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EFFECTS OF MARINATION ON *SALMONELLA*
PENETRATION AND MUSCLE STRUCTURE
OF TURKEY BREAST

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EFFECTS OF MARINATION ON *SALMONELLA* PENETRATION AND MUSCLE STRUCTURE OF TURKEY BREAST

By

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ABSTRACT

EFFECTS OF MARINATION ON *SALMONELLA* PENETRATION AND MUSCLE STRUCTURE OF TURKEY BREAST

By

Vareemon Tuntivanich

The effects of marinade composition, microbial load, and point of exposure on the multidirectional penetration of *Salmonella* into intact whole muscle turkey during marination were evaluated. Irradiated whole muscle turkey breasts were vacuum tumbled for 20 min with marinade solutions containing an 8–strain *Salmonella*. Each sample was dissected to collect multidirectional *Salmonella* concentration data. Three 1 cm-thick slices (cranial, middle, and caudal) were removed from the turkey breast longitudinal to the muscle fibers. Within these three slices, 1-cm cubes were excised using a self-cauterizing electrosurgical unit and enumerated for surviving cells.

The highest and lowest *Salmonella* counts were observed in the area immediately below the inoculated surface and in the center segment, respectively. There was a slight difference in *Salmonella* distribution for the cranial and caudal slices as compared to the middle slice, which may be due to the typical structure and orientation of turkey breast muscle. With higher inoculation levels (10^8 vs. 10^4 CFU/mL), higher numbers of salmonellae ($P \leq 0.05$) were recovered from the turkey breast. In contrast, marinade containing 3.2% salt and 0.8% phosphate (typical concentration, TC) or 10.2% salt and 4.05% phosphate (high concentration, HC) showed no differences ($P > 0.05$) in *Salmonella* populations after penetration into whole muscle. Greater *Salmonella* penetration ($P \leq 0.05$) into marinated turkey breast was observed when the pathogen was introduced during vacuum tumbling with a contaminated marinade, compared to pre- and

post-tumbling process. This may be due to the lower bacterial levels to start with in pre- and post- process treatments, as opposed to the during process treatment.

The changes in turkey muscle microstructure after marination were also documented. Salt and phosphate significantly contributed to changes in muscle microstructural changes. Muscle samples immersed in HC demonstrated smaller extracellular areas as compared to control and TC samples. Swelling of myofibrils in TC and HC were observed under transmission electron microscopy. Cryostat sectioning and fluorescence microscopy were used to determine the location of marker bacteria (GFP-transformed *Salmonella*) in fresh turkey breast muscle. In cross-sectional areas of muscle cells, GFP-labeled salmonellae were seen between muscle fibers and bundles and tended to be located near endomysial and perimysial supporting tissue.

These results suggest the potential for *Salmonella* to penetrate and reside at specific locations in value-added or marinated products. The results provide important information concerning product and process safety, which is necessary to develop a quantitative model for pathogen penetration into whole muscle poultry products during marination.

To my family who are giving me the endless love.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Turkey production in the United States has more than doubled since 1970. Per capita consumption of turkey has increased from 2.9 kilograms in 1970 to 5.9 kilograms in 2005 (USDA 2007). In 2004, turkey was the number four protein choice for American consumers because of taste and nutritional value (National Turkey Federation 2004). Additionally, consumer trends show an increasing demand for ready-to-eat products, which include marinated whole muscle products (Russell 2002). Many poultry products sold in retail markets are value added by means of marination and mechanical tenderization.

Vacuum tumbling is a popular method for tenderization. During the process, whole muscle meat is tumbled either with a marinade solution or after it is injected. Salt and phosphates are commonly incorporated into the marinade to increase product yield and palatability. They function by inducing changes in muscle structure and contributing to increased water absorption. However, if the product surface and/or marinade were contaminated, bacterial pathogens may be introduced along with the water portion of the marinade into the interior of value-added meat products (Phebus and others 1999).

