed differences in recovery of heat-injured (62.8°C, 7.5 min) *L. monocytogenes* with different concentrations of Oxyrase® in Pennsylvania State University (PSU) broth. Oxyrase® was measured in units (U), with 1 U equivalent to the amount that decreased 1% of the dissolved oxygen per second per mL of broth as instructed by the manufacturer. Following incubation for 72 h, 5, 10, 20, 40 and 50 U/mL detected 26.7%, 50%, 43.7%, 23.3% and 13.3% of heat-injured cells,

respectively. The conclusions of the current work could be further examined using a wider range of concentrations for both sodium pyruvate and Oxyrase®.

Suh and Knabel (2000) also observed that Oxyrase® at any activity (or concentration) did not enhance the detection of *L. monocytogenes* heated at 62.8°C for 10 min. Results pertaining to Oxyrase® in the literature are mixed, with reports for both increased recovery (Stephens et al. 2000, Yu and Fung 1991, Baylis et al. 2000b) and no effect (Tran 1995, Suh and Knabel 2000) with Oxyrase®, the latter of which was also seen in our study. Oxyrase® is the oxygen-reducing membrane fraction from *E. coli*. The membrane electron transport chain that it contains could transfer electrons to residual oxygen in the media, contributing to the generation of $O_2^{\cdot-}$ (Suh and Knabel 2000). Guidot et al. (1993) determined that the electron transport chain is responsible for significant production of $O_2^{\cdot-}$, which could hinder recovery of injured cells. Injured microorganisms, particularly those that are heat-injured, are often deficient in enzymes required to neutralize toxic oxygen species. This may be one reason why healthy microorganisms that exhibit superior growth in Oxyrase®-supplemented media have not been recovered as well in the same media when injured.

The results presented in Tables 3.1, 3.2 and 3.3 include another dimension, namely a treatment effect. Heat shock significantly decreased the *Salmonella* populations on all media tested. More work is needed to determine how much of this effect is due to the inability to recover injured cells and how much is due to the potential lethality of the heat shock treatment.

Cold shock also decreased the recovery of *Salmonella*, with this decrease attributed to the inability to recover injured cells. Since TSA-YE + 0.1% NaPyr, TSA-

YE + 0.6% NaPyr and TSA-YE + Oxyrase® did not recover more heat- or cold-stressed cells than unsupplemented TSA-YE, the effectiveness of other concentrations or other reducing agents or oxygen-scavengers warrants further investigation. For starvation, neither the choice of non-selective recovery media nor starvation itself was significant, indicating, at the very least, that work involving the given starvation treatment is not limited by recovery methods.

3.6 Conclusion

Overall, TSA-YE with 0.1% or 0.6% sodium pyruvate or Oxyrase® was no more effective than unsupplemented TSA-YE at recovering heat-, cold- or starvation-stressed *Salmonella*. However, the effects of toxic oxygen species and neutralizing supplements on the recovery of injured microorganisms are complex. Differences can be observed with different bacterial species, strains, inoculum levels, stress treatments, the type, degree and site of injury, type and concentration of supplement, media ingredients and media preparation and storage conditions (including exposure to light, oxygen and high temperatures). Great care should be taken to include as many of these factors as possible when attempting to recover injured microorganisms, because the safety of thermally and otherwise processed foods is based on the detection of all pathogenic organisms, including those sublethally injured cells that retain their pathogenicity.

CHAPTER 4

THERMAL RESISTANCE OF HEAT-, COLD- AND STARVATION-STRESSED *SALMONELLA* IN IRRADIATED COMMINUTED TURKEY

4.1 Abstract

To investigate the effects of sublethal stress on *Salmonella* thermal inactivation kinetics, an eight-strain *Salmonella* cocktail was subjected to heat shock (30 min / 54°C), cold shock (2 h / 4°C), and starvation stress (10 d in phosphate buffer at 4°C), harvested by centrifugation and inoculated into irradiated comminuted turkey. Immediately after stress treatment, the *Salmonella* cocktails contained 89.02% heat-shocked, 44.71% cold-shocked, and 67.72% starved cells, respectively. $D_{60°C}$-values for the heat-shocked cocktail (0.64 min on Trypticase soy agar with 0.6 % yeast extract (TSA-YE), 0.35 min on xylose lysine deoxycholate (XLD) agar) were significantly higher ($P<0.05$) than for the unshocked control (0.41 min on TSA-YE, 0.17 min on XLD), whereas those for the cold-shocked cocktail (0.38 min on TSA-YE, 0.17 min on XLD) were not significantly different from the control. Starved cells had the same $D_{60°C}$-value on TSA-YE as the unshocked cocktail, but the $D_{60°C}$-value on XLD was significantly lower (0.14 min). Although starvation and cold shock were unable to provide a protective effect, heat shock increased thermal resistance, making it important for the product history and physiological state of *Salmonella* to be considered when developing and validating thermal processes. In addition, $D_{60°C}$-values observed on selective media were significantly lower than those observed on non-selective media for all stress treatments as well as the control. As a result, non-selective culture media should be used to assess the

response of microorganisms to a thermal challenge, as selective media can underestimate the number of cells remaining in a population.

4.2 Introduction

In 1999, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) finalized regulations for the production of certain fully and partially cooked meat and poultry products (FSIS 1999). Rather than dictate step-by-step processing methods with specific endpoint temperatures, the regulations establish lethality levels for pathogen reduction as well as set limits for pathogen growth based on performance standards for lethality, stabilization and product handling. *Salmonella* reductions of 6.5 logs in cooked beef, roast beef and cooked corn beef and 7.0 logs in certain fully and partially cooked poultry products are now required (FSIS 1999), with a comparable reduction proposed for all other ready-to-eat (RTE) meat and poultry products (FSIS 2001).

FSIS lethality standards reflect the destruction of "reference" organisms, whose elimination or reduction will indicate the elimination or reduction of other pathogenic organisms. Except for thermally-processed, commercially sterile products, the lethality performance standards are based on *Salmonella*. An estimated 1.4 million cases of salmonellosis occur each year in the United States (Frenzen et al. 1999) at a cost of $2.4 billion (Frenzen 2002). Outbreaks associated with *Salmonella* have been reported in a wide range of foods, including meat and poultry. The conditions required for an appropriate reduction of *Salmonella* in raw meat and poultry will simultaneously reduce most other vegetative pathogenic microorganisms, and although *Listeria monocytogenes*

is typically more heat resistant than *Salmonella*, *Listeria*-related recalls of RTE meat and poultry products have almost invariably been traced to post-processing contamination rather than survival during processing.

To verify compliance with performance standards, FSIS supports the use of *Salmonella* cocktails containing relatively heat resistant strains and particularly those implicated in outbreaks (FSIS 2001). While these cultures are normally prepared under optimal laboratory conditions, a cocktail containing stressed cells more truly represents the physiological state of organisms that may enter products from the food processing environment, with the latter being a far better choice for thermal inactivation and other challenge studies (Johnson 2003, Juneja and Novak 2003).

Raw meat and poultry is generally perceived as an ideal growth medium for bacteria; however, almost all such contaminating microorganisms have been subjected to environmental and processing stresses. Physical, chemical and nutritional stresses can occur at various stages within the farm-to-fork continuum (Yousef and Courtney 2003). Fermentation, the addition of preservatives and the use of acid washes to decontaminate carcasses can lead to acid stress (Abee and Wouters 1999, Dickson and Siragusa 1994). Starvation stress can occur in low nutrient or nutrient competitive environments, which can include animal carcasses, food, equipment surfaces, walls, floors and water (Lou and Yousef 1996, Dickson and Frank 1993). Rapid rehydration of foods and exposure to high salt concentrations can cause osmotic injury (Van Schothorst and Maggie Duke 1984, Bremer and Krämer 2000), with exposure to certain sanitizers leading to oxidative stress (Yousef and Courtney 2003). Microorganisms inhabiting refrigerated foods are subject to cold stress. The cold shock phenomenon occurs when growing bacteria are exposed to

a sudden temperature decrease of approximately 15°C or greater (Mackey 1984, Jones et al. 1996).

Briefly shifting an organism from lower to higher temperatures within or above their normal growth range (Bunning et al. 1990, Mackey and Derrick 1986, Pagán et al. 1997, Farber and Brown 1990) can lead to heat shock. Conditions favorable for the development of heat-stressed cells include slow heating (e.g., pasteurization of egg products), slow cooking (e.g., meats and roasts), holding products on warming trays or under heat lamps, interrupted cooking cycles due to equipment failure and exposure to hot acid sprays used on carcasses (Mackey and Derrick 1987b, Murano and Pierson 1993, Bunning et al. 1990, Juneja and Novak 2003, Yousef and Courtney 2003).

Heat shock (Bunning et al. 1990, Juneja et al. 1998, Linton et al. 1992, Murano and Pierson 1993, Pagán et al. 1999), as well as starvation (Leenanon and Drake 2001, Lou and Yousef 1996, Rowe and Kirk 2000, Tolker-Nielsen and Molin 1996) and acid stress (Farber and Pagotto 1992, Leenanon and Drake 2001, Leyer and Johnson 1993, Lou and Yousef 1996), reportedly enhance the thermotolerance of *S.* Typhimurium, *Escherichia coli* O157:H7 and *L. monocytogenes*. In addition to this enhanced or acquired thermotolerance, exposure to sublethal stresses may potentiate the virulence of foodborne pathogens, as the expression of many virulence factors is dependent upon environmental cues such as low pH and elevated temperatures (Mekalanos 1992, Knøchel and Gould 1995).

Potentially enhanced survival of stressed pathogens in foods greatly impacts the reliability of current lethality standards, risk assessment, predictive models and HAACP programs in meeting food safety goals (Johnson 2003). Studies addressing the thermal

inactivation of stressed pathogens in meat products rather than laboratory broth systems are needed to better assess the adequacy of the current USDA-FSIS lethality standards. Hence, the aim of this study was to investigate the effects of heat-, cold- and starvation-stress on the thermal inactivation kinetics of *Salmonella* in comminuted turkey.

4.3 Materials and Methods

4.3.1 Bacterial Strains

The following eight strains of *Salmonella* were used: *S.* Thompson FSIS 120 (chicken isolate), *S.* Enteritidis H3527 (clinical isolate phage type 13A), *S.* Enteritidis H3502 (clinical isolate phage type 4), *S.* Typhimurium H3380 (DT104 clinical isolate), *S.* Hadar MF60404 (turkey isolate), *S.* Copenhagen 8457 (pork isolate) and *S.* Heidelberg F5038BG1 (clinical isolate), all obtained from V.K. Juneja (Agricultural Research Service, Eastern Regional Research Center, USDA-ARS, Philadelphia, PA), and *S.* Typhimurium 420, a DT104 strain isolated from a pork processing facility. All strains were maintained at –70°C in Trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol (J.T. Baker, Mallinckbrodt Baker, Inc., Phillipsburg, NJ).

4.3.2 Culture Preparation

Each culture was propagated by transferring one loopful of frozen cell suspension to 9 mL of TSB containing 0.6% yeast extract (Difco) (TSB-YE). Cultures were subjected to a minimum of two consecutive overnight transfers (37°C, 18-24 h) in TSB-YE before use.

4.3.3 Preparation of the Unshocked *Salmonella* Cocktail

An 8-strain *Salmonella* cocktail was prepared by combining 2 mL of each overnight (stationary phase) culture in a sterile 50 mL centrifuge tube (Clear Propylene, Plug Seal Cap, Corning Inc., Corning, NY), centrifuging at 10,000 rpm for 15 min at 4°C (Super T21, Sorvall® Products, L.P., Newtown, CT) and re-suspending the pellet in 1 mL 0.1% peptone (Difco) so as to contain approximately 10^{10} CFU/mL. The unshocked *Salmonella* culture, designed to serve as the control, was placed on ice a maximum of 2 h until it was inoculated into comminuted turkey.

4.3.4 Heat Shock Treatment

An 8-strain *Salmonella* cocktail was prepared by combining 3 mL of the individual overnight (stationary phase) cultures, centrifuging at 10,000 rpm for 15 min at 4°C and re-suspending the pellet in 3 mL 0.1% peptone to a concentration of approximately 10^{10} CFU/mL. Heat-injured cells were obtained using the method of Mathew and Ryser (2002) and Busch and Donnelly (1992), with minor modifications. In this procedure, 200 mL of TSB-YE (in a 2800 mL wide-mouth Fernbach flask) was tempered to 54°C in a shaking (30 rpm) water bath (Reciprocal Shaking Bath, Precision Scientific, Winchester, VA), inoculated with 2 mL (~10^{10} CFU/mL) of the *Salmonella* cocktail and heated at 54°C for 30 min.

The initial temperature for heat shock was 48°C, which was also used by Mackey and Derrick (1986, 1987a, 1987b) and Bunning et al. (1990) for *Salmonella*. However, for the individual *Salmonella* strains in the cocktail, the extent of injury observed at 48°C (6-54%), as well as at 52°C (23-68%), was decided to be insufficient for the current study (see Appendix B, Table B.1). At 56°C, a 30 min heat shock was lethal for 4 of the 8

strains. As 54°C resulted in approximately 90% injury in preliminary studies, it was this temperature that was incorporated into the heat shock procedure.

After 30 min, heat-shocked cells were immediately cooled in an ice-water bath for 3 to 5 min. Thereafter, the suspension was transferred from the Fernbach flask to a sterile 250 mL polypropylene centrifuge bottle (Sorvall® Products, L.P., Newtown, CT), pelleted by centrifugation at 10,000 rpm for 15 min at 4°C and re-suspended in 1 mL 0.1% peptone to obtain a heat-shocked cocktail containing approximately 10^9 CFU/mL. The cocktail was then placed on ice a maximum of 2 h until inoculation into comminuted turkey.

4.3.5 Cold Shock Treatment

The 8-strain *Salmonella* cocktail was prepared as previously described for heat shock, except the pellet was re-suspended in 4 mL 0.1% peptone to obtain a culture containing approximately 10^{10} CFU/mL. Cold-stressed cells were obtained by inoculating 200 mL TSB-YE (in a 500 mL Erlenmeyer flask) previously tempered to 4°C with 3 mL (~10^{10} CFU/mL) of the *Salmonella* cocktail. This cell suspension was then gently agitated for 1 min and held quiescently at 4°C for 2 h. The 2 h time period for the cold shock treatment was chosen because preliminary work showed, that by 4 h, injury had decreased for the individual *Salmonella* strains in the cocktail (see Appendix B, Table B.2).

The cold-shocked suspension was prepared for inoculation into comminuted turkey using the same method as for heat shock, producing a culture containing approximately 10^{10} CFU/mL.

4.3.6 Starvation Treatment

The method for starvation was similar to that of Dickson and Frank (1993). The 8-strain *Salmonella* cocktail was prepared as previously described for heat shock, except the pellet was re-suspended in 24 mL Butterfield's phosphate buffer (BPB), pH 7.18 (prepared according to the Bacteriological Analytical Manual (FDA 1998)). To prevent carry-over of nutrients, the culture was centrifuged again at 10,000 rpm for 15 min at 4°C and the pellet re-suspended in 3 mL BPB to obtain a cocktail containing approximately 10^{10} CFU/mL. Cells were starved by transferring 1 mL of the *Salmonella* cocktail into 99 mL BPB in a 160-mL screw-capped glass bottle, gently agitating the cell suspension for 1 min and holding it quiescently at 4°C for 10 d. The starved suspension was prepared for inoculation into comminuted turkey using the same method as previously described for heat shock, with the resulting culture containing approximately 10^9 CFU/mL.

Preliminary work showed that 5 d of starvation resulted in insufficient injury for the individual *Salmonella* strains in the cocktail and by 14 d, injury had begun to decrease from those levels observed at 10 d (see Appendix B, Table B.3). As a result, the 10 d period was incorporated into the starvation procedure. To verify that the injury observed at 10 d was due to starvation in BPB and not to the effects of low temperature, injury was also determined after 10 d at 4°C for cultures of the *Salmonella* strains grown and held in TSB-YE (see Appendix B, Table B.4).

4.3.7 Comminuted Turkey

Skinless turkey breast meat was acquired from Michigan Turkey Producers, Inc. (Wyoming, MI) and transported at 0°C to the Michigan State University Meat Laboratory. The breast meat was chopped in a bowl chopper (Hobart Manufacturing Co.,

Model 841810, Troy, OH) until the temperature increased from 0°C to 13°C. The comminuted turkey was double-bagged in polyethylene-laminated nylon pouches, vacuum packaged in approximately 30 g portions, frozen at -12°C and irradiated (12 kGy) at Iowa State University to eliminate indigenous microflora.

Triplicate samples of irradiated comminuted turkey were tested for sterility by enriching 1 g of turkey in 9 mL TSB-YE at 37°C for 18-24 h. Following incubation, three plates of Trypticase soy agar (Difco) containing 0.6 % yeast extract (TSA-YE) were inoculated by spread plating with 0.33 mL and incubated at 37°C for 18-24 h.

Fat, moisture and protein contents were determined by AOAC (1996) methods 991.36, 950.46B, and 981.1, respectively. For determination of pH, 10 g of comminuted turkey were added to 90 g of distilled water and homogenized using a Polytron homogenizer (Model PT 10/35, Brinkman Instruments, Westbury, NJ) at a speed setting of 3 for 30s. The pH of the homogenized comminuted turkey was measured with a combination electrode (Model 145, Corning, Medfield, MA). Analyses were carried out in triplicate.

4.3.8 Inoculation of Comminuted Turkey

Pouches of comminuted turkey were thawed under cold running water approximately 1 h prior to performing thermal inactivation studies. Using aseptic procedures, 0.3 mL of the unshocked or stressed *Salmonella* cocktail were added dropwise to 10 g of comminuted turkey to achieve an approximate concentration for unshocked, cold-shocked and starved cells of 10^8 CFU/g and 10^7 CFU/g for heat-shocked cells. The meat was manually mixed in a sterile beaker for 3 to 5 min using sterile

gloves. Even distribution was verified by replacing the inoculum with green food dye (McCormick and Company, Inc., Hunt Valley, MD) and plating sub-samples of the meat.

One g of inoculated meat was transferred aseptically to 5 x 25.5 cm polyethylene laminated nylon bags (Butcher and Packer Supply Co., Detroit, MI). The bags were tested for sterility by depositing 9mL TSB-YE in 10 random bags that were then incubated at 37°C for 18-24 h. Following incubation, 0.33 mL of this enrichment was spread-plated on each of three plates of TSA-YE. Background microflora were not detected.

Bags containing the meat were rolled, with a large glass test tube, between two pre-measured templates to a thickness of 1mm and then heat sealed using a soldering iron or the heat selector switch of a Freshlock Turbo II™ Premium Home-use Vacuum Sealer (Model 1830, Keystone Manufacturing Company, Inc., Buffalo, NY).

4.3.9 Thermal Inactivation

The heat-sealed bags were fit into a rack and submerged in a temperature-controlled waterbath (NESLAB Instruments, Inc., Newington, NH) set at 60.5°C to obtain an actual water temperature of 60°C (DuaLogR™ thermocouple thermometer, Cole Parmer Instrument Co., Model No. 01100-50, Vernon Hills, IL).

Thermal lag time (the time for the meat to reach within 0.5°C of the waterbath temperature) was determined by inserting a T-type thermocouple into the geometric center of a 1-g comminuted turkey sample, submerging the sample in the waterbath and logging the temperature (DuaLogR™ thermocouple thermometer, Model No. 01100-50). Following 7 trials, the lag time was determined to be 14.57 ± 1.27 s, which was adjusted

to 15 s for the thermal inactivation studies. The end of the lag time was identified as "time zero", the beginning of the inactivation time period.

4.3.10 Enumeration

Bags of comminuted turkey were removed from the 60°C waterbath at 13 or 25 s intervals and submerged in an ice-water bath. Thereafter, the cooked comminuted turkey was aseptically transferred to sterile Whirlpak™ bags (Nasco, Ft. Atkinson, WI) and manually homogenized for 1 min in 9 mL 0.1% peptone. Samples were placed in an ice-water bath and plated within 6 h. Samples removed at 25 and 13 s intervals were serially diluted in 0.1% peptone and spread plated in duplicate on TSA-YE (a medium that will recover both healthy and injured cells) and xylose lysine deoxycholate (XLD) agar (Difco) (a medium that recovers uninjured cells), respectively, with the plates counted after 18-24 h of incubation at 37°C.

Counts also were determined for each cocktail before and after stressing and after inoculation into comminuted turkey before cooking. Cells were enumerated by serial dilution in 0.1% peptone followed by spiral plating (Autoplate® 4000, Spiral Biotech, Inc., Norwood, MA) in duplicate on TSA-YE and XLD. All plates were counted after 18-24 h of incubation at 37°C.

Percent injury after the individual stress treatments and after inoculation into comminuted turkey was determined for each replication according to the formula: % injury = [(count on non-selective agar – count on selective agar)/count on non-selective agar] x 100, with the results reported as averages.

4.3.11 Data Analysis

Thermal inactivation of the unshocked *Salmonella* cocktail was tested six times, while the heat-, cold- and starve-stressed *Salmonella* cocktails were tested four times. The raw data were analyzed with an analysis of covariance model (Proc GLM, SAS® Version 8.02, SAS Institute, Inc., Cary, NC), with independent variables being treatment (heat, cold, starvation, unshocked), medium (TSA-YE, XLD), time (thermal inactivation treatment) and their interactions, where time is considered to be a continuous predictor (or covariate). For each trial, D-values (the time required to reduce the microbial population by 90%) were calculated for each treatment, using at least seven data points on TSA-YE and four on XLD, by linear regression of surviving bacteria vs. time using Microsoft Excel 2000 (Microsoft Corp, Redmond, VA). Assuming linear inactivation kinetics, the D-value is the negative reciprocal of the slope of the regression line. Results are reported as averages.

4.4 Results

4.4.1 Composition of Comminuted Turkey

The fat, moisture and protein content of comminuted turkey was 1.50 ± 0.27%, 70.46 ± 0.17% and 23.47 ± 0.61%, respectively. The pH was 5.79 ± 0.04. The background microflora in irradiated comminuted turkey was determined to be present at <1 CFU/g, with none of the enrichments showing growth.

4.4.2 Sublethal Injury

The three stress treatments produced *Salmonella* cocktails containing 89.02 ± 13.18% heat-injured, 44.71 ± 20.07% cold-injured and 67.72 ± 19.54% starvation-injured

cells, respectively. After inoculating the comminuted turkey approximately 1 h later, 88.79 ± 12.39%, 72.19 ± 7.14% and 72.35 ± 8.14% of these previously stressed cells were heat-, cold- and starve-injured, respectively. Except for cold shock, the initial injuries and the injuries after inoculation into comminuted turkey were not significantly different ($P>0.05$). All comminuted turkey samples were plated within 6 h, with injury remaining the same during this time, indicating absence of repair.

4.4.3 Thermal Resistance of *Salmonella* Cocktails

Analysis of Variance (ANOVA) results are shown in Appendix B (Table B.7). Analysis of the raw data showed a significant effect ($P<0.05$) of treatment, medium, time and the interactive effects of these factors on thermal inactivation. Thermal inactivation curves for each treatment (unshocked, heat-injured, cold-injured, starvation-injured) are presented in Figures 4.1, 4.2, 4.3 and 4.4. Thermal inactivation was carried out over a period of 175 and 91 s for samples plated on TSA-YE and XLD, respectively. TSA-YE thermal inactivation curves are plotted with all 8 data points sampled (up to 175 s), while fewer data points were available for XLD curves. By 91 s, there were no detectable (<10 CFU/g) unshocked and heat-shocked survivors on XLD, and no cold-shocked and starved survivors by 78 and 52 s, respectively. Overall, for all treatments, XLD yielded significantly lower recovery ($P<0.05$) compared to TSA-YE, indicating that the population of healthy cells alone appears less thermotolerant compared to the total population of healthy and injured cells.

For all treatments and media, the data, as evidenced by the thermal inactivation curves, are clearly non-linear. The deviations from linearity are most likely because

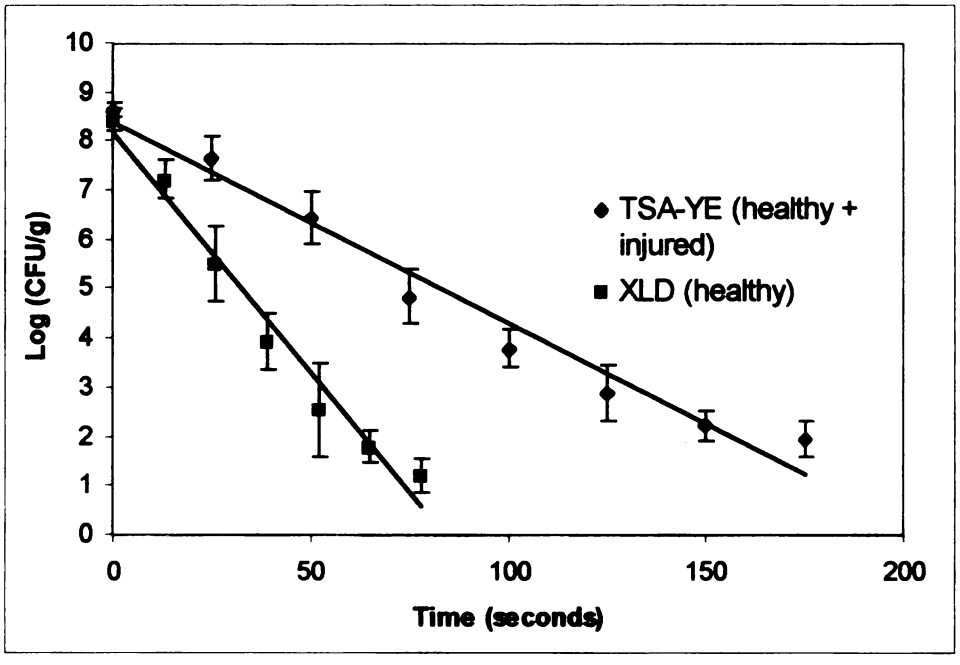

Fig. 4.1 Thermal inactivation at 60°C of the unshocked *Salmonella* cocktail. Values plotted are the arithmetic means of six trials, with standard deviations shown as error bars.

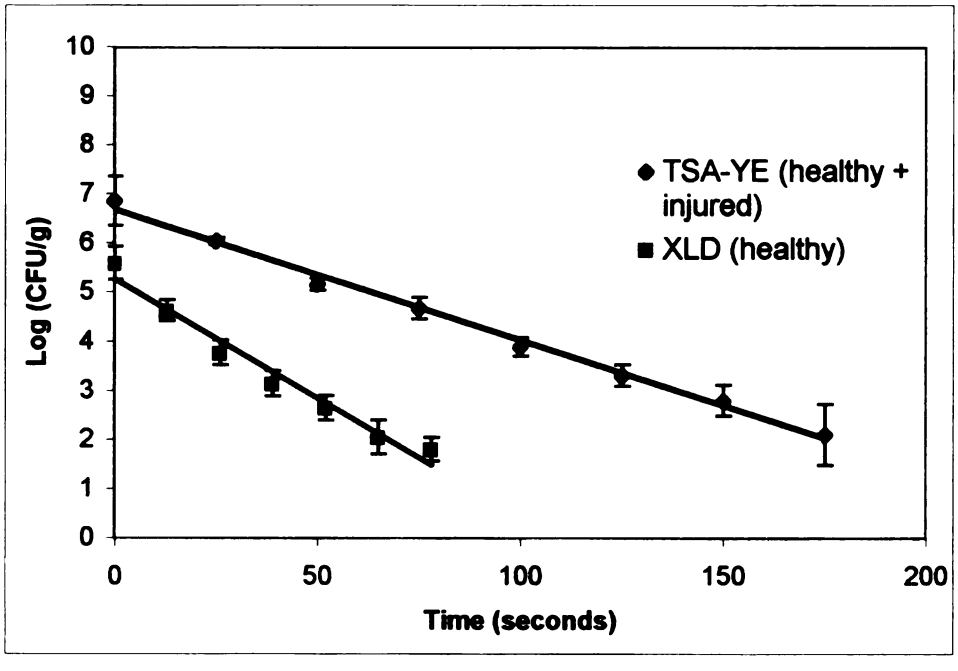

Fig. 4.2 Thermal inactivation at 60°C of the heat-shocked *Salmonella* cocktail. Values plotted are the arithmetic means of four trials, with standard deviations shown as error bars.

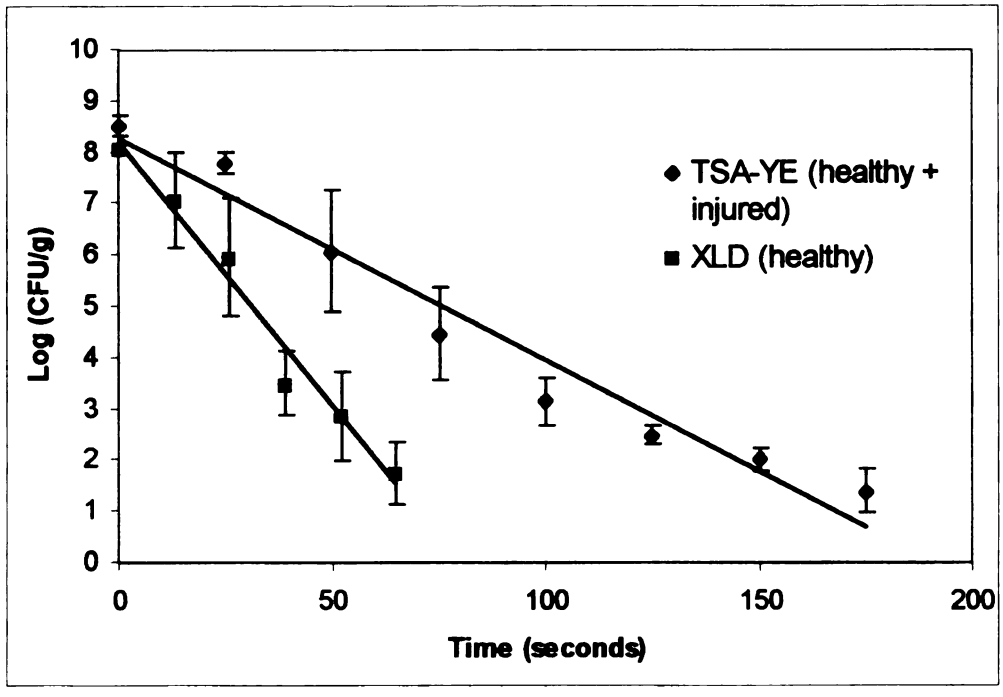

Fig. 4.3 Thermal inactivation at 60°C of the cold-shocked *Salmonella* cocktail. Values plotted are the arithmetic means of four trials, with standard deviations shown as error bars.

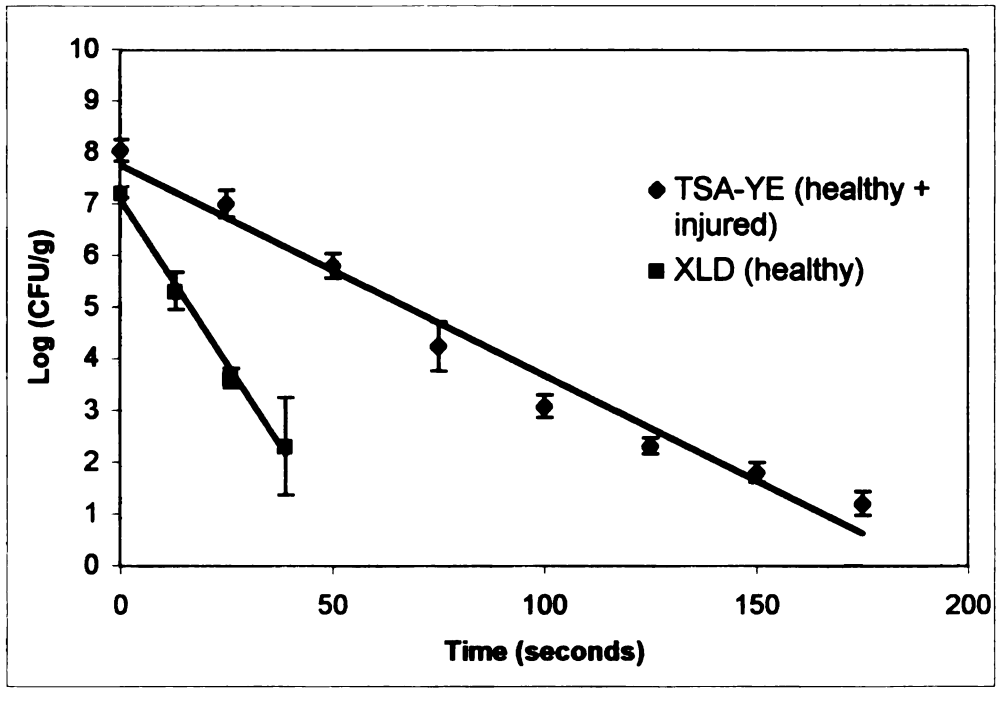

Fig. 4.4 Thermal inactivation at 60°C of the starved *Salmonella* cocktail. Values plotted are the arithmetic means of four trials, with standard deviations shown as error bars.

thermal inactivation curves represent a population of bacterial cells (including different strains), which have varying degrees of thermal resistance (Whiting 1995, Peleg and Cole 1998). However, as the emphasis was on testing and comparing treatments and there was a relatively good fit (R^2 values >0.95), the log-linear model was used for all analyses.

$D_{60°C}$-values and regression parameters are presented in Table 4.1 for TSA-YE and Table 4.2 for XLD. The differences, for each treatment, between $D_{60°C}$-values on non-selective and selective media (injury index) are shown in Table 4.3. Slopes and $D_{60°C}$-values on XLD for the heat-shocked and starved *Salmonella* cocktails were significantly different ($P<0.05$) than for the unshocked control, but not for the cold-shocked cocktail ($P>0.05$). On TSA-YE, only the heat-shocked cocktail had a significantly different slope and $D_{60°C}$-value than the unshocked cocktail (see Appendix B, Table B.8).

Table 4.1 $D_{60°C}$-values and regression parameters on TSA-YE for unshocked and heat-, cold- and starvation-stressed Salmonella.