Once these bacteria have penetrated into meat products, they may exhibit enhanced thermal resistance, which depends on various factors, including meat species, muscle type, pH, fat content, and additives used in processing. As a result, thermal processing using proper cooking time and internal temperature is important to assure product safety. Foodborne outbreaks associated with poultry products have involved

several pathogens, such as *Listeria monocytogenes*, *Salmonella* and *Campylobacter*. *Salmonella* is the specific organism targeted in this research since it is responsible for an estimated 1.4 million cases of foodborne illness each year in the U.S. and is commonly found in poultry products (CDC 2005c). According to the USDA Food Safety Inspection Service (FSIS), raw turkey is a common source of *Salmonella* in the U.S. food supply (USDA-FSIS 2002). About 20.3% of ground turkey sampled in 2006 was positive for *Salmonella* (USDA-FSIS 2007). An infective dose as little as 10 to 100 cells can lead to symptoms of salmonellosis (nausea, abdominal cramps, diarrhea and vomiting) in susceptible persons.

To improve the microbiological safety of marinated poultry products, the overall goal of this project was to determine the basic pathways by which pathogens penetrate into marinated whole muscle poultry products. The term "penetration" is used throughout the study when the microorganisms are observed or recovered from the interior of turkey breast due to the migration and/or invasion of the bacteria. The hypotheses for this project are:

1. Salmonellae follow a non-random penetration pathway, and the degree of penetration depends on several factors, such as marinade composition, microbial population, and point at which the product is exposed to *Salmonella*.
2. Once salmonellae penetrate into the interior of the muscle, the pathogen preferentially locates within the muscle bundles after marination.
3. Marinade ingredients, such as salt and phosphates, contribute to structural changes facilitating microbial penetration into the inner portion of muscle.

To test these hypotheses, the specific objectives were to: (1) evaluate the effects of marinade composition, pathogen load, and contamination stage on the multidirectional penetration of *Salmonella* into intact whole muscle turkey during vacuum tumbling marination; (2) document changes in turkey muscle microstructure after marination; and (3) determine the location of *Salmonella* in whole muscle turkey after marination with a *Salmonella*-inoculated marinade. In addition to these specific objectives, the effects of turkey physical structure on the thermal resistance of *Salmonella* were quantified and *Salmonella* viability in marinades containing salt and phosphate was also determined. These last studies are addressed in the appendices.

CHAPTER 2

LITERATURE REVIEW

Turkey consumption

More than 256 million turkeys were raised in the U.S. in 2005. According to the National Turkey Federation, 41.1% were sold to grocery stores and other retail outlets and 21.6% were distributed to foodservice outlets. From 1970 to 2005 consumption of turkey has more than doubled (USDA 2007). Nearly half of the U.S. population consumes turkey at least once every two weeks, with more than a quarter eating turkey lunchmeat. Although, whole turkey continues to be the most popular, ground turkey has high appeal among all ages, genders, and economic levels.

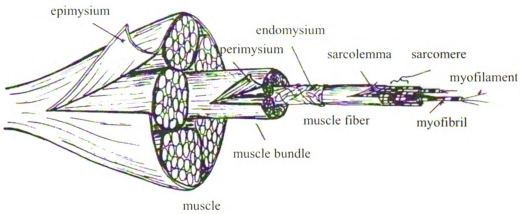
White and dark meat differ nutritionally. White meat has fewer calories and less fat than dark meat and is generally preferred in the U.S. rather than dark meat. A turkey typically has about 70% white meat and 30% dark meat. Today's improvements in genetics, feed, and management practices have produced domesticated turkeys with more breast meat and meatier thighs.

The "Go Lean with Protein" section of the food guide pyramid recommends eating two to three servings from the meat and poultry group each day. Therefore when comparing many other foods within this group, turkey can be a choice to provide high quality protein, low in fat, and fewer calories. A nutritional fact of boneless skinless cooked turkey breast contains 26 g of protein, which is about 8% higher than the same serving size of boneless skinless chicken breast or trimmed top loin beefsteak.