Treatment	y-intercept	Slope	R^2	D-value (min)
Unshocked (n=6)	8.37 ± 0.33	-0.04 ± 0.002	0.97	0.41 ± 0.02
Heat (n=4)	6.67 ± 0.26	-0.03 ± 0.003	0.99	0.64 ± 0.08[a]
Cold (n=4)	8.32 ± 0.51	-0.04 ± 0.004	0.96	0.38 ± 0.03
Starvation (n=4)	7.74 ± 0.17	-0.04 ± 0.002	0.97	0.41 ± 0.02

Arithmetic Means ± Standard Deviations
[a]Significantly different (P<0.05) from the unshocked control

Table 4.2 $D_{60°C}$-values and regression parameters on XLD for unshocked and heat-, cold- and starvation-stressed *Salmonella*.

Treatment	y-intercept	Slope	R^2	D-value (min)
Unshocked (n=6)	8.25 ± 0.10	-0.10 ± 0.01	0.98	0.17 ± 0.02
Heat (n=4)	5.25 ± 0.20	-0.05 ± 0.003	0.97	0.35 ± 0.02[a]
Cold (n=4)	8.06 ± 0.52	-0.10 ± 0.01	0.98	0.17 ± 0.02
Starvation (n=4)	7.04 ± 0.14	-0.13 ± 0.02	0.99	0.14 ± 0.02[a]

Arithmetic Means ± Standard Deviations
[a]Significantly different (P<0.05) from the unshocked control

Table 4.3 Injury index[a] following thermal treatment (60°C) of *Salmonella*.

Treatment	Injury Index
Unshocked	0.24
Heat	0.29
Cold	0.21
Starvation	0.27

[a]Injury index = D-value (non-selective) – D-value (selective)

4.5 Discussion

While comparisons in comminuted turkey are limited, the $D_{60°C}$-value (on TSA-YE) reported here for the unshocked *Salmonella* cocktail (0.41 ± 0.02 min) is in close agreement with the $D_{60°C}$-value of 0.46 min for *S.* Thompson in ground beef reported by Mackey and Derrick (1987b). Similarly, Maurer (2001) reported a D-value at 61°C of 0.44 min for an 8-strain *Salmonella* cocktail containing 7 of our 8 strains (*S.* Montevideo FSIS 051 used in place of *S.* Typhimurium 420) in ground beef, and Smith (2000) reported $D_{61°C}$-values of 0.43 and 0.41 min for two strains of *S.* Typhimurium DT104 in ground beef.

Looking at $D_{60°C}$-values for all treatments, the population of healthy cells alone on XLD was less thermotolerant compared to the entire population of healthy and injured cells as enumerated on TSA-YE. On XLD, *Salmonella* was non-detectable (<10 CFU/g) by 91 s of cooking, whereas 175 s of cooking still resulted in detectable levels on TSA-YE. As this is true for unstressed as well as stressed *Salmonella*, cooking appears to injure a portion of the population, altering its resistance profile. McClements et al. (2001) reported the same phenomenon in *L. monocytogenes* using high hydrostatic pressure, a treatment known to cause sublethal injury (Wuytack et al. 2003), with an 8-log reduction on *Listeria* selective agar (Oxford formulation) after 24 min of exposure to 400 MPa compared to a 4-log reduction on TSA-YE. The shifting of cells from the healthy to injured state is not reflected when using a selective medium or a non-selective medium alone, as non-detectable cells (on selective media) are often incorrectly assumed to be dead.

Differences between D-values on non-selective and selective media have been used to assess bacterial injury following thermal stress, with similar D-values indicating no sublethal injury. Murano and Pierson (1993), who refer to this relationship as an Injury Index, found that the injury index for heat-shocked *E. coli* O157:H7 was greater than for non-heat-shocked controls, suggesting that exposure to a heat shock did not protect the cells against further thermal injury and that pre-heat shock enhanced sublethal injury. The same could be considered true for heat shock and starvation in the current work, as both have a higher injury index than the unshocked control.

The $D_{60°C}$-values for control and stressed salmonellae, on both selective and non-selective media, should also be examined within the context of the FSIS lethality performance standards. A 7-log reduction applies to poultry products, with processors either validating the lethality of their treatment or following pre-determined time/temperature requirements (safe harbor guidelines). At 60°C, the minimum processing time required to achieve a 7-log reduction is 12 min (FSIS 1999), which corresponds to a 1-log reduction in 1.71 min. For all treatments tested here, this lethality is exceeded in comminuted turkey ($D_{60°C}$-values less than 1.71 min).

Although thermal inactivation of *Salmonella* in comminuted turkey exceeded the lethality compliance guidelines, safe harbor requirements are based on the processing time after the minimum temperature is reached. They do not account for long come-up times or slow-cooking of meat products (e.g. large roasts) that could heat shock pathogens. Results presented in this study indicate that heat shock enhances the thermal resistance of *Salmonella*.

Our heat-shocked *Salmonella* had a higher $D_{60°C}$-value on XLD than did the unshocked *Salmonella*, indicating that more time is needed for the healthy fraction of the heat-shocked population to lose tolerance to selective agents (due to injury by the cooking process) compared to the primarily healthy unshocked population. As bacterial cells are assumed to go from the healthy to injured state before dying, this effect of heat shock may be the first stage in acquired thermotolerance.

Heat shock produced a significantly higher $D_{60°C}$-value on TSA-YE, indicating that a certain degree of thermal resistance was conferred on the *Salmonella* population. In experiments with *S.* Typhimurium, Bunning et al. (1990) characterized the degree of thermal resistance by the ratio of the $D_{57.8°C}$-value after heat shock to the $D_{57.8°C}$-value of the control. They found that this ratio was 1.1, 1.3 and 3.0 for *S.* Typhimurium heat-shocked in TSB-YE for 30 min at 52°C, 42°C and 48°C, respectively, before thermal inactivation. While investigating the thermotolerance of heat-shocked (48°C for 30 min) and non-heat-shocked *S.* Thompson in minced beef, Mackey and Derrick (1987b) calculated D-value ratios of 2.38 and 2.74 at 54°C and 60°C, respectively. The $D_{60°C}$-value ratio in our study was 1.56, which is in the range reported by Bunning et al. (1990) for *S.* Typhimurium, but somewhat lower than that observed for *S.* Thompson. Differences in the degree of protection afforded by heat shock may be related to the inoculum level and serotype of *Salmonella*, as well as the heat shock temperature, medium and heating menstra. Given that Mackey and Derrick (1987b) also heat-shocked *S.* Thompson directly in minced beef, the results and implications of our current work could be expanded if the *Salmonella* cocktail were heat-shocked in comminuted turkey rather than in a broth prior to inoculation.

Acquired thermotolerance following heat shock has been reported in other species. Juneja et al. (1998) sublethally heated samples of beef gravy and ground beef previously inoculated with a four-strain cocktail of *E. coli* O157:H7 at 46°C for 15 to 30 min. Heat-shocked cells were more thermotolerant than unshocked cells, with a 1.56- and 1.50-fold increase in the time to achieve a 4-log reduction at 60°C in beef gravy and ground beef, respectively. When treated at 62°C, cells of *L. monocytogenes* heat-shocked in TSB-YE at 45°C for 180 min were six-fold more heat resistant than unshocked cells (Pagán et al. 1999), with heat shocking in TSB-YE at 48°C for 10 min increasing the $D_{55°C}$-value of *L. monocytogenes* more than two-fold (Linton et al. 1992).

Stress responses, including general and heat shock specific responses, may prepare cells for additional thermal stress with the synthesis of heat shock proteins (HSPs). Many HSPs are chaperones that serve primarily to bind to and stabilize proteins, monitor protein folding to prevent misfolding or aggregation of proteins and re-fold improperly folded or denatured proteins (Juneja and Novak 2003). However, the presence of HSPs does not always correlate with acquired thermotolerance (Watson 1990). Heat shock may also act by enhancing repair (including DNA repair) mechanisms of cells (Murano and Pierson 1993) and promoting the accumulation of osmolytes such as trehalose, which may stabilize proteins, enzymes and membranes (Abee and Wouters 1999, Rowbury 2003).

Starvation-stressed cells also had a higher injury index than did unstressed cells. However, in contrast to heat shock, starvation did not increase the $D_{60°C}$-values on TSA-YE or XLD. In fact, the $D_{60°C}$-value on XLD was significantly lower for starved *Salmonella* than for the control, suggesting that the healthy fraction of the starved

population is more readily injured during cooking compared to the primarily healthy unshocked population. The significance of this observation is not yet clear, because the $D_{60°C}$-value of the entire population on TSA-YE was unaffected by starvation, indicating acquired thermotolerance was not a consequence of the given starvation treatment.

L. monocytogenes starved in phosphate buffer for 156 h at 30°C exhibited a 13-fold increase in the $D_{56°C}$-value on non-selective media, with starvation apparently protecting injured cells from the lethal effect of extended heating (Lou and Yousef 1996). Leenanon and Drake (2001) reported $D_{56°C}$-values for starved (0.85% NaCl, 48 h, 37°C) and non-starved *E. coli* O157:H7 of 9.7 and 7.1 min, respectively. Differences in the protective effect of starvation may be due to the length of starvation. Maximum thermal resistance for *L. monocytogenes* was achieved between 138 and 156 h of starvation (Lou and Yousef 1996), while starvation for 4 h provided maximum thermal resistance for *E. coli* (Jenkins et al. 1988). In our work, 10 d may have exceeded the starvation period for maximum thermal resistance in *Salmonella*, with Smith (2000) reporting that starvation in peptone water for 14 d (4°C) actually reduced thermal resistance of *Salmonella* at 55°C, 58°C, 61°C and 63°C in ground beef.

Additionally, our overnight cultures were in stationary phase, the phase that predominates in natural settings (Rowe and Kirk 2000). The stationary phase is accompanied by a general state of stress (including heat) resistance (Matin 1991, Givskov et al. 1994, Hengge-Aronis 1993, Lazazzera 2000), which may be due to the synthesis of protective proteins that facilitate resistance to chemical and physical challenges (Rowe and Kirk 2000). These proteins include enzymes for trehalose synthesis and uptake, H_2O_2 degradation, DNA repair and glycogen synthesis (Rowbury 2003).

Inability to detect an increase in thermal resistance in our starved cocktail may be due to a thermal protective effect from the stationary phase control culture. The significantly increased $D_{56°C}$-value reported by Lou and Yousef (1996) for starved *L. monocytogenes* was based on a comparison to control cells in the exponential phase. While Tolker-Nielsen and Molin (1996) observed a significant increase in heat resistance with starved *S.* Typhimurium, they also were using exponential phase control cells. While, some authors have used stationary phase control cells, the results have been variable. Rowe and Kirk (2000) reported enhanced thermotolerance in two of three starved strains of *E. coli* O157:H7, and Bang and Drake (2002) reported a significant increase in the $D_{47°C}$-value of only one of three strains of *Vibrio vulnificus*.

In our work, similar $D_{60°C}$-values were obtained on TSA-YE and XLD for both the cold-shocked and control cocktails. However, reports of decreased heat resistance with cold stress can be found in the literature. Leenanon and Drake (2001) cold-stressed (TSB, 1 week, 5°C) *E. coli* O157:H7 and non-pathogenic *E. coli* and reported $D_{56°C}$-values of 6.4 and 5.1 min, respectively, compared to 6.8 and 5.5 min for the non-cold-stressed controls. Additionally, Miller et al. (2000) observed a 25% reduction in the $D_{60°C}$-value for *L. monocytogenes* inoculated onto frankfurter skins following 3 h of cold shocking at 0°C. A 15% reduction was observed when *L. monocytogenes* was inoculated into UHT milk and cold shocked. Storing homogenized whole egg at 4°C for as little as 4 h significantly increased the thermal sensitivity of *S.* Enteritidis at 60°C (Humphrey 1990), with maximum thermal sensitivity achieved within 24 h. In the case of the current work, extending the cold shock period beyond 2 h might also result in enhanced thermal sensitivity.

While the mechanism of thermal sensitivity is unknown, alterations in membrane permeability, reductions in ATP levels and RNA damage have been proposed (Humphrey 1990). Given that no thermal protection was afforded by cold shock in our work and the apparent thermal sensitivity reported by other authors, cold shock (e.g., refrigeration) prior to heating may be an effective pathogen intervention strategy (Miller et al. 2000). Heat sensitive cells of *S.* Enteritidis rapidly regain heat resistance when incubated at 37°C, with their recovery far less rapid at 20°C (Humphrey 1990). Thermal sensitivity in *L. monocytogenes* is sustained even if cells are shifted to 28°C for 3 h before heating (Miller et al. 2000). Therefore, it seems unlikely that brief exposure to ambient temperatures between refrigeration and thermal challenge would affect the heat resistance of sensitized cells.

The cold shock response and its relationship to other stresses are complex. Cold stress can reportedly enhance freeze and freeze/thaw resistance (Jeffreys et al. 1998, Bollman et al. 2001, Bang and Drake 2002, Leenanon and Drake 2001), and exposure to refrigeration temperatures may also maintain the heat shock response and its associated thermotolerance. Heat-shocked (48°C, 30 min) cells of *L. monocytogenes* shifted to 4°C retained their thermotolerance in a sausage mix at least 24 h after initial heat shock (Farber and Brown 1990). While thermotolerance was lost for heat-shocked (46°C, 15-30 min) *E. coli* O157:H7 after storage at 4°C for 14 h, it was sustained after at least 24 h of storage at 15°C or 28°C (Juneja et al. 1998). Bunning et al. (1990) reported that the $D_{57.8°C}$-value for *L. monocytogenes* heat-shocked at 48°C returned to control levels within 1 h when the cells were shifted to 35°C, while the elevated heat resistance reported by Mackey and Derrick (1986) for heat-shocked (42°C, 45°C or 48°C) *S.* Typhimurium

persisted for at least 10 h of incubation at the heat shock temperatures. Duration of thermotolerance is likely impacted by the temperatures for growth, heat shock and post-heat shock storage. The acquisition of thermotolerance associated with heat shock as observed in our study should be further investigated to identify conditions that allow it to persist and those that do not, with the latter used to develop pathogen intervention strategies applicable in thermally processed foods.

4.6 Conclusion

Only heat shock resulted in elevated thermotolerance of the *Salmonella* cocktail. Starvation did not protect against heat, although the physiological state of the control culture may have affected the comparisons. Because starvation results in cells entering the stationary phase, thermal resistance of the starved culture did not differ significantly from the stationary phase control cells. Cold shock also did not impact thermal resistance, and further studies will indicate whether different cold shock treatments can actually sensitize *Salmonella* to thermal challenges. Given the outcome with heat shock, great care should be taken to include the product history and physiological state of *Salmonella* when conducting thermal inactivation and challenge studies and when developing guidelines for thermal processing. Also, non-selective culture media should be used to assess the response of microorganisms to physical and chemical challenges, as selective media can underestimate the number of cells remaining in a population. This is especially important, as the sublethally injured organisms that do not proliferate on selective media still retain their pathogenicity.

CHAPTER 5

CONCLUSIONS AND FUTURE RECOMMENDATIONS

The safety of thermally and otherwise processed foods is based on the detection of all pathogenic organisms, including those sublethally injured cells that retain their pathogenicity. Recovery of injured organisms is often highly sensitive to factors that do not affect, to the same degree, the recovery of uninjured organisms, especially oxidative stress. Several studies have successfully enhanced recovery of sublethally injured microorganisms by supplementing various media with reducing agents, O_2 scavengers and/or H_2O_2-degrading agents. However, our work showed that TSA-YE with 0.1% or 0.6% sodium pyruvate or Oxyrase® was no more effective than unsupplemented TSA-YE at recovering heat-, cold- or starvation-stressed *Salmonella*.

The effects of toxic oxygen species and neutralizing supplements on the recovery of injured microorganisms are complex. A number of factors can affect the outcome, including the type of supplement and the concentration at which it is used. As such, the effectiveness of other concentrations or other reducing agents or oxygen-scavengers warrants further investigation.

Physical, chemical and nutritional stresses can occur at various stages within the farm-to-fork continuum, and exposing *Salmonella* to various stresses in the meat processing environment can induce sublethal injury and stress response mechanisms that may alter the microorganism's thermotolerance during cooking. Although starvation and cold shock did not protect against heat, thermotolerance of *Salmonella* was elevated by heat shock, making it important for the product history and physiological state of

Salmonella to be considered when developing and validating thermal processes. In addition, different inactivation kinetics were observed on non-selective and selective media. As a result, non-selective culture media should be used to assess the response of microorganisms to physical and chemical challenges, as selective media can underestimate the number of cells remaining in a population. This is especially important, as the sublethally injured organisms that do not proliferate on selective media still retain their pathogenicity.

The duration of thermotolerance may depend on a number of factors, including the growth temperature, the heat shock temperature and the post-heat shock storage temperature. The acquired thermotolerance associated with heat shock in this study should be investigated in more detail to identify conditions that allow it to persist and those that do not. The latter could be used to develop pathogen control strategies applicable to a food processing environment. One of these control strategies may be cold storage. While cold shock did not affect the thermal resistance of *Salmonella* in this study, other authors have indicated that it may increase the thermal sensitivity of bacterial cells. Therefore, further studies should be undertaken to identify different cold shock treatments that may sensitize the *Salmonella* cocktail to a thermal challenge.

The differential plating results indicate that repair of sublethal injury did not occur during the time between the stress treatments and inoculation into ground turkey or plating of the uncooked control samples (~ 6 h in ice-water). Additional research on the timeframe (and conditions) required for optimal repair of sublethal injury should be conducted, as repair of injury will correlate with proliferation of bacteria within a food

product. The relationship between repair time and the duration of enhanced thermotolerance would also be of interest.

Finally, as there were significant differences in thermal inactivation observed on non-selective and selective media, models and D-values developed with a selective medium should be identified and compared to results obtained using a non-selective medium.

APPENDIX A

DEVELOPMENT OF A NOVEL GRADIENT PLATE METHOD FOR ASSESSING SUBLETHAL INJURY

A.1 Abstract

Currently, assessment of sublethal injury in a bacterial population following preservation treatments relies on standard differential plating with non-selective and selective media, a method that is both time and labor-intensive. This study determined the effectiveness of two novel gradient plates, poured with either selective BSA or XLD as the bottom layer and non-selective TSA-YE as the top layer, for determining sublethal injury. The diffusion of selective components into the non-selective medium affected the ability of the gradient plates to recover heat-injured *Salmonella*. Because of this, it was determined that the gradient plates were inappropriate in the quantitative assessment of injury. However, the gradient plates did recover a range of cells across the plate and the technique is a viable one-plate screening method for qualitatively identifying injury.

A.2 Introduction

Heat treatment is the most widely applied method of food preservation (Wuytack et al. 2003), with many foods dependent upon thermal processing for their stability and safety. Temperatures and treatments meant to be lethal will not necessarily destroy all microorganisms, as a potentially significant number of cells actually survive a given heat process and are merely injured (Murano and Pierson 1993). Injured cells are considered capable of causing disease if ingested by susceptible individuals (Reissbrodt et al. 2002).

Heat-injured bacteria exhibit increased sensitivity to selective agents such as bile salts, tellurite, sodium desoxycholate, bismuth sulfite, sodium chloride, crystal violet and brilliant green (Ray 2001). Because of this characteristic, growth on selective media is the primary method of assessing sublethal injury (Hurst 1984, Gilbert 1984, Czechowicz et al. 1996, Semanchek and Golden 1998, Brashears et al. 2001). The difference in plate counts between selective and non-selective media is used to quantify sublethal injury as a proportion or percentage of the entire population (Kang and Siragusa 1999, Semanchek and Golden 1998, Baylis et al. 2000a, Dickson and Frank 1993, Restaino et al. 1980, Brashears et al. 2001).

Gradient plates have been used to identify bacterial mutagens (Rexroat et al. 1995) and antibiotic resistant mutants, with mutants appearing in areas of higher antibiotic concentration. Two-dimensional gradient plates have been used to characterize the simultaneous bacterial growth response to two different environmental parameters, such as pH and NaCl (Caldwell and Hirsch 1973, Wimpenny and Waters 1984, Thomas et al. 1992). Use of the gradient plate technique to characterize injured bacterial populations is limited to one report in which the growth limits for heat-injured *Brochothrix thermosphatica* were assessed on two-dimensional pH/NaCl gradient plates using image analysis (Rattanasomboon et al. 2001).

The present study tested the ability of gradient plates, prepared with non-selective and selective media, to quantify thermal injury in *Salmonella*. Since the bacterial population may be best represented as a continuum of cells ranging from mildly to severely injured, along with potentially unaffected and dead cells following heat

treatment (Hurst 1984, Mackey 2000, Stephens et al. 1997), the gradient plates were also examined for their ability to characterize this range and distribution of injury.

A.3 Materials and Methods

A.3.1 Bacterial Strains

The following eight strains of *Salmonella* were used: *S.* Thompson FSIS 120 (chicken isolate), *S.* Enteritidis H3527 (clinical isolate phage type 13A), *S.* Enteritidis H3502 (clinical isolate phage type 4), *S.* Typhimurium H3380 (DT104 clinical isolate), *S.* Hadar MF60404 (turkey isolate), *S.* Copenhagen 8457 (pork isolate) and *S.* Heidelberg F5038BG1 (clinical isolate), all obtained from V.K. Juneja (Agricultural Research Service, Eastern Regional Research Center, USDA-ARS, Philadelphia, PA) and *S.* Typhimurium 420, a DT104 strain isolated from a pork processing facility. All strains were maintained at –70°C in Trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol (J.T. Baker, Mallinckbrodt Baker, Inc., Phillipsburg, NJ).

A.3.2 Culture Preparation

Each culture was propagated by transferring one loopful of the frozen cell suspension into 9 mL of TSB containing 0.6% yeast extract (TSB-YE) (Difco). Cultures were subjected to a minimum of two consecutive overnight transfers (37°C, 18-24 h) in TSB-YE before use.

A.3.3 Heat Shock Treatment

An 8-strain *Salmonella* cocktail was prepared by combining 3 mL of the individual overnight cultures in a sterile 50 mL centrifuge tube (Clear Propylene, Plug

Seal Cap, Corning Inc., Corning, NY), centrifuging at 10,000 rpm for 15 min at 4°C (Super T21, Sorvall® Products, L.P., Newtown, CT) and re-suspending the pellet in 3 mL of 0.1% peptone (Difco) to obtain a culture containing approximately 10^{10} CFU/mL. Heat-injured cells were obtained using the method of Mathew and Ryser (2002) and Busch and Donnelly (1992), with minor modifications. In this procedure, 200 mL of TSB-YE (in a 2800 mL wide-mouth Fernbach flask) was tempered to 54°C in a shaking (30 rpm) water bath (Reciprocal Shaking Bath, Precision Scientific, Winchester, VA), inoculated with 2 mL (~10^{10} CFU/mL) of the *Salmonella* cocktail and heated at 54°C for 30 min. After 30 min, heat-shocked cells were immediately cooled in an ice-water bath for 3 to 5 min.

The initial temperature for heat shock was 48°C, which was also used by Mackey and Derrick (1986, 1987a, 1987b) and Bunning et al. (1990) for *Salmonella.* However, for the individual *Salmonella* strains in the cocktail, the extent of injury observed at 48°C (6-54%), as well as at 52°C (23-68%), was decided to be insufficient for the current study (see Appendix B, Table B.1). At 56°C, a 30 min heat shock was lethal for 4 of the 8 strains. As 54°C resulted in approximately 90% injury in preliminary studies, it was this temperature that was incorporated into the heat shock procedure.

A.3.4 Gradient Plate Production

Gradient plates were prepared in 100 x 100 x 15-mm square petri dishes (Integrid, BDFalcon, Franklin Lakes, NJ) divided into an equal 6 x 6 grid (Figure A.1), with numerical columns and alphabetical rows. The following media were used: Trypticase soy agar (Difco) containing 0.6% yeast extract (TSA-YE), bismuth sulfite agar (Difco) (BSA) and xylose lysine deoxycholate (XLD) agar (Difco). For the bottom layer, 25 mL

of either XLD or BSA were pipetted into the petri dishes. The plates were elevated to a height of 0.6 cm (creating an angle with the bench of ~ 4°) (Figure A.2) and the agar allowed to harden (~15 min). After solidification, the plates were placed flat and 25 mL of non-selective TSA-YE were pipetted over the selective medium (Figure A.3). The plates were allowed to harden, stored overnight and then dried for 30 min before use.

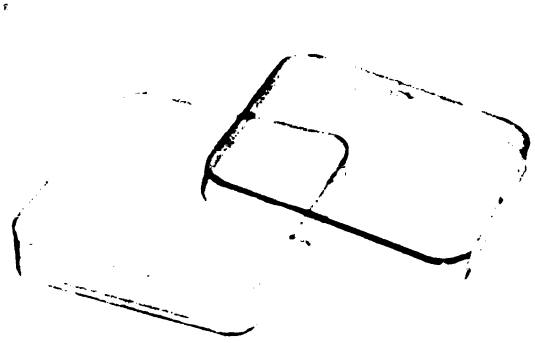

Fig. A.1 Integrid 100 x 100 x 15 mm square plates with 6 x 6 grid.

Fig. A.2 Preparation of the bottom (selective) layer of gradient plates.
* Angle measurement: ~ 4°

Fig. A.3 Completed gradient plate, with a selective (bottom) and non-selective (top) layer.

A.3.5 Recovery of Cells

Before and after heat stressing, the cells were enumerated by serial dilution in 0.1% peptone followed by spiral plating (Autoplate® 4000, Spiral Biotech, Inc., Norwood, MA) in duplicate on TSA-YE, XLD and BSA in standard 100 x 15-mm round petri dishes. Plates were counted after 18 to 24 h of incubation at 37°C. Counts on the non-selective medium (TSA-YE) represent the total viable population, while those on the selective media represent the uninjured fraction. Percent injury following heat treatment was determined according to the formula: % injury = [(count on non-selective agar – count on selective agar)/count on non-selective agar] x 100. Percent injury was calculated for each replication, with the results reported as averages.

Cells were also recovered before and after heat stressing by spread plating in duplicate on XLD/TSA-YE and BSA/TSA-YE gradient plates. Plates were counted after 18 to 24 h of incubation at 37°C. Populations recovered on the entire plate and in the six vertical columns representing the six different gradations of selectivity were calculated, the latter according to the formula: CFU/mL = [(# of colonies in column)(dilution factor)] ÷ 0.022. The denominator is the assumed volume deposited within each column, or 1/6 of the entire volume (0.13 mL) spread on the gradient plate. With selectivity decreasing across the plate, column 1 is assumed to be equal in selectivity to the selective medium and column 6 to the non-selective medium. Percent injury on the gradient plates was calculated as follows: % injury = [(count in column 6 – count in column 1)/count in column 6] x 100. Percent injury was calculated for each replication, with the results reported as averages.

A.3.6 Data Analysis

All experiments were repeated six times, with the results reported as averages. An unpaired Student's t-test (Proc TTEST, SAS® Version 8.02, SAS Institute, Inc., Cary, NC) was used to compare column 1 and column 6 data to the selective and non-selective media, respectively. A one-way Analysis of Variance (ANOVA) (Proc GLM, SAS® Version 8.02, SAS Institute, Inc., Cary, NC) was used for all other analyses.

A.4 Results

A.4.1 Sublethal Injury

Based on traditional differential plating on TSA-YE and XLD or BSA, 93.48 ± 6.61% and 76.72 ± 27.47% of the original *Salmonella* cocktail was sublethally injured, respectively. When determined by gradient plating, injury on XLD/TSA-YE and BSA/TSA-YE gradient plates was 65.62 ± 15.29% and 46.53 ± 16.95%, respectively. The two XLD injuries are significantly ($P<0.05$) different, while the two BSA injuries are not ($P=0.045$). The BSA injury results were affected by a potential outlier from standard differential plating (only 24.70% injury as compared to 94.50%, 98.10%, 93.20%, 77.80% and 72% for the other trials), which is likely the cause of the large standard deviation (27.47%). When this outlier is excluded, the BSA injuries also become significantly different.

A.4.2 XLD/TSA-YE Gradient Plates

Before heat shock, no significant ($P>0.05$) differences were seen between the numbers of *Salmonella* recovered on TSA-YE and column 6 (non-selective) of the gradient plates or between XLD and column 1 (selective) of the gradient plates (Table

A.1). After heat shock, standard XLD plates and column 1 did not differ, while standard TSA-YE plates and column 6 were significantly different ($P<0.05$). The averages for each individual column of the gradient plates are presented in Table A.2. While initial counts did not differ from column 1 to column 6, post-heat shock counts gradually increased across the plate (Figure A.4).

A.4.3 BSA/TSA-YE Gradient Plates

Both before and after heat shock, the numbers of *Salmonella* recovered on TSA-YE and column 6 were not significantly different ($P>0.05$), as was also true of BSA and column 1 (Table A.3). As with the analysis of BSA injury, if the potential outlier is removed, TSA-YE and column 6 counts after heat shock are significantly different ($P<0.05$) (Table A.4). The data for each individual column of the gradient plates are found in Table A.5. Initial *Salmonella* levels in each column were the same, while those following heat shock were not (Figure A.5).

Table A.1 Growth of *Salmonella* on standard TSA-YE and XLD plates and in selected columns of XLD/TSA-YE gradient plates.

	Initial	Post-Heat Shock
TSA-YE (NS)	10.74 ± 0.41	9.51 ± 0.22[a]
XLD (S)	10.71 ± 0.40	8.14 ± 0.35
Column 1 (S)	10.25 ± 0.40	8.23 ± 0.48
Column 6 (NS)	10.38 ± 0.36	8.74 ± 0.31[b]

NS-non-selective, S-selective
Arithmetic Means [Log (CFU/mL)] ± Standard Deviations, n=6
Means for the same treatment and medium selectivity with different superscripts are significantly ($P<0.05$) different.

Table A.2 Recovery of *Salmonella* on XLD/TSA-YE gradient plates

	Initial	Post-Heat Shock
Column 1 (S)	10.25 ± 0.40	8.23 ± 0.48
Column 2	10.09 ± 0.39	8.22 ± 0.42
Column 3	10.13 ± 0.37	8.23 ± 0.48
Column 4	10.08 ± 0.41	8.40 ± 0.46
Column 5	10.22 ± 0.40	8.53 ± 0.34
Column 6 (NS)	10.38 ± 0.36	8.74 ± 0.31

NS-non-selective, S-selective
Arithmetic Means [Log (CFU/mL)] ± Standard Deviations, n=6

Table A.3 Growth of *Salmonella* on standard TSA-YE and BSA plates and in selected columns of BSA/TSA-YE gradient plates.

	Initial	Post-Heat Shock
TSA-YE (NS)	10.74 ± 0.41	9.51 ± 0.22
BSA (S)	10.74 ± 0.41	8.60 ± 0.44
Column 1 (S)	10.53 ± 0.26	9.02 ± 0.17
Column 6 (NS)	10.50 ± 0.35	9.29 ± 0.07

NS-non-selective, S-selective
Arithmetic Means [Log (CFU/mL)] ± Standard Deviations, n=6
Means for the same treatment and medium selectivity with different superscripts are significantly ($P<0.05$) different.

Table A.4 Growth of *Salmonella* on standard TSA-YE and BSA plates and in selected columns of BSA/TSA-YE gradient plates.

	Initial	Post-Heat Shock
TSA-YE (NS)	10.78 ± 0.44	9.60 ± 0.06[a]
BSA (S)	10.78 ± 0.44	8.53 ± 0.46
Column 1 (S)	10.55 ± 0.29	9.02 ± 0.19
Column 6 (NS)	10.53 ± 0.38	9.31 ± 0.06[b]

NS-non-selective, S-selective
Arithmetic Means [Log (CFU/mL)] ± Standard Deviations, n=5
Means for the same treatment and medium selectivity with different superscripts are significantly ($P<0.05$) different.

Table A.5 Recovery of *Salmonella* on BSA/TSA-YE gradient plates

	Initial	Post-Heat Shock
Column 1 (S)	10.53 ± 0.26	9.02 ± 0.17
Column 2	10.45 ± 0.33	8.89 ± 0.22
Column 3	10.17 ± 0.48	8.88 ± 0.21
Column 4	10.18 ± 0.38	8.93 ± 0.22
Column 5	10.42 ± 0.37	9.10 ± 0.20
Column 6 (NS)	10.50 ± 0.35	9.29 ± 0.07

NS-non-selective, S-selective
Arithmetic Means [Log (CFU/mL)] ± Standard Deviations, n=6

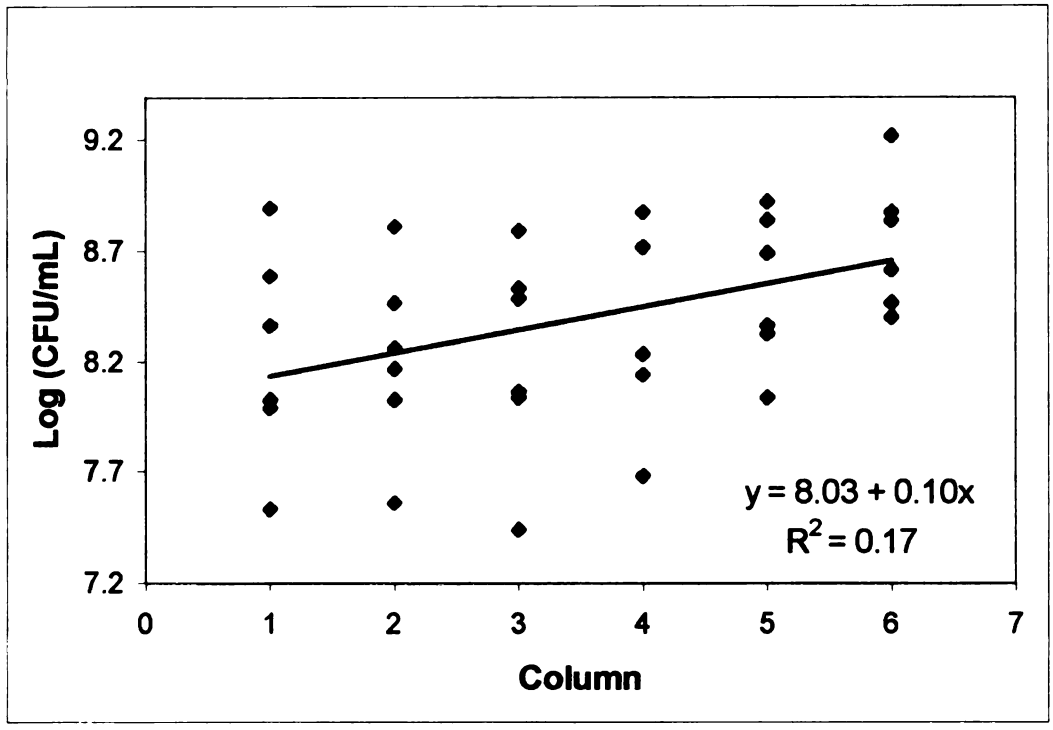

Fig. A.4 Recovery on XLD/TSA-YE gradient plates following heat shock.