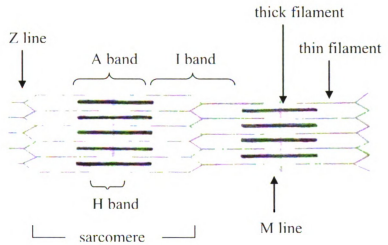
In a progress report on *Salmonella* testing of raw poultry products from 1998 to 2006 (USDA-FSIS 2007), approximately 7.1% of 2785 turkey carcasses and 20.3% of 444 ground turkey samples yielded *Salmonella*. Because food safety is a top priority of the U.S. meat and poultry industry, the pathogen reduction strategies in the workplace must coincide with the current regulations, process controls, and hazard analysis and critical control point (HACCP) programs to achieve the safest products possible for consumer.

Skeletal muscle structure and biochemistry

Three types of muscle tissues are distinguished based on functional and morphological characteristics: smooth muscle, cardiac muscle, and skeletal muscle. Skeletal muscle is responsible for body position and posture maintenance. Figure 2.1 a illustrates the structure of skeletal muscle, which is attached to bone via tendon and surrounded by a dense collagen called epimysium. Within a muscle bundle, muscle cells or muscle fibers are arranged in parallel and bound together by collagenous supporting tissue, the perimysium. A muscle fiber, which is surrounded by endomysium, is composed of longitudinally structural and functional subunits/organelles called myofibrils. The myofibrils are composed of myofilament polymers, referred to as thick and thin filaments. The predominant proteins in thick and thin filaments are myosin and actin, respectively and the overlapping of actin and myosin filaments gives rise to a characteristic banding called striated pattern of skeletal muscle cell and myofibril as shown in Figure 2.1 b (Ross and others 2003).



(a)



(b)

Figure 2.1 Diagrams of skeletal muscle organization (a) transverse section of muscle showing fascicles, muscle fibers, myofibrils, and myofilaments (b) myofilaments identifying specific bands and lines (adapted from Aberle and others 2001).

Over 20 different proteins are associated with myofibril. The major proteins are myosin, actin, titin, tropomyosin, troponin, and nebulin (Aberle and others 2001). Actin and myosin comprise more than half of the protein of skeletal muscle and are the main factors in postmortem onset and resolution of rigor, tenderness, water holding capacity, emulsification and binding properties of meat (Richardson and Mead 1999).

The pH of muscle during life is about 7.2. After death, the muscle acidifies to pH 6 or less through the accumulation of lactic acid, which derives from the postmortem breakdown of glycogen by glycolysis. Acidification may affect color and water holding capacity of meat. The development of rigor mortis also occurs when adenosine triphosphate (ATP) levels decline in the postmortem animal. The muscle consequently transforms from being soft and pliable to a more rigid and inextensible state, which affects textural qualities of meat after cooking (Foegeding and others 1996). By understanding the mechanism and key factors that influence muscle protein functionality, it is possible to manipulate product formulations and processing conditions to improve the quality of existing or new products.

Non-meat ingredients and meat marination

Other than the meat block, which is the major part of the product, non-meat ingredients can also be applied to achieve unique characteristics in the final product. They can be classified based upon their functional properties. These ingredients include spices and flavorings, sweeteners, salts including curing compounds, water, extenders and binders, starter cultures, alkaline phosphates, and synthetic antioxidants. Of these non-meat ingredients, salt and alkaline phosphates are of interest for this research.

Many of the physical properties of meat and poultry, including color, texture, firmness, juiciness, and tenderness of cooked meat, are partially dependent on the meat water-holding capacity. Higher water holding improves meat quality by contributing to consumer perception of juiciness. In addition, from the processor's viewpoint, increased water holding capacity is associated with increased product yield and therefore profits (Smith and Acton 2001).

Marination is a process in which liquid and other non-meat ingredients are added to meat before cooking. Many commercial meat and poultry products are marinated by still marination, tumbling, blending and mechanical injection. Benefits from marination include increased product juiciness, tenderness, and yield. In addition, flavor is enhanced both by the addition of spices and flavorings and by reduction in rancidity that may develop during storage or after cooking.

Salt and alkaline phosphates are basic non-meat ingredients that are commonly incorporated into the marinade solution. Sodium chloride is not only used for preservation and flavor enhancement but also for myofibrillar protein solubilization. Solubilized protein is important for processed meat products because it enhances binding and stabilizes water and fat in emulsified products. Salt also increases water holding capacity of processed meat products (Foegeding and others 1996). However, quality of the salt is an important concern because some salts might contain heavy metals, which accelerate lipid oxidation. Xiong and Kupski (1999) demonstrated that 8% salt promoted moisture absorption in chicken broiler breast. They explained that salt enhanced moisture retention because of an increase in capillary force that enabled water to be chemically bound or physically entrapped in the muscle. In addition, the effect of salt on

microstructure of pork was studied by Bocker and other (2005). They observed expansion of muscle tissue when salt was added up to 3%. However, the structure started to shrink when salt concentration was doubled to 6%. They concluded that salt affects the secondary structure of myofibrillar protein.

Alkaline phosphates also contribute to the increase in water retention in processed meat products. Alkaline pyrophosphates act as a fluidizing agent in muscle, dissociating actin and myosin, changing ionic strength and pH, which leads to increased water uptake (Xiong and Kupski 1999). The end result is less moisture loss during the cooking along with increased tenderness and juiciness. The final product yield is dependent on the type of alkaline phosphate, for instance, tetrasodium pyrophosphate, tetrapotassium pyrophosphate, sodium tripolyphosphate, and hexametaphosphate (Dziezak 1990). Alkaline pyrophosphates contribute to the highest final product yield and increase the rate of marinade absorption compared to tripolyphosphate and hexametaphosphate (Xiong and Kupski 1999, Zheng and others 1999). The action of phosphates in improving water holding capacity appears to be twofold: firstly, increasing the pH, and secondly, causing an unfolding of muscle proteins, consequently creating more space among protein molecules and sites available for additional water to bind (Pearson and Gillett 1999). Froning and Sackett (1985) suggested that phosphates increased the extractability of myofibrillar proteins, particularly the combination of tripolyphosphate and hexametaphosphate. The myofibrillar proteins have the ability to attract and bind water because of the increase in their negative charges and space between myofilaments.

Based on USDA regulations, the total amount of phosphate added to the finished product can not exceed 0.5% (Smith and Acton 2001). Marinade pickup by the muscle

thus determines the amount of allowable phosphate in the marinade solution. The target level of salt in the marinade or muscle is not regulated and is determined by the processor. Marinade pickup is calculated as follows:

$$\% \text{ marinade pickup} = \frac{\text{marinated weight} - \text{initial weight}}{\text{initial weight}} \times 100$$

A combination of salts and polyphosphates in marinade solution are commonly used to enhance the tenderness of meat products (Richardson and Mead 1999). The effect of various combinations of salt (0-2.25% meat weight basis) and sodium tripolyphosphate (0-0.5% meat weight basis) was observed in restructured pork (Schwartz and Mandigo 1976). Use of these non-meat ingredients together at levels of 0.75% salt and 0.125% alkaline phosphate was recommended and desirable for water holding capacity, cooking yield, and juiciness of restructured pork.

Salmonella spp.

Foodborne disease is caused by consuming contaminated foods and beverages. The causative agents can range from microbial pathogens, which include bacteria, viruses, and parasites, to harmful toxins and chemicals. *Campylobacter*, *Salmonella*, and *Escherichia coli* O157:H7 are the most commonly recognized foodborne infections caused by bacteria (CDC 2005b).

Salmonella has been known to cause illness for over 100 years. It was discovered by an American bacteriologist named D.E. Salmon after whom it is named. *Salmonella* is widespread in the intestines of birds, reptiles, and mammals. In addition, it can also enter natural environments through human or animal excretion. Humans can acquire

Salmonella through various meat and poultry products that have been mishandled or improperly processed. Controlling and minimizing bacterial contamination is of interest among processors and food safety researchers because of the increase in foodborne outbreaks (FDA-CFSAN 1992).