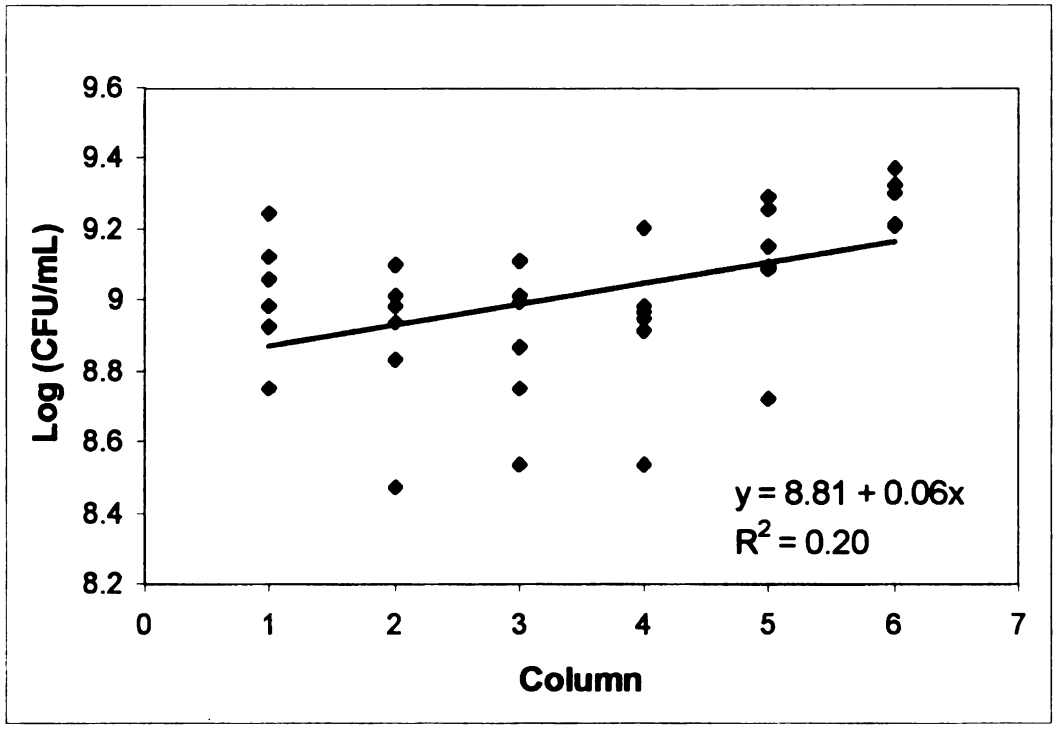

Fig. A.5 Recovery on BSA/TSA-YE gradient plates following heat shock.

A.5 Discussion

Analysis of the XLD/TSA-YE gradient plates was straightforward, while analysis of the BSA/TSA-YE gradient plates considers the exclusion of the potential outlier associated with standard BSA plates. The presence of this outlier, and the overall variability associated with BSA injury, is likely due to the medium itself. Additional measurements of injury on BSA and XLD following heat shock were compared (see Appendix B, Table B.9). From this analysis, it is apparent that the range of injuries (and standard deviation) associated with BSA is much larger than for XLD and the average injury is lower.

Moriñigo et al. (1989) also observed greater injury with XLD following seawater stress, reporting a 50-60% reduction in *Salmonella* levels on XLD and only a 25% reduction on BSA. Of the different media (BSA, XLD, brilliant green agar, brilliant green-phenol red-lactose sucrose-agar, eosin-methylene blue agar, Hektoen enteric agar, Salmonella-Shigella agar, tryptic soy-brilliant green agar and tryptic soy-xylose-lysine agar) tested, Moriñigo et al. (1989) found that XLD and brilliant green-phenol red-lactose-sucrose agars were the most selective for *Salmonella*, with the selective agents in XLD perhaps being more inhibitory to heat-injured *Salmonella* than those in BSA. In addition, problems with preparation, storage and aging of BSA before use can affect selectivity and overall performance (Warburton et al. 1994).

Recovery of fewer salmonellae after heat treatment in column 6 (non-selective) of the XLD/TSA-YE and BSA/TSA-YE gradient plates (n=5) than on standard TSA-YE plates suggests a problem with diffusion of the selective media. Diffusion of the selective agents in XLD and BSA apparently inhibited heat-injured *Salmonella* that were

able to recover on TSA-YE plates. The selective components would not be expected to affect healthy cells, which explains why diffusion was not a problem for initial plate counts.

The gradient plates could be improved by replacing XLD and BSA with a selective medium that would allow measurement of the gradient, with the relationship of concentration as a function of distance along the plate. However, even plates with a pre-determined gradient are affected by diffusion and must be used in a timely manner. A major limitation of the gradient plate technique is that experiments must be performed over a relatively short period of time due to the diffusive breakdown of the gradients (Rattanasomboon et al. 2001, McClure et al. 1989).

Because of problems with diffusion and the inability to measure a true selective gradient, the gradient plates developed here cannot replace standard differential plating for the quantitative assessment of bacterial injury. However, these plates show potential for qualitative assessment of sublethal injury. According to the statistical analysis, initially all six columns across the BSA/TSA-YE and XLD/TSA-YE gradient plates did not differ significantly. However, following the heat treatment, the six columns on each plate were not the same. The slope of the regression line for both types of gradient plates was >1, indicating a general trend of increasing salmonellae numbers across the plates (Figures A.4 and A.5).

The numerical value of the slope may prove useful as an estimation of the degree of injury sustained by a population. However, the graphical representation, with a slope >1, of bacteria across the gradient plate can be used to visualize the distribution of cells, with a larger number of injured cells able to grow as one proceeds from column 1 to

column 6, with the additional bacteria recovered in each column hypothetically more injured than those in the previous columns. If the gradient plates were prepared with a measurable gradient (e.g., NaCl) and used before the diffusive breakdown of that gradient, the ability to proliferate in the presence of a certain concentration (or column) could be used to classify and/or quantify injury. Subtracting the total number of colonies in previous columns would give the number of injured cells in a given column.

Perhaps the most promising aspect of the gradient plates is the possibility for the development of a screening assay for sublethal injury. The six columns yielded similar results before heat shock but not after, generally exhibiting a gradual increase instead. Although this method requires some refinement, the observation of a slope >1 can be used as a simple indicator of injury. If injury is identified, further analyses (e.g., standard differential plating, protein analysis, thermal inactivation studies to assess cross-protection) can be conducted to quantify and characterize this injury. Such gradient plates may be especially useful for determining the potential for non-thermal and non-traditional (e.g., pulsed electric field) processing methods to cause sublethal injury. For example, using differential plating with TSA and three selective media (TSA with 3% NaCl, TSA adjusted to pH 5.5 and violet red bile glucose agar), Wuytack et al. (2003) found that high-pressure homogenization and pulsed electric field treatments caused little or no injury. Confirmation of such results using the gradient plate technique would conserve both time and resources.

A.6 Conclusion

While the current XLD/TSA-YE and BSA/TSA-YE gradient plates cannot yet replace standard differential plating for quantitative assessment of bacterial injury, these plates do show potential as a screening method to identify sublethal injury in a bacterial population. In addition, if advancements are made to the technique, including the preparation of plates with a measurable gradient, the method could be used to quantify or classify injury. Sublethal injury has many consequences, such as cross-protection against other, more severe stresses and the potential for increased virulence of injured cells. The ability of a single-plate method to identify injured populations could become a crucial first step in characterizing the bacterial response to microbial reduction strategies and preservation treatments. Based on the qualitative identification of injury, future efforts should address environmental conditions that protect injured cells and processing parameters that control injury and lethality.

APPENDIX B

SUPPLEMENTARY DATA AND STATISTICS

Table B.1 Preliminary heat shock data comparing percent injury (on BSA) at different temperatures.

Salmonella Strain	48°C (n=2)[a]	52°C (n=2)[a]	54°C (n=3)[ab]	56°C (n=1)
S. Typhimurium 420		61.20	91.93	ND[c]
S. Thompson FSIS 120	23.20	67.60	91.13	59.60
S. Enteritidis H3527	6.13	66.45	90.80	98.40
S. Enteritidis H3502	42.05	58.25	93.53	88.40
S. Typhimurium H3380	54.05	56.22	91.43	100
S. Hadar MF60404	35.82	35.90	88.67	90.90
S. Copenhagen 8457	42.10	49.30	92.47	100
S. Montevideo FSIS 051	100	98.70	ND[c]	100
S. Heidelberg F5038BG1	16.05	22.60	91.40	100

[a]Arithmetic mean
[b]For at least three trials, *S.* Montevideo FSIS 051 was unable to grow on BSA, even if not heat-treated (i.e., plated after being grown overnight at 37°C). As a result, this strain was replaced with *S.* Typhimurium 420, a DT104 environmental isolate.
[c]ND-not determined

Table B.2 Preliminary cold shock (4°C) data comparing percent injury (on BSA) at different time intervals.

Salmonella Strain	30 minutes	2 hours	4 hours
S. Typhimurium 420	29.23 ± 12.81	75.90 ± 2.72	30.97 ± 18.00
S. Thompson FSIS 120	61.73 ± 19.90	85.10 ± 12.18	43.53 ± 22.70
S. Enteritidis H3527	45.10 ± 32.57	76.90 ± 4.17	38.90 ± 20.14
S. Enteritidis H3502	55.90 ± 19.32	74.87 ± 8.28	38.23 ± 16.77
S. Typhimurium H3380	54.63 ± 16.72	81.83 ± 3.43	18.93 ± 19.36
S. Hadar MF60404	52.40 ± 20.65	73.20 ± 6.51	19.23 ± 13.40
S. Copenhagen 8457	53.00 ± 18.76	79.33 ± 1.16	16.23 ± 4.09
S. Heidelberg F5038BG1	44.60 ± 11.13	79.47 ± 1.86	58.17 ± 2.60

Percentages are arithmetic mean ± standard deviation for n=3.

Table B.3 Preliminary starvation data comparing percent injury (on BSA) at different time intervals.

Salmonella Strain	5 days (n=1)	10 days (n=6)[ab]	14 days (n=1)
S. Typhimurium 420	ND[c]	84.15	ND[c]
S. Thompson FSIS 120	0	84.85	74.90
S. Enteritidis H3527	0	83.77	55.50
S. Enteritidis H3502	18.06	79.58	74.70
S. Typhimurium H3380	0	79.31	27.10
S. Hadar MF60404	0	79.92	74.40
S. Copenhagen 8457	0	84.98	40.20
S. Montevideo FSIS 051	0	ND[c]	39.30
S. Heidelberg F5038BG1	0	86.23	40.10

[a]Arithmetic mean
[b]For at least three trials, *S.* Montevideo FSIS 051 was unable to grow on BSA, even if not heat-treated (i.e., plated after being grown overnight at 37°C). As a result, this strain was replaced with *S.* Typhimurium 420, a DT104 environmental isolate.
[c]ND-not determined

Table B.4 Injury (on BSA) for 8 individual *Salmonella* strains following cold storage in TSB-YE at 4°C for 10 d.

Salmonella Strain	% Injury
S. Typhimurium 420	0 ± 0
S. Thompson FSIS 120	0 ± 0
S. Enteritidis H3527	0 ± 0
S. Enteritidis H3502	3.23 ± 3.30
S. Typhimurium H3380	2.40 ± 4.16
S. Hadar MF60404	0.33 ± 0.58
S. Copenhagen 8457	2.10 ± 2.05
S. Heidelberg F5038BG1	3.00 ± 5.20

Percentages are arithmetic mean ± standard deviation for n=3.

Table B.5 Analysis of variance (ANOVA) table for injury of 8 individual *Salmonella* strains following heat shock (54°C/30 min), cold shock (4°C/2 h) or starvation (4°C/10 d) (Proc Mixed, SAS® Version 8.02, SAS Institute, Inc., Cary, NC).

Effect	Num DF	Den DF	F value	Pr>F[a]
Heat Shock (n=3)				
Strain	8	44	1.18	0.3348
Treatment (Heat)	1	44	8108.69	<.0001
Strain*Treatment	8	44	0.63	0.7456
Cold Shock (n=3)				
Strain	8	42	0.34	0.9439
Treatment (Cold)	1	42	1938.59	<.0001
Strain*Treatment	8	42	0.63	0.7452
Starvation (n=3)				
Strain	8	42	1.64	0.1438
Treatment (Starvation)	1	42	5093.37	<.0001
Strain*Treatment	8	42	0.79	0.6171

[a]Pr>F values less than 0.05 indicates significance of the given effect.

Table B.6 ANOVA table for recovery of *Salmonella* following heat shock, cold shock or starvation (Proc Mixed, SAS® Version 8.02, SAS Institute, Inc., Cary, NC).

Effect	Num DF	Den DF	F value	Pr>F[a]
Heat Shock				
Medium	3	18	0.65	0.5945
Treatment	1	8	134.20	<.0001
Medium*Treatment	3	18	2.47	0.0947
Cold Shock				
Medium	3	12	0.82	0.5099
Treatment	1	5	33.02	0.0022
Medium*Treatment	3	12	0.52	0.6781
Starvation				
Medium	3	12	0.99	0.4319
Treatment	1	5	0.01	0.9094
Medium*Treatment	3	12	0.74	0.5458

[a]Pr>F values less than 0.05 indicates significance of the given effect.

Table B.7 ANOVA table for the effects of treatment, medium, time and their interactions on the number of survivors following thermal (60°C) treatment of *Salmonella* (Proc GLM, SAS® Version 8.02, SAS Institute, Inc., Cary, NC).

Source	DF	Sum of Squares	Mean Square	F value	Pr>F[a]
Treatment	3	68.01	22.67	72.06	<0.0001
Medium	1	8.28	8.28	26.31	<0.0001
Treatment*Medium	3	4.92	1.64	5.22	0.0017
Time	1	682.39	682.39	2169.07	<0.0001
Time*Medium	1	112.53	112.53	357.69	<0.0001
Time*Treatment	3	60.24	20.08	63.83	<0.0001
Time*Trt*Medium	3	20.68	6.89	21.91	<0.0001

[a]Pr>F values less than 0.05 indicates significance of the given effect.

Table B.8 Statistical analysis (Proc GLM, SAS® Version 8.02, SAS Institute, Inc., Cary, NC) of the thermal inactivation of *Salmonella*, sorted by medium (Proc Sort, SAS® Version 8.02), with unshocked as the control on each medium.

Parameter		Estimate	Standard Error	t value	Pr>|t|[a]
TSA-YE					
Time		-0.04083	0.00132	-30.98	<0.0001
Time*Treatment	Unshocked	0.00000			
Time*Treatment	Heat	0.01406	0.00214	6.57	<0.0001
Time*Treatment	Cold	-0.00312	0.00214	-1.46	0.1473
Time*Treatment	Starvation	0.00017	0.00208	0.08	0.9362
XLD					
Time		-0.09873	0.00353	-27.94	<0.0001
Time*Treatment	Unshocked	0.00000			
Time*Treatment	Heat	0.05119	0.00509	10.06	<0.0001
Time*Treatment	Cold	0.00227	0.00606	0.38	0.7083
Time*Treatment	Starvation	-0.02294	0.00870	-2.64	0.0099

[a]Pr>|t| values less than 0.05 indicates significance of the given effect.

Table B.9 Percent injury on three different selective media following heat shock.

	XLD[a]	BSA[a]	BG[a]
Observations[b]	19	35	9
Average	92.12	79.20	70.57
Minimum	70.80	24.70	7.10
Maximum	98.90	98.10	81.30
Standard Deviation	6.97	18.08	23.90

XLD- xylose lysine deoxycholate, BSA-bismuth sulfite agar, BG-brilliant green
[a]Media ingredients. Per liter of water: XLD (3 g yeast extract, 3.75 g xylose, 7.5 g lactose, 7.5 g sucrose, 5 g L-Lysine, 5 g NaCl, 6.8 g sodium thiosulfate, 0.8 g ferric ammonium citrate, 15 g agar, 0.08 g phenol red), BSA (5 g beef extract, 10 g peptone, 5 g dextrose, 4 g disodium phosphate, 0.3 g ferrous sulfate, 2 g bismuth ammonium citrate, 6 g sodium sulfite, 0.025 g brilliant green, 20 g agar), BG (10 g proteose peptone, 3 g yeast extract, 10 g lactose, 10 g saccharose, 5 g NaCl, 20 g agar, 0.0125 g brilliant green, 0.08 g phenol red).
[b]Observations were collected from several different projects, including work involving gradient plates, media supplementation and thermal inactivation in ground turkey.

Table B.10 Percent injury on three different selective media following cold shock.

	XLD	BSA	BG
Observations[a]	14	26	9
Average	59.67	60.07	67.04
Minimum	20.10	0	56.20
Maximum	90.70	92.50	89.30
Standard Deviation	20.49	22.59	10.83

XLD- xylose lysine deoxycholate, BSA-bismuth sulfite agar, BG-brilliant green
[a]Observations were collected from several different projects, including work involving gradient plates, media supplementation and thermal inactivation in ground turkey.

Table B.11 Percent injury on three different selective media following starvation.