Characteristic of Salmonella

The genus *Salmonella* belongs to the family *Enterobacteriaceae*, known as enteric bacteria. *Salmonella* is a rod-shaped, nonsporeforming, Gram negative, facultative bacterium, measuring 0.5 μ x 2-3 μ that exists singly, in pairs, or in short chains. *Salmonella* is a motile organism (with nonmotile exceptions of *S. gallinarum* and *S. pullorum*) that typically possesses 1-5 flagella. Over 2500 serotypes of *Salmonella* have been identified of which approximately 2000 serotypes are pathogenic to humans. *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Enteritidis (*S.* Enteritidis) are the most common in the U.S. with half of all salmonellosis cases caused by these two serotypes (CDC 2005c).

Salmonellae live in the intestinal tracts of warm and cold blooded animals including humans and birds and are widespread particularly in poultry and swine. Reptiles are likely to harbor *Salmonella*. Therefore, those who come into contact with these animals should follow good practices including handling and cleaning. *Salmonella* may be found in the feces of some pets, especially those with diarrhea. Environmental sources of the organism include water, soil, animal feces, insects, as well as commercial and home food preparation areas (FDA-CFSAN 1992).

Transmission and manifestation of salmonellosis

Salmonellosis is an infection caused by *Salmonella*. Transmission of the organism is through contaminated food, water, or contact with infected animals or their feces. Contaminated foods are often meat and poultry products, milk, eggs, and produce. Food may become contaminated by the unwashed hands of an infected food handler.

Most persons infected with *Salmonella* have symptoms of diarrhea, fever, and abdominal cramps. The illness develops 12 to 72 h after infection and usually lasts 4 to 7 days. The infective dose can be as low as 15-20 cells depending on the health of the host. Most persons recover without treatment; however, the elderly, infants, and those with weakened immune systems are more likely to have a severe illness (FDA-CFSAN 1992). Those who tend to have severe diarrhea may need to be hospitalized. In these patients, *Salmonella* infection may spread from the gastrointestinal tract to the blood stream and then to other organs, even resulting in death unless the patients are treated with antibiotics.

Salmonellosis outbreaks

Approximately 1.4 million cases of salmonellosis occur per year in the U.S.; of these about 30,000 are culture-confirmed cases reported to the Centers for Disease Control and Prevention (CDC), more than 500 are fatal cases, and 2% are complicated by chronic arthritis (CDC 2005c). Serotypes Typhimurium and Enteritidis have predominated in outbreaks with Typhimurium the most commonly isolated serotype since 1997. The three other most common serotypes are Enteritidis, Newport, and Heidelberg (CDC 2005a).

Poultry is an important source of human *Salmonella* infections. The following are examples of several outbreaks associated with poultry consumption:

- In 1982, outbreaks of foodborne salmonellosis caused by improperly cooked and stored poultry giblets for Thanksgiving occurred in Maine. One hundred-twelve culture-confirmed cases of *Salmonella* Enteritidis were identified in patients after consuming turkey from a restaurant.

- In 1985, an estimated 351 children and staff at a Georgia elementary school developed gastroenteritis. *Salmonella* Enteritidis was isolated from more than 100 children. The illness was strongly associated with eating turkey salad from the school cafeteria.

- In 1986, 2130 students and employees of an Oklahoma public school system developed symptoms of salmonellosis including diarrhea, nausea, vomiting, abdominal cramps, and fever. While consumption of chicken was associated with this outbreak, the exact cause remains unclear with other contributing factors including improper thawing, cooking, and/or storing processes.

- In 1990, an outbreak classic salmonellosis symptoms occurred during the holiday season in a Connecticut hospital. Consumption of improperly thawed and cooked turkey used for turkey salad, turkey sandwiches, and chef's salad served to patients was suspected as the cause.