	XLD	BSA	BG
Observations[a]	8	20	6
Average	65.76	61.10	78.12
Minimum	31.60	0	64.80
Maximum	80.2	94.30	91.60
Standard Deviation	19.25	33.14	11.25

XLD- xylose lysine deoxycholate, BSA-bismuth sulfite agar, BG-brilliant green
[a]Observations were collected from several different projects, including work involving media supplementation and thermal inactivation in ground turkey.

REFERENCES

Abee, T. and Wouters, J.A. 1999. Microbial stress response in minimal processing. Int. J. Food Microbiol. 50: 65-91

Abshire, K.Z. and Neidhardt, F.C. 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. 175: 3734-3743

Adler, H.I. 1990. The use of microbial membranes to achieve anaerobiosis. Crit. Rev. Biotechnol. 10: 119-127

Adler, H.I., Carrasco, A., Crow, W. and Gill, J.S. 1981. Cytoplasmic membrane-fraction that promotes septation in an *Escherichia coli lon* mutant. J. Bacteriol. 147: 326-332

Ahmed, M.N., Conner, D.E. and Huffman, D.L. 1995. Heat-resistance of *Escherichia coli* O157:H7 in meat and poultry as affected by product composition. J. Food Sci. 60: 606-610

Allen, L.A., Pasley, S.M. and Pierce, M.S.F. 1952. Conditions affecting the growth of *Bacterium coli* on bile salts media. Enumeration of this organism in polluted waters. J. Gen. Microbiol. 7: 257-267

AMI. 2000. The role of meat and poultry industry in the U.S. economy. American Meat Institute. http://www.meatami.org/indupg02.htm

Amin, V. and Olson, N. 1968. Influence of catalase activity on resistance of a coagulase-positive staphylococci to hydrogen peroxide. Appl. Microbiol. 16: 267-270

Andrews, G.P. and Martin, S.E. 1979. Catalase activity during recovery of heat-stressed *Staphylococcus aureus* MF-31. Appl. Environ. Microbiol. 38: 390-394

Andrews, W.H. 1986. Resuscitation of injured *Salmonella* spp. and coliforms from foods. J. Food Prot. 49: 62-75

AOAC. 1996. Official Methods of Analysis, 16th ed. Association of Official Analytical Chemists, Gaithersburg, MD

Archer, D.L. 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Technol. 7: 91-95

Archer, J., Jervis, E.T., Bird, J. and Gaze, J.E. 1998. Heat resistance of *Salmonella weltevreden* in low-moisture environments. J. Food Prot. 61: 969-973

Audia, J.P., Webb, C.C. and Foster, J.W. 2001. Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. Int. J. Med. Microbiol. 291: 97-106

Baird-Parker, A.C. and Davenport, E. 1965. The effect of recovery medium on isolation of *Staphylococcus aureus* after heat treatment and after the storage of frozen or dried cells. J. Appl. Bacteriol. 28: 390-402

Bang, W. and Drake, M.A. 2002. Resistnace of cold- and starvation-stressed *Vibrio vulnificus* to heat and freeze-thaw exposure. J. Food Prot. 65: 975-980

Bang, I.S., Audia, J.P., Park, Y.K. and Foster, J.W. 2002. Autoinduction of the *ompR* response regulator by acid shock and control of the *Salmonella enterica* acid tolerance response. Mol. Microbiol. 44: 1235-1250

Bayles, D.O., Annous, B.A. and Wilkinson, B.J. 1996. Cold stress proteins induced in *Listeria monocytogenes* in response to temperature downshock and growth at low temperatures. Appl. Environ. Microbiol. 62: 1116-1119

Baylis, C.L., MacPhee, S. and Betts, R.P. 2000a. Comparison of two commercial preparations of buffered pepton water for the recovery and growth of *Salmonella* bacteria from foods. J. Appl. Microbiol. 89: 501-510

Baylis, C.L., MacPhee, S. and Betts, R.P. 2000b. Comparison of methods for the recovery and detection of low levels of injured *Salmonella* in ice cream and milk powder. Lett. Appl. Microbiol. 30: 320-324

Bayne, H.G., Garibaldi, J.A. and Lineweaver, H. 1965. Heat resistance of *Salmonella Typhimurium* and *Salmonella Seftenberg* 775W in chicken meat. Poultry Sci. 44: 1281-1284

Bearson, S.M., Benjamin, W.H., Jr., Swords, W.E. and Foster, J.W. 1996. Acid shock induction of RpoS is mediated by the mouse virulence gene *mviA* of *Salmonella typhimurium*. J. Bacteriol. 178: 2572-2579

Bell, C. and Kyriakides, A. 2002. *Salmonella*. A Practical Approach to the Organism and its Control in Food. Blackwell Science, Oxford

Berlutti, F., Casalino, M., Zagaglia, C., Fradiani, P.A., Visca, P. and Nicoletti, M. 1998. Expression of the virulence plasmid-carried apyrase gene (*apy*) of enteroinvasive *Escherichia coli* and *Shigella flexneri* is under the control of H-NS and the VirF and VirB regulatory cascade. Infect. Immun. 66: 4957-4964

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.W. and McCarthy, J.D. 2000. Modifications to methods for enumeration and detection of injured *Escherichia coli* O157:H7 in foods. Int. J. Food Microbiol. 55: 285-290

Bollman, J., Ismond, A. and Blank, G. 2001. Survival of *Escherichia coli* O157:H7 in frozen foods: impact of the cold shock response. Int. J. Food Microbiol. 64: 127-138

Boziaris, I.S. and Adams, M.R. 2001. Temperature shock, injury and transient sensitivity to nisin in Gram negatives. J. Appl. Microbiol. 91: 715-724

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

Brashears, M.M., Amezquita, A. and Stratton, J. 2001. Validation of methods used to recover *Escherichia coli* O157:H7 and *Salmonella* spp. subjected to stress conditions. J. Food Prot. 64: 1466-1471

Bremer, E. and Krämer, R. 2000. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes. *In* Storz and Hengge-Aronis (ed.), Bacterial Stress Responses. ASM Press, Washington D. C., p. 79-97.

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

Bromberg, R., George, S.M. and Peck, M.W. 1998. Oxygen sensitivity of heated cells of *Escherichia coli* O157:H7. J. Appl. Microbiol. 85: 231-237

Buchmeier, N.A. and Heffron, F. 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science. 248: 730-732

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

Busch, S.V. and Donnelly, C.W. 1992. Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria innocua*. Appl. Environ. Microbiol. 58: 14-20

Busta, F.F. 1976. Practical implications of injured microorganisms in food. J. Milk Food Technol. 39: 138-145

Busta, F.F. 1994. Importance of injured food-borne microorganisms in minimal processing. *In* Singh and Oliveira (ed.), Minimal Processing of Foods and Process Optimization: An Interface. CRC Press, Boca Raton, p. 227-231

Buzby, J.C., Frenzen, P.D., Rasco, B. 2001. Product Liability and Microbial Foodborne Illness. ERS Agricultural Economic Report No. 799, April 2001

Calabrese, J.P. and Bissonnette, G.K. 1990. Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria. Appl. Environ. Microbiol. 56: 3558-3564

Caldwell, D.E. and Hirsch, P. 1973. Growth of microorganisms in two-dimensional steady-state diffusion gradients. Can. J. Microbiol. 19: 53-58

Carlson, T.R. 2002. Effect of water activity and humidity on the thermal inactivation of *Salmonella* during heating of meat. M.S. Thesis. Michigan State University, East Lansing, MI

Carmel, P. 2000. The Meat and Poultry Industry. October 2000 report for B2B Electronic Commerce lecture. Management of Global Information Technology, Kogod School of Business, American University, Washington D.C. http://www.American.edu/carmel/ma5901a/industry.htm

Casadei, I.R., Hitchings, E., Archer, J. and Gaze, J.E. 2001. Heat resistance of *Bacillus cereus*, *Salmonella typhimurium* and *Lactobacillus delbrueckii* in relation to pH and ethanol. Int. J. Food Microbiol. 63: 125-134

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

CDC. 1997a. *Salmonella* Surveillance: Annual Summary, 1996. Atlanta, Georgia: U.S. Department of Health and Human Services.

CDC 1997b. Foodborne Outbreak Response and Surveillance Unit, Foodborne Disease Outbreak Line Listing, 1997. Atlanta, Georgia: U.S. Department of Health and Human Services. http://www.cdc.gov/foodborneoutbreaks/us_outbpdf/fbofinal1997.pdf

CDC. 1998. *Salmonella* Surveillance: Annual Summary, 1997. Atlanta, Georgia: U.S. Department of Health and Human Services.

CDC. 1999. *Salmonella* Surveillance: Annual Summary, 1998. Atlanta, Georgia: U.S. Department of Health and Human Services.

CDC. 2000. *Salmonella* Surveillance: Annual Summary, 1999. Atlanta, Georgia: U.S. Department of Health and Human Services.

CDC. 2001a. FoodNet Surveillance Report for 2000: Final Report. Atlanta: Centers for Disease Control and Prevention

CDC. 2001b. *Salmonella* Surveillance: Annual Summary, 2000. Atlanta, Georgia: U.S. Department of Health and Human Services.

CDC. 2001c. Preliminary FoodNet Data on the Incidence of Foodborne Illnesses-Selected Sites, United States, 2000. MMWR. 50: 241-246

CDC. 2002a. Preliminary FoodNet Data on the Incidence of Foodborne Illnesses-Selected Sites, United States, 2001. MMWR. 51: 325-329

CDC 2002b. Multistate outbreaks of *Salmonella* serotype Poona infections associated with eating cataloupe from Mexico-United States and Canada, 2000-2002. MMWR. 51: 1044-1047

CDC 2003. Outbreaks of *Salmonella* serotype Enteritidis infection associated with eating shell eggs-United States, 1999-2001. MMWR. 51: 1149-1152

Chantarapanont, W., Slutsker, L., Tauxe, R.V. and Beuchat, L.R. 2000. Factors influencing inactivation of *Salmonella enteritidis* in hard-cooked egg. J. Food Prot. 63: 36-43

Cheng, H.-Y., Yang, H.-Y. and Chou, C.-C. 2002. Influence of acid adaptation on the tolerance of *Escherichia coli* O157:H7 to some subsequent stresses. J. Food Prot. 65: 260-265

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. 1995. Microbial biofilms. Annu. Rev. Microbiol. 49: 711-745

Craig, J.E., Boyle, D., Francis, K.P. and Gallagher, M.P. 1998. Expression of the cold-shock gene *cspB* in *Salmonella typhimurium* occurs below a threshold temperature. Microbiol. 144: 697-704

Crutchfield, S. 2001. ERS Briefing Room-Economics of foodborne disease: overview. http://www.ers.usda.gov/briefing/FoodborneDisease/overview.htm

Crutchfield, S.R., Buzby, J.C., Roberts, T., Ollinger, M., and Jordan Lin, C.-T. 1997. An economic assessment of food safety regulations: the new approach to meat and poultry inspection. ERS Agricultural Economic Report No. 755, July 1997

Czechowicz, S.M., Santos, O. and Zottola, E.A. 1996. Recovery of thermally-stressed *Escherichia coli* O157:H7 by media supplemented with pyruvate. Int. J. Food Microbiol. 33: 275-284

Dallmier, A.W. and Martin, S.E. 1988. Catalase and superoxide dismutase activities after heat injury of *Listeria monocytogenes*. Appl. Environ. Microbiol. 54: 581-582

D'Aoust, J.-Y. 1981. Update on preenrichment and selective enrichment conditions for detection of *Salmonella* in foods. J. Food Prot. 44: 369-374

D'Aoust, J.-Y. 1997. *Salmonella* Species. *In* Doyle, Beuchat and Montville (ed.), Food Microbiology. Fundamentals and Frontiers. ASM Press, Washington D.C., p. 129-158

D'Aoust, J.-Y., Warburton, D.W. and Sewell, A.M. 1985. *Salmonella* and food safety. J. Food Prot. 48: 1062-1066

Datta, A.R. and Kothary, M.H. 1993. Effects of glucose, growth temperature, and pH on listeriolysin O production in *Listeria monocytogenes*. Appl. Environ. Microbiol. 59: 3495-3497

Dickson, J.S. and Frank, J.F. 1993. Bacterial starvation stress and contamination of beef. Food Microbiol. 10: 215-222

Dickson, J.S. and Siragusa, G.R. 1994. Survival of *Salmonella typhimurium, Escherichia coli* 0157:H7 and *Listeria monocytogenes* Scott A during storage on beef sanitized with organic acids. J. Food Safety.14: 313-327

Dillon, J.R. and Bezanson, G.S. 1984. Microbial response to injuries from host defense mechanisms and antibiotics. *In* Hurst and Nasim (ed.), Repairable Lesions in Microorganisms. Academic Press, London, p. 187-215

Dodd, C.E. and Aldsworth, T.G. 2002. The importance of RpoS in the survival of bacteria through food processing. Int. J. Food Microbiol. 74: 189-194

Dorman, C.J., Bhriain, N.N. and Higgins, C.F. 1990. DNA supercoiling and environmental regulation of virulence gene expression in *Shigella flexneri*. Nature. 344: 789-792

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

ERS. 2001. Statistical Indicators, Summary Data. Economic Research Service, Agricultural Outlook Bulletin, April 2001, AGO-280, p. 28-56

ERS. 2002. Statistical Indicators, Summary Data. Economic Research Service, Agricultural Outlook, August 2002, AGO-293, p. 35-64

Farber, J.M. and Brown, B.E. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. Appl. Environ. Microbiol. 56: 1584-1587

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

Farr, S.B. and Kogoma, T. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimuium*. Microbiol. Rev. 55: 561-585

FDA 1998. Bacteriological Analytical Manual, 8th Edition, Revision A, 1998 http://www.cfsan.fda.gov/~ebam/r11.html

Flowers, R.S., D'Aoust, J.-Y., Andrews, W.H. and Bailey, J.S. 1992. *Salmonella. In* VanderZant and Splittstoesser (ed.), Compendium of Methods for the Microbiological Examination of Foods. 3rd ed. APHA, Washington, D.C., p. 371-422

Flowers, R.S., Martin, S.E., Brewer, D.G. and Ordal, Z.J. 1977. Catalase and enumeration of stressed *Staphylococcus aureus* cells. Appl. Environ. Microbiol. 33: 1112-1117

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

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

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

Foster, J.W. and Moreno, M. 1999. Inducible acid tolerance mechanisms in enteric bacteria. Novartis Found. Symp. 221: 55-69

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

Frenzen, P.D., Riggs, T.L., Buzby, J.C., Breuer, T., Roberts, T., Voetsch, D. and Reddy, S. 1999. *Salmonella* cost estimate updated using FoodNet data. Food Safety. 22: 10-15

Frenzen, P.D. 2002. ERS Briefing Room-Economics of foodborne disease: *Salmonella*. http://www.ers.usda.gov/briefing/FoodborneDisease/Salmonella/index.htm

FSIS. 1999. Performance standards for the production of certain meat and poultry products. Federal Register. Vol. 64, No. 3, p. 732-749

FSIS. 2001. Performance standards for the production of processed meat and poultry products. Federal Register. Vol. 66, No. 39, p. 12590-12636

FSIS. 2002a. United States Department of Agriculture Food Safety and Inspection Service, Recall Information Center. http://www.fsis.usda.gov/OA/recalls/rec_summ.htm

FSIS. 2002b. Progress report on *Salmonella* testing of raw meat and poultry products, 1998-2001. United States Department of Agriculture, Office of Public Health and Science, Biosciences Division

Gahan, C.G.M. and Hill, C. 1999. The relationship between acid stress responses and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. Int. J. Food Microbiol. 50: 93-100

Genther, F.J. and Martin, S.E. 1990. Ribosome assembly in *Salmonella typhimurium* 7136 during recovery from heat shock. Food Microbiol. 7: 53-67

Gerris, C.V. 2000. Use of lactic acid for carcass decontamination. Meat & Poultry. 1 April, 2000

Gilbert, P. 1984. The revival of micro-organisms sublethally injured by chemical inhibitors. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 175-197

Gilbert, R.J. and Humphrey, T.J. 1998. Food-Borne Bacterial Gastroenteritis. *In* Hausler Jr. and Sussman (ed.), Topley & Wilson's Microbiology and Microbial Infections. Vol. 3 Bacterial Infections. 9th ed. Arnold, London, p. 539-565

Girgis, H.S., Smith, J., Luchansky, J.B. and Klaenhammer, T.R. 2003. Stress adaptations of lactic acid bacteria. *In* Yousef and Juneja (ed.), Microbial Stress Adaptation and Food Safety. CRC Press, Boca Raton, p. 159-211

Givskov, M., Eberl, L., Møller, S., Poulsen, L.K. and Molin, S. 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J. Bacteriol. 176: 7-14

Goldstein, J., Pollitt, N.S. and Inouye, M. 1990. Major cold-shock protein of *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 87: 283-287

Gomez, R.F. and Sinskey, A.J. 1975. Effect of aeration on minimal medium recovery of heated *Salmonella typhimurium*. J. Bacteriol. 122: 106-109

Gôrt, A.S. and Imlay, J.A. 1998. Balance between endogenous superoxide stress and antioxidant defenses. J. Bacteriol. 180: 1402-1410

Gould, G.W. 1984. Injury and repair mechanisms in bacterial spores. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 199-220

Graumann, P. and Marahiel, M.A. 1996. Some like it cold: response of microorganisms to cold shock. Arch. Microbiol. 166: 293-300

Graumann, P., Schröder, K., Schmid, R. and Marahiel, M.A. 1996. Cold shock stress-induced proteins in *Bacillus subtilis*. J. Bacteriol. 178: 4611-4619