In addition to poultry, other sources of salmonellosis include eggs, produce (e.g. tomatoes, leafy greens), other meats (e.g. ground beef, beef jerky), milk, as well as direct contact with animals and their environment. A recent multistate outbreak of salmonellosis in the U.S. emerged in a new food source, peanut butter, in 2007. The causative

organism, *Salmonella* serotype Tennessee, was reported in 47 states since August 2006. As of May 2007, a total of 628 persons were infected after consuming peanut butter that was traced to one manufacturing facility (CDC 2007).

Salmonella serotypes Typhimurium and Enteritidis have both declined substantially (28% and 34%, respectively) since 1995. The total number of *Salmonella* isolates has also declined during this period, though not as substantially as serotypes Typhimurium and Enteritidis (CDC 2005a).

Food safety guidelines and regulations

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) adopted a document entitled, "HACCP Principles for Food Production," in 1989 and defined HACCP as "a systematic approach to be used in food production as a means to assure food safety." Therefore, to produce safe food, a management system under HACCP program has to cover the analysis and control of biological, chemical, and physical hazards from raw material production, procurement and handling, to manufacturing, distribution and consumption of the finished product (Scott and Stevenson 2006).

A final Food Safety and Inspection Service (FSIS) rule for sanitation performance standards was established in 1999, which is applicable to all meat and poultry plants. Establishments that follow this guide can be certain that they are meeting the sanitation performance standards. However, it is not a requirement to perform the specific sanitary practices described in this document (USDA-FSIS 1999).

In 1999, FSIS required that certain ready-to-eat (RTE) products (cooked/roast beef products, fully cooked, uncured meat patties, and certain fully cooked poultry products) meet three performance standards: lethality, stabilization, and handling. For the lethality standard, the FSIS did not require that any specific technique or process be used to meet the standard; however, for cooked products, the FSIS did propose to require a heat treatment (USDA-FSIS 1999). In 2005, the FSIS documented time and temperature tables used for cooking ready-to-eat poultry products that the industry may follow (USDA-FSIS 2005).

Pathogen reduction performance standards are based on the prevalence of *Salmonella* found by the agency's nationwide microbiological baseline studies. FSIS selected *Salmonella* as the target organism because, firstly, it is one of the most common causes of foodborne illness associated with meat and poultry products. Secondly, *Salmonella* lives in the intestinal tract of many animals destined for human consumption. Thirdly, current methods can recover *Salmonella* from various meat and poultry products, and finally, reducing the presence of *Salmonella* will also eliminate or adequately reduce other vegetative pathogenic microorganisms in the product. In August 2006, the FSIS published the first "Compliance Guideline for Controlling *Salmonella* in Poultry". This guideline, which targets small and very small poultry plants, describes concerns and validates controls for each step in the broiler slaughter process (USDA-FSIS 2006).

Bacterial penetration

It is often assumed that the interior of intact, undamaged whole muscle is free from microorganisms. Sources of contamination of meat are through evisceration,

slaughtering instruments, and harvest environment, animal hide and intestines, water, and subsequent handling (Elmossalami and Wassef 1971). Several studies have demonstrated bacterial migration into the inner portion of whole muscle during processing.

Penetration of microorganisms into muscle depends on various factors such as humidity, temperature, histological structure of meat, and characteristics of the bacteria. Gill and Penney (1977, 1982) concluded that significant migration was due to proteolytic activity and mobility of organisms and incubation temperature with the rate of penetration increasing with incubation temperature. *S.* Typhimurium, a motile and proteolytic pathogen, was found to travel further into beef muscle at 37 °C as opposed to 20 °C. *S.* Enteritidis also migrated greater distances below the surface as time increased (Elmossalami and Wassef 1971). Work done by Thomas and others (1987) supported these results and they also indicated that water availability in the extracellular space contributed to an increase in penetration rate. Bacterial migration into the interior portion of meat is related to the water holding capacity of meat proteins. Moreover, meat that has undergone freeze-thaw cycles tends to be more susceptible to bacterial penetration (Sikes and Maxcy 1980). Fiber orientation is another factor that plays an important role in the depth of penetration (Sikes and Maxcy 1980). The penetration is more extensive when the inoculum was applied vertically to the meat grain as opposed to horizontal application.