Guidot, P.M., McCord, J.M., Wright, R.M. and Repine, J.E. 1993. Absence of electron transport restores growth of a manganese-superoxide dismutase deficient *Saccharomyces cerevisiae*. J. Biol. Chem. 68: 26699-26703

Hansen, N.-H. and Riemann, H. 1963. Factors affecting the heat resistance of nonsporing organisms. J. Appl. Bacteriol. 26: 314-333

Harris, N.D. 1963. The influence of the recovery medium and the incubation temperature on the survival of damaged bacteria. J. Appl. Bacteriol. 26: 387-397

Hecker, M., Schumann, W. and Völker, U. 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. 19: 417-428

Hengge-Aronis, R. 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in E. coli. Cell. 72: 165-168

Hengge-Aronis, R. 2000. The general stress response in *Escherichia coli*. *In* Storz and Hengge-Aronis (ed.), Bacterial Stress Responses. ASM Press, Washington D.C., p. 161-178

Humphrey, T.J. 1988. Peroxide sensitivity and catalase activity in *Campylobacter jejuni* after injury and during recovery. J. Appl. Bacteriol. 64: 337-343

Humphrey, T.J. 1990. Heat resistance in *Salmonella enteritidis* phage type 4: the influence of storage temperatures before heating. J. Appl. Bacteriol. 69: 493-497

Humphrey, T.J., Wilde, S.J. and Rowbury, R.J. 1997. Heat tolerance of *Salmonella typhimurium* DT104 isolates attached to muscle tissue. Lett. Appl. Microbiol. 25: 265-268

Humphrey, T.J., Williams, A., McAlpine, K., Lever, M.S., Guard-Petter, J. and Cox, J.M. 1996. Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. Epidemiol. Infect. 177: 79-88

Hurst, A. 1977. Bacterial injury: a review. Can. J. Microbiol. 23: 935-944

Hurst, A. 1984. Revival of vegetative bacteria after sublethal heating. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 77-103

Hurst, A., Hendry, G.S., Hughes, A. and Paley, B. 1976. Enumeration of sublethally heated *Staphylococci* in some dried foods. Can. J. Microbiol. 22: 677-683

Hurst, A. and Hughes, A. 1981. Repair of salt tolerance and repair of lost D-alanine and magnesium following sublethal heating of *Staphylococcus aureus* are independent events. Can. J. Microbiol. 27: 627-632

IFT. 2002. Expert Report on Emerging Microbiological Food Safety Issues. Implications for Control in the 21st Century. Institute of Food Technologists, International Food Safety and Quality Conference and Exposition, Atlanta, GA. 20 February 2002

Jackson, H. and Woodbine, M. 1963. The effect of sublethal heat treatment on the growth of *Staphylococcus aureus*. J. Appl. Bacteriol. 26: 152-158

Jay, J.M. 1998. Modern Food Microbiology. 5th ed. Aspen Publishers, Inc., Gaithersburg

Jeffreys, A.G., Hak, K.M., Steffan, R.J., Foster, J.W. and Bej, A.K. 1998. Growth, survival and characterization of *cspA* in *Salmonella enteritidis* following cold shock. Curr. Microbiol. 36: 29-35

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

Johnson, E.A. 2003. Microbial adaptation and survival in foods. *In* Yousef and Juneja (ed.), Microbial Stress Adaptation and Food Safety. CRC Press, Boca Raton, p. 75-103

Johnson, K.M. and Busta, F.F. 1984. Detection and enumeration of injured bacterial spores in processed foods. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 241-256

Jones, K. 2001. ERS Agricultural Economist, Personal Communication

Jones, P.G., Mitta, M., Kim, Y., Jiang, W. and Inouyi, M. 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 93: 76-80

Jones, P.G., VanBogelen, R. and Neidhardt, F.C. 1987. Induction of proteins in response to low temperature in *Escherichia coli*. J. Bacteriol. 169: 2092-2095

Juneja, V.K. and Eblen, B.S. 1999. Predictive thermal inactivation model for *Listeria monocytogenes* with temperature, pH, NaCl, and sodium pyrophosphate as controlling factors. J. Food Prot. 62: 986-993

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

Juneja, V.K., Klein, P.G. and Marmer, B.S. 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

Juneja, V.K. and Novak, J.S. 2003. Adaptation of foodborne pathogens to stress from exposure to physical intervention strategies. *In* Yousef and Juneja (ed.), Microbial Stress Adaptation and Food Safety. CRC Press, Boca Raton, p. 31-53

Kang, D.H. and Siragusa, G.R. 1999. Agar underlay method for recovery of sublethally heat-injured bacteria. Appl. Environ. Microbiol. 65: 5334-5337

Kapoor, S., Singh, R.D., Sharma, P.C. and Khullar, M. 2002. Anaerobiosis induced virulence of *Salmonella typhi*. Indian J. Med. Res. 115: 184-188

Kaufmann, S.H. 1989. Stress proteins: virulence factors of intracellular disease agents? Immun. Infekt. 17: 124-128

Kennedy, J.E., Oblinger, J.L. and West, R.L. 1980. Fate of *Salmonella infantis*, *Staphylococcus aureus*, and *Hafnia alvei* in vacuum packaged beef plate pieces during refrigerated storage. J. Food Sci. 45: 1273-1277, 1300

Knabel, S.J. and Thielen, S.A. 1995. Enhanced recovery of severely heat-injured, thermotolerant *Listeria monocytogenes* from FDA primary enrichment media using a novel, simple, strictly anaerobic method. J. Food Prot. 58: 29-34

Knabel, S.J., Walker, H.W., Hartman, P.A. and Mendonca, A.F. 1990. Effects of growth temperature and strictly anaerobic recovery on the survival of *Listeria monocytogenes* during pasteurization. Appl. Environ. Microbiol. 56: 370-376

Knøchel, S. and Gould, G. 1995. Preservation microbiology and safety: Quo vadis? Trends Food Sci. Technol. 6: 127-131

Kushal, R. and Anand, S.K. 1999. Repair and recovery of thermally injured cells of *Yersinia enterocolitica* in milk. J. Food Prot. 62: 1203-1205

Lazazzera, B.A. 2000. Quorum sensing and starvation: signals for entry into stationary phase. Curr. Opin. Microbiol. 3: 177-182

Lee, L.S., Lin, J., Hall, H.K., Bearson, B. and Foster, J.W. 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

Leenanon, B. and Drake, M.A. 2001. Acid stress, starvation, and cold stress affect poststress behavior of *Escherichia coli* O157:H7 and nonpathogenic *Escherichia coli*. J. Food Prot. 64: 970-974

Leimeister-Wachter, M., Domann, E. and Chakraborty, T. 1992. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. J. Bacteriol. 174: 947-952

Le Minor, L. 1992. The Genus *Salmonella*. *In* Balows, Trüper, Dworkin, Harder and Schleifer (ed.), The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Vol. 3, 2nd ed. Springer-Verlag, New York. p. 2760-2774

Levine, P., Rose, B., Green, S., Ransom, G. and Hill, W. 2001. Pathogen testing of Ready-to-Eat meat and poultry products collected at Federally inspected establishments in the United States, 1990 to 1999. J. Food Prot. 64: 1188-1193

Levinson, R. 2000. Testimony on Food Safety and Microbial Contamination presented by Richard Levinson, Associate Executive Director of the American Public Health Association to the Senate Committee on Agriculture, Nutrition, and Forestry. September 20, 2000

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

Lindquist, S. 1992. Heat-shock proteins and stress tolerance in microorganisms. Curr. Opin. Gen. Develop. 2: 748-755

Line, J.E., Fain, A.R., Moran, A.B., Martin, L.M., Lechowich, R.V., Carosella, J.M. and Brown, W.L. 1991. Lethality of heat to *Escherichia coli* O157:H7: D-value and z-value determinations in ground beef. J. Food Prot. 54: 762-766

Linton, R.H., Webster, J.B., Pierson, M.D., Bishop, J.R. and Hackney, C.R. 1992. The effect of sublethal heat shock and growth atmosphere on the heat resistance of *Listeria monocytogenes* ScottA. J. Food Prot. 55: 84-87

Loewen, P.C., Hu, B., Strutinsky, J. and Sparling, R. 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. Can. J. Microbiol. 44: 707-717

Lottering, E.A. and Streips, U.N. 1995. Induction of cold shock proteins in *Bacillus subtilis*. Curr. Microbiol. 30: 193-199

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

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

Mackey, B.M. 1984. Lethal and sublethal effects of refrigeration, freezing and freeze-drying on micro-organsisms. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 45-75

Mackey, B.M. 2000. Injured bacteria. *In* Land, Baird-Parker and Gould (ed.), The Microbiological Safety and Quality of Food. Aspen Publishers, Inc., Gaithersburg, p. 315-341

Mackey, B.M., Boogard, E., Hayes, C.M. and Baranyi, J. 1994. Recovery of heat-injured *Listeria monocytogenes*. Int. J. Food Microbiol. 22: 227-237

Mackey, B.M. and Derrick, C.M. 1982. The effect of sublethal injury by heating, freezing, drying and gamma-radiation on the duration of the lag phase of *Salmonella typhimurium*. J. Appl. Bacteriol. 53: 243-251

Mackey, B.M. and Derrick, C.M. 1986. Elevation of the heat resistance of *Salmonella typhimurium* by sublethal heat shock. J. Appl. Bacteriol. 61: 389-393

Mackey, B.M. and Derrick, C.M. 1987a. Changes in the heat resistance of *Salmonella typhimurium* during heating at rising temperatures. Lett. Appl. Microbiol. 4: 13-16

Mackey, B.M. and Derrick, C.M. 1987b. The effect of prior heat shock on the thermoresistance of *Salmonella thompson* in foods. Lett. Appl. Microbiol. 5: 115-118

Mackey, B.M. and Seymour, D.A. 1987. The effect of catalase on recovery of heat-injured DNA-repair mutants of *Escherichia coli*. J. Gen. Microbiol. 133: 1601-1610

Madigan, M.T., Martinko, J.M., and Parker, J. 1997. *Brock* Biology *of* Microorganisms. 8th ed. Prentice Hall, Upper Saddle River

Mañas, P., Pagan, R., Leguérinel, I., Condon, S., Mafart, P. and Sala, F. 2001. Effect of sodium chloride concentration on the heat resistance and recovery of *Salmonella typhimurium*. Int. J. Food Microbiol. 63: 209-216

Martin, S.E., Flowers, R.S. and Ordal, Z.J. 1976. Catalase: its effect on microbial enumeration. Appl. Environ. Microbiol. 32: 731-734

Mathew, F.P. and Ryser, E.T. 2002. Competition of thermally injured *Listeria monocytogenes* with a mesophilic lactic acid starter culture in milk for various heat treatments. J. Food Prot. 65: 643-650

Matin, A. 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol. Microbiol. 5: 3-10

Mattick, K.L., Jørgensen, F., Legan, J.D., Lappin-Scott, H.M. and Humphrey, T.J. 2000. Habituation of *Salmonella* spp. at reduced water activity and its effect on heat tolerance. Appl. Envrion. Microbiol. 66: 4921-4925

Mattick, K.L., Jørgensen, F., Wang, P., Pound, J., Vandeven, M.H., Ward, L.R., Legan, J.D., Lappin-Scott, H.M. and Humphrey, T.J. 2001. Effect of challenge temperature and solute type on heat tolerance of *Salmonella* serovars at low water activity. Appl. Environ. Microbiol. 67: 4128-4136

Maurelli, A.T. 1989. Regulation of virulence genes in *Shigella*. Mol. Biol. Med. 6: 425-432

Maurer, J.L. 2001. Environmental effects on the thermal resistance of *Salmonella*, *Escherichia coli* O157:H7, and triose phosphate isomerase in ground turkey and beef. M.S. Thesis. Michigan State University, East Lansing, MI

Mayr, B., Kaplan, T., Lechner, S. and Scherer, S. 1996. Identification and purification of a family of dimeric major cold shock protein homologs from the psychrotrophic *Bacillus cereus* WSBC 10201. J. Bacteriol. 178: 2916-2925

Mazzotta, A.S. 2001. Thermal inactivation of stationary-phase and acid-adapted *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in fruit juices. J. Food Prot. 64: 315-320

McClements, J.M.J, Patterson, M.F. and Linton, M. 2001. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. J. Food Prot. 64: 514-522

McClure, P.J., Roberts, T.A. and Oguru, P.O. 1989. Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in a liquid medium. Lett. Appl. Microbiol. 9: 95-99

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

McGovern, V.P. and Oliver, J.D. 1995. Induction of cold responsive proteins in *Vibrio vulnificus*. J. Bacteriol. 177: 4131-4133

McMahon, C.M.M., Byrne, C.M., Sheridan, J.J., McDowell, D.A., Blair, I.S. and Hegarty, T. 2000. The effect of culture growth phase on induction of the heat shock response in *Yersinia enterocolitica* and *Listeria monocytogenes*. J. Appl. Microbiol. 89: 198-206

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

Mekalanos, J.J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. 174: 1-7

Meyer, D.H. and Donnelly, C.W. 1992. Effect of incubation temperature on repair of heat-injured *Listeria* in milk. J. Food Prot. 55: 579-582

Miller, A.J., Bayles, D.O., and Eblen, B.S. 2000. Cold shock induction of thermal sensitivity in *Listeria monocytogenes*. Appl. Environ. Microbiol. 66: 4345-4350

Mitchell, D.O. and Starzyk, M.J. 1975. Survival of *Salmonella* and other indicator microorganisms. Can. J. Microbiol. 21: 1420-1421

Mizunoe, Y., Wai, S.N., Takade, A. and Yoshida, S. 1999. Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H_2O_2-degrading compounds. Arch. Microbiol. 172: 63-67

Moats, W.A., Dabbah, R. and Edwards, V.M. 1971. Survival of *Salmonella anatum* heated in various media. Appl. Microbiol. 21: 476-481

Montville, T.J. 1997. Principles which influence microbial growth, survival, and death in foods. *In* Doyle, Beuchat and Montville (ed.), Food Microbiology, Fundamentals and Frontiers. ASM Press, Washington D.C., p. 13-29

Moriñigo, M.A., Martinez-Manzanares, E., Muñoz, A., Cornax, R., Romero, P. and Borrego, J.J. 1989. Evaluation of different plating media used in the isolation of salmonella from environmental samples. J. Appl. Bacteriol. 66: 353-360

Mossell, D.A.A., and van Netten, P. 1984. Harmful effects of selective media on stressed micro-organisms: nature and remedies. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 329-369

Musarrat, J. and Ahmad, M. 1988. pH induced damage and repair in *E. coli*. Mutat. Res. 193: 219-227

Murano, E.A. and Pierson, M.D. 1992. Effect of heat shock and growth atmosphere on the heat resistance of *Escherichia coli* O157:H7. J. Food Prot. 55: 171-175

Murano, E.A. and Pierson, M.D. 1993. Effect of heat shock and incubation atmosphere on injury and recovery of *Escherichia coli* O157:H7. J. Food Prot. 56: 568-572

Murphy, R.Y., Marks, B.P., Johnson, E.R., and Johnson, M.G. 1999. Inactivation of *Salmonella* and *Listeria* in ground chicken breast meat during thermal processing. J. Food Prot. 62: 980-985

Murphy, R.Y., Duncan, L.K., Johnson, E.R., Davis, M.D. and Smith, J.N. 2002. Thermal inactivation D- and z-values of *Salmonella* serotypes and *Listeria innocua* in chicken patties, chicken tenders, franks, beef patties, and blended beef and turkey patties. J. Food Prot. 65: 53-60

Nebra, Y., Jofre, J. and Blanch, A.R. 2002. The effect of reducing agents on the recovery of injured *Bifidobacterium* cells. J. Microbiol. Methods. 49: 247-254

Neidhardt, F.C. and VanBogelen, R.A. 1987. Heat Shock Response. *In* Neidhardt, Ingraham, Low, Magasanik, Schaechter and Umbarger (ed.), *Escherichia coli* and *Salmonella Typhimurium*: Cellular and Molecular Biology. ASM Press, Washington D.C., p. 1334-1345

Novak, J.S., Tunick, M.H. and Juneja, V.K. 2001. Heat treatment adaptations in *Clostridium perfringens* vegetative cells. J. Food Prot. 64: 1527-1534

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

Oerskov, J.L. 1925. Les bacilles appartenant aux groupes typhique, paratyphique, paradysentérie, et coli, deviennent, aprés chauffagé hypersensibles à l'action de l'oxygène atmosphérique. C.R. Soc. Biol., Paris. 92: 402

Oliver, J.D. 1981. Lethal cold stress of *Vibrio vulnificus* in oysters. Appl. Environ. Microbiol. 41: 710-717

Pagán, R., Condon, S., and Sala, F.J. 1997. Effects of several factors on the heat-shock-induced thermotolerance of *Listeria monocytogenes*. Appl. Environ. Microbiol. 63: 3225-3232

Pagán, R., Mañas, P., Palop, A. and Sala, F.J. 1999. Resistance of heat-shocked cells of *Listeria monocytogenes* to mano-sonication and mano-thermo-sonication. Lett. Appl. Microbiol. 28: 71-75

Palumbo, S.A. 1984. Heat injury and repair in *Campylobacter jejuni*. Appl. Environ. Microbiol. 48: 477-480

Pascual, C., Robinson, T.P., Ocio, M.J., Aboaba, O.O. and Mackey, B.M. 2001. The effect of inoculum size and sublethal injury on the ability of *Listeria monocytogenes* to initiate growth under suboptimal conditions. Lett. Appl. Microbiol. 33: 357-361

Patel, J.R. and Beuchat, L.R. 1995. Enrichment in Fraser broth supplemented with catalase of Oxyrase®, combined with the microcolony immunoblot technique, for detecting heat-injured *Listeria monocytogenes* in foods. Int. J. Food Microbiol. 26: 165-176

Patel, J.R., Hwang, C.-A., Beuchat, L.R., Doyle, M.P. and Brackett, R.E. 1995. Comparison of oxygen scavengers for their ability to enhance resuscitation of heat-injured *Listeria monocytogenes*. J. Food Prot. 58: 244-250

Pearson, A.M. and Gillett, T.A. 1996. Processed Meats. 3rd ed. Chapman and Hall, New York

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

Pellon, J.R. and Sinskey, A.J. 1984. Heat-induced damage to the bacterial chromosome and its repair. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 106-125

Poppe, C. 1999. Epidemiology of *Salmonella enterica* Serovar Enteritidis. *In* Saeed, Potter and Wall (ed.), *Salmonella enterica* Serovar Enteritidis in Humans and Animals. Epidemiology, Pathogenesis and Control. Iowa State University Press, Ames, p. 3-18

Postgate, J.R. and Hunter, J.R. 1963. The survival of starved bacteria. J. Appl. Bacteriol. 26: 295-306

Przybylski, K.S. and Witter, L.D. 1979. Injury and recovery of *Escherichia coli* after sublethal acidification. Appl. Environ. Microbiol. 37: 261-265

Quesnel, L.B. 1984. Biological indicators and sterilization processes. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 257-291

Quintavalla, S. and Campanini, M. 1991. Effect of rising temperature on the heat resistance of *Listeria monocytogenes* in meat emulsion. Lett. Appl. Microbiol. 12: 184-187

Quintavalla, S., Larini, S., Mutti, P. and Barbuti, S. 2001. Evaluation of the thermal resistance of different Salmonella serotypes in pork meat containing curing additives. Int. J. Food Microbiol. 67: 107-114

Rattanasomboon, N., Bellara, S.R., Fryer, P.J., Thomas, C.R. and McFarlane, C.M. 2001. The gradient plate technique as a means of studying the recovery of heat-injured *Brochothrix thermosphacta*. Int. J. Food Sci. Technol. 36: 369-376

Ray, B. 1984. Reversible freeze-injury. *In* Hurst and Nasim (ed.), Repairable Lesions in Microorganisms. Academic Press, London, p. 237-271

Ray, B. 1986. Impact of bacterial injury and repair in food microbiology: its past, present and future. J. Food Prot. 49: 651-655

Ray, B. 1989. Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water and Feeds. CRC Press, Inc. Boca Raton

Ray, B. 2001. Fundamental Food Microbiology. 2nd ed. CRC Press, Boca Raton

Rayman, M.K., Aris, B. and El Derea, H.B. 1978. The effect of compounds which degrade hydrogen peroxide on the enumeration of heat-stressed cells of *Salmonella seftenberg*. Can. J. Microbiol. 24: 883-885

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

Reichart, O. 1994. Modelling the destruction of Escherichia coli on the base of reaction kinetics. Int. J. Food Microbiol. 23: 449-465

Reissbrodt, R., Rienaecker, I., Romanova, J.M., Freestone, P.P.E., Haigh, R.D., Lyte, M., Tschäpe, H. and Williams, P.H. 2002. Resuscitation of *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* from the viable but nonculturable state by heat-stable enterobacterial autoinducer. Appl. Environ. Microbiol. 68: 4788-4794

Restaino, L., Jeter, W.S. and Hill, W.M. 1980. Thermal injury of *Yersinia enterocolitica*. Appl. Environ. Microbiol. 40: 939-949

Rexroat, M.A., Oberly, T.J., Bewsey, B.J. and Garriott, M.L. 1995. The gradient plate assay: a modified Ames assay used as a prescreen for the identification of bacterial mutagens. Mutat. Res. 341: 185-192

Ribot, E., Wierzba, R., Angulo, F. and Barrett, T. 2002. *Salmonella enterica* serotype Typhimurium DT104 isolated from humans, United States, 1985, 1990, and 1995. Emerg. Infect. Dis. 8: 387-391

Romans, J.R., Costello, W.J., Wendell Carlson, C., Greaser, M.L., and Jones, K.W. 2001. The Meat We Eat. 14th ed. Interstate Publishers, Inc., Danville

Rowbury, R.J. 2003. Physiology and molecular basis of stress adaptation, with particular reference to the subversion of stress adaptation, and to the involvement of extracellular components in adaptation. *In* Yousef and Juneja (ed.), Microbial Stress Adaptation and Food Safety. CRC Press, Boca Raton, p. 247-302

Rowe, M.T. and Kirk, R.B. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157:H7 to subsequent heat stress. J. Food Prot. 63: 1745-1748

Russell, A.D. 1984. Potential sites of damage in micro-organisms exposed to chemical or physical agents. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 1-18

Sato, M. and Takahashi, H. 1970. Cold-shock of bacteria. IV. Involvement of DNA-ligase reaction in recovery of *Escherichia coli* from cold shock. J. Gen. Appl. Microbiol. 16: 279-290

Schellhorn, H.E. and Hassan, H.M. 1988. Response of hydroperoxidase and superoxide dismutase deficient mutants of *Escherichia coli* K-12 to oxidative stress. Can. J. Microbiol. 34: 1171-1176

Semanchek, J.J. and Golden, D.A. 1998. Influence of growth temperature on inactivation and injury of *Escherichia coli* O157:H7 by heat, acid, and freezing. J. Food Prot. 61: 395-401

Shallow, S., Vugia, D., Fiorentino, T., Mshar, P., Farley, M., Lance-Parker, S., Pass, M., Karchmer, T., Carter, M., Smith, K., Zansky, S., and Cassidy, M. 2000. Preliminary FoodNet data on the incidence of foodborne illnesses-selected sites, United States, 1999. MMWR Weekly. 49: 201-205

Simpson, J.A., Narita, S., Gieseg, S., Gebicki, S., Gebicki, J.M. and Dean, R.T. 1992. Long-lived reactive species on free-radical-damaged proteins. Biochem. J. 282: 621-624

Singh, A. and McFeters, G.A. 1987. Survival and virulence of copper- and chlorine-stressed *Yersinia enterocolitica* in experimentally infected mice. Appl. Environ. Microbiol. 53: 1768-1774

Skurnik, M. and Toivanen, P. 1992. LcrF is the temperature-regulated activator of the *yadA* gene of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. J. Bacteriol. 174: 2047-2051

Sleator, R.D. and Hill, C. 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. 26: 49-71

Smirnova, G.V., Zakirova, O.N. and Oktiabr'skii, O.N. 2001a. The role of antioxidant systems in response of *Escherichia coli* to heat shock. Mikrobiol. 70: 595-601

Smirnova, G.V., Zakirova, O.N. and Oktiabr'skii, O.N. 2001b. Role of the antioxidant system in response of *Escherichia coli* to cold stress. Mikrobiol. 70: 55-60

Smith, J.L. and Dell, B.J. 1990. Capability of selective media to detect heat-injured *Shigella flexneri*. J. Food Prot. 45: 360-365

Smith, J.L. and Palumbo, S.A. 1978. Injury to *Staphylococcus aureus* during sausage fermentation. Appl. Environ. Microbiol. 36: 857-860

Smith, S.E. 2000. R-phycoerythrin as a time-temperature integrator, and triose phosphate isomerase as a temperature indicator of thermal processing in beef patties. M.S. Thesis. Michigan State University, East Lansing, MI

Snyder, L. and Champness, W. 1997. Molecular Genetics of Bacteria. ASM Press, Washington D.C.

Sokolovic, Z., Fuchs, A. and Goebel, W. 1990. Synthesis of species-specific stress proteins by virulent strains of *Listeria monocytogenes*. Infect. Immun. 58: 3582-3587

Sokolovic, Z., Riedel, J., Wuenscher, M. and Goebel, W. 1993. Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. 8: 219-227

Southard, L., Gustafson, R., Miller, J., and Harvey, D. 2000. Meat and poultry production to continue record-setting pace. ERS Agricultural Outlook Bulletin, June-July 2000, p. 5-6

Stead, D. and Park, S.F. 2000. Roles of superoxide dismutase and catalase in resistance of *Campylobacter coli* to freeze-thaw stress. Appl. Environ. Microbiol. 66: 3110-3112

Stephens, P.J., Cole, M.B. and Jones, M.V. 1994. Effect of heating rate on the thermal inactivation of *Listeria monocytogenes*. J. Appl. Bacteriol. 77: 702-708

Stephens, P.J., Druggan, P. and Nebe-von Caron, G. 2000. Stressed *Salmonella* are exposed to reactive oxygen species from two independent sources during recovery in conventional culture media. Int. J. Food Microbiol. 60: 269-285

Stephens, P.J., Joynson, J.A., Davies, K.W., Holbrook, R., Lappin-Scott, H.M. and Humphrey, T.J. 1997. The use of an automated growth analyzer to measure recovery times of single heat-injured *Salmonella* cells. J. Appl. Microbiol. 83: 445-455

Storz, G. and Hengge-Aronis, R. 2000. Preface. *In* Storz and Hengge-Aronis (ed.), Bacterial Stress Responses. ASM Press, Washington D.C., p. xii-xiv

Storz, G. and Zheng, M. 2000. Oxidative stress. *In* Storz and Hengge-Aronis (ed.), Bacterial Stress Responses. ASM Press, Washington D.C., p. 47-59

Suh, J.-H. and Knabel, S.J. 2000. Comparison of different reducing agents for enhanced detection of heat-injured *Listeria monocytogenes*. J. Food Prot. 63: 1058-1063

Sunil, B. 2000. The true impact of food borne infections. J. Indian Vet. Assoc., Kerala. vol. 5. http://www.jivaonline.com

Sykes, G. 1963. The phenomenon of bacterial survival. J. Appl. Bacteriol. 26: 287-294

Takeda, Y. and Avila, H. 1986. Structure and gene expression of the *E. coli* Mn-superoxide dismutase gene. Nucleic Acids Res. 14: 4577-4589

Taormina, P.J. and Beuchat, L.R. 2001. Survival and heat resistance of *Listeria monocytogenes* after exposure to alkali and chlorine. Appl. Environ. Microbiol. 67: 2555-2563

Teo, A.Y. and Knabel, S.J. 2000. Development of a simple recovery-enrichment system for enhanced detection of heat-injured *Listeria monocytogenes* in pasteurized milk. J. Food Prot. 63: 462-472

Thanomsub, B., Anupunpisit, V., Chanphetch, S., Watcharachaipong, T., Poonkhum, R. and Srisukonth, C. 2002. Effects of ozone treatment on cell growth and ultrastructural changes in bacteria. J. Gen. Appl. Microbiol. 48: 193-199

Thippareddi, H., Phebus, R.K., Fung, D.Y.C. and Kastner, C.L. 1995. Use of universal preenrichment medium supplemented with Oxyrase™ for the simultaneous recovery of *Escherichia coli* O157:H7 and *Yersinia enterocolitica*. J. Rap. Methods Auto. Microbiol. 4: 37-50

Thomas, L.V., Wimpenny, J.W.T. and Peters, A.C. 1992. Testing multiple variables on the growth of a mixed inoculum of *Salmonella* strains using gradient plates. Int.J. Food Microbiol. 15: 165-175

Tolker-Nielsen, T. and Molin, S. 1996. Role of ribosome degradation in the death of heat-stressed *Salmonella typhimurium*. FEMS Microbiol. Lett. 142: 155-160

Tompkin, R.B. 1986. Microbiology of Ready-to-Eat Meat and Poultry Products. Adv. Meat Research. 2: 89-121

Toomey, K.E., Blake, P.A. and Ralston, M. 2001. Foodborne outbreaks associated with eating pork barbecue-Georgia, 1996-2000. Georgia Epidemiology Report. 17: 1-3

Tran, T.T. 1995. Evaluation of Oxyrase® enrichment method for isolation of *Campylobacter jejuni* from inoculated foods. Lett. Appl. Microbiol. 21: 345-347

Tsuchido, T., VanBogelen, R.A. and Neidhardt, F.C. 1986. Heat shock response in *Escherichia coli* influences cell division. Proc. Natl. Acad. Sci. USA. 83: 6959-6963

Valentine, J.S., Foote, C.S., Greenberg, A. and Liebman, J.F. 1995. Active Oxygen in Biochemistry. Blackie Academic & Professional, London

Valone, S.E., Chikami, G.K. and Miller, V.L. 1993. Stress induction of the virulence proteins (SpvA, -B, and -C) from native plasmid pSDL2 of *Salmonella dublin*. Infect. Immun. 61: 705-713

VanBogelen, R.A. and Neidhardt, F.C. 1990. Ribosomes as sensors of heat and cold shock in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 87: 5589-5593

Van Schothorst, M. and Maggie Duke, A. 1984. Effect of sample handling on microbial limits laid down in standards. *In* Andrew and Russell (ed.), The Revival of Injured Microbes. Academic Press, London, p. 309-328

Vasconcelos, J.A. and Deneer, H.G. 1994. Expression of superoxide dismutase in *Listeria monocytogenes*. 60: 2360-2366

Völker, U., Mach, H., Schmid, R. and Hecker, M. 1992. Stress proteins and cross-protection by heat shock and salt stress in *Bacillus subtilis*. J. Gen. Microbiol. 138: 2125-2135

Warburton, D.W., Bowen, B., Konkle, A., Crawford, C., Durzi, S., Foster, R., Fox, C., Gour, L., Krohn, G., LaCasse, P., Lamontagne, G., McDonagh, S., Arling, V., Mackenzie, J., Todd, E.C.D., Oggel, J., Plante, R., Shaw, S., Tiwari, N.P., Trottier, Y. and Wheeler, B.D. 1994. A comparison of six different plating media used in isolation of *Salmonella*. Int. J. Food Microbiol. 22: 277-289

Watson, K. 1990. Microbial Stress Proteins. Adv. Microb. Pysiol. 31: 183-223

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

Wimpenny, J.W.T. and Waters, P. 1984. Growth of microorganisms in gel-stabilised two-dimensional diffusion. J. Gen. Microbiol. 130: 2921-2926

Wong, H.-C., Peng, P.-Y., Lan, S.-L., Chen, Y.-C., Lu, K.-H., Shen, C.-T. and Lan, S.-F. 2002. Effects of heat shock on the thermotolerance, protein composition, and toxin production of *Vibrio parahaemolyticus*. J. Food Prot. 65: 499-507

Wouters, J.A., Rombouts, F.M., Kuipers, O.P., de Vos, W.M. and Abee, T. 2000. The role of cold-shock proteins in low-temperature adaptation of food-related bacteria. Syst. Appl. Microbiol. 23: 165-173

Wouters, J.A., Sanders, J.-W., Kok, J., De Vos, W.M., Kuipers, O.P. and Abee, T. 1998. Clustered organization and transcriptional analysis of a family of five *csp* genes of *Lactococcus lactis* MG1363. Microbiol. 144: 2885-2893

Wright, C.T. and Klaenhammer, T.R. 1981. Calcium-induced alterations in cellular morphology affecting the resistance of *Lactobacillus acidophilus* to freezing. Appl. Environ. Microbiol. 41: 807-815

Wuytack, E.Y., Phuong, L.D.T., Aertsen, A., Reyns, K.M.F., Marquenie, D., De Ketelaere, B., Masschalck, B., Van Opstal, I., Diels, A.M.J. and Michiels, C.W. 2003. Comparison of sublethal injury induced in *Salmonella enterica* serovar Typhimurium by heat and by different nonthermal treatments. J. Food Protection. 66: 31-37

Xavier, I.J. and Ingham, S. 1993. Increased D-values for *Salmonella enteritidis* resulting from the use of anaerobic enumeration methods. Food Microbiol. 10: 223-228

Yamamoto, T., Hanawa, T. and Ogata, S. 1994. Induction of *Yersinia enterocolitica* stress proteins by phagocytosis with macrophage. Microbiol. Immunol. 38: 295-300

Yousef, A.E. and Courtney, P.D. 2003. Basics of stress adaptation and implications in new-generation foods. *In* Yousef and Juneja (ed.), Microbial Stress Adaptation and Food Safety. CRC Press, Boca Raton, p. 1-30

Yu, L.S.L. and Fung, D.Y.C. 1991. Effect of Oxyrase™ enzyme on *Listeria monocytogenes* and other facultative anaerobes. J. Food Safety. 11: 163-175

Zemser, R.B. and Martin, S.E. 1998. Heat stability of virulence-associated enzymes from *Listeria monocytogenes* SLCC 5764. J. Food Prot. 61: 899-902

Zhao, T. and Doyle, M.P. 2001. Evaluation of universal preenrichment broth for growth of heat-injured pathogens. J. Food Prot. 64: 1751-1755

Zook, C.D., Busta, F.F. and Brady, L.J. 2001. Sublethal sanitizer stress and adaptive response of *E. coli* O157:H7. J. Food Prot. 64: 767-769

Zugel, U. and Kaufmann, S.H. 1999. Role of heat shock proteins in protection from pathogenesis of infectious diseases. Clin. Microbiol. Rev. 12: 19-39