If a mechanical means is used to tenderize and/or infuse flavor into meat products, a potential risk may be posed through this physical action by carrying contaminants from the surface into the interior. The transferred bacteria may proliferate and increase in number inside the tenderized meat products. Phebus and others (1999) showed that up to

4% of an *E. coli* O157:H7 inoculum was carried by the blades into the interior of surface-inoculated beef sirloins during tenderization. Interior portions of blade tenderized beef roasts have shown measurable aerobic plate counts with greater contamination on the exterior portions (Raccach and Henrickson 1979). Aerobic and anaerobic plate counts demonstrated the translocation of bacteria after four passes through a needle tenderizer (Boyd and others 1978). However, without blade tenderization, a significant microorganism penetration was also observed. Warsow and others (2007) evaluated the potential for *Salmonella* migration into intact whole muscle turkey breasts during still marination. *Salmonella* counts in samples increased with application of vacuum and decreased with a below the inoculated surface increased.

The hydrodyne process (patent number 5,273,766 and 5,328,403) is an emerging, non-thermal process for improving meat tenderness with this process reducing shear force as much as 72% (Solomon and others 1997). While hydrostatic pressure (100-1000 MPa) from high pressure processing (HPP) is used as a non-thermal pasteurization approach (NACMCF 2004), the hydrodyne process is a hydrodynamic pressure processing that uses only a small amount of a high energy explosive to generate supersonic shock waves upon detonation in a liquid medium. The mechanical force of the shock waves that produce tenderization may also cause mechanical stress on bacteria. However, only a slight reduction in the *E. coli* O157:H7 population was found in ground beef when this process was applied (Podolak and others 2006). Moreover, the tenderization achieved by the hydrodyne process allowed *E. coli* to move from the outer inoculated surface into the beef muscle due to disruption of muscle fibers by the wave

force (Lorca and others 2002, 2003). This technique is now commercially used to eliminate pathogens from processed RTE meat products (Hayman and others 2004).

Bacterial attachment

The tissues under the hide of healthy animals are essentially sterile prior to slaughter (Elmonssalami and Wassef 1971; Niven 1987); however, contamination often occurs during processing. During slaughter, surface tissue can be exposed to microorganisms, including pathogens, and become contaminated. Cut surfaces are susceptible to bacterial penetration because they have no membranous surface to hold its cells together and protect the interior (Anderson and others 1992). There are several processing procedures that facilitate bacterial migration. The attachment of bacteria to meat after migration or contamination is a very complex process (Notermans and Kampelmacher 1983). Bacterial structures involved in attachment include extracellular polymers, fimbriae (pilli) and flagellae. In addition to bacterial structures, attachment also depends on bacterial strain, bacterial concentration, type of meat surface or microtopography of the surface, and temperature (Firstenberg-Eden 1981).

Gill and Penney (1977) indicated that, after surface inoculation, *Salmonella* likely penetrated into tissue between the muscle fibers and later confirmed that organisms are usually located between the muscle fibers and the surrounding endomysium (collagen fiber layers) (Gill and Penney 1982). In addition, some bacteria were also observed within the endomysium. Lorca and others (2002) located bacterial cells in muscle after hydrodynamic treatment by using a green fluorescent protein (GFP)-labeled *E. coli* strain coupled with laser scanning confocal microscopy (LSCM). These GFP-labeled cells were

seen in crevices between muscle bundle fibers. Their work is consistent with the work of Prachaiyo and McLandsborough (2000), who indicated that, after inoculated meat surface for 5 min and washed 3 times with phosphate-buffered saline, GFP-labeled *E. coli* associated within the surface or sarcolemma of individual muscle fibers or within crevices of raw beef muscle (in the spaces between and on the surface of muscle fibers).

Bacterial thermal inactivation

Thermal inactivation of a microorganism has been previously expressed in terms of *D* and *z* values. These values are calculated assuming microbial inactivation follows first order kinetics. *D* value or decimal reduction time is defined as the time required to destroy 90% of the population, which indicates the thermal stability/resistance of a specific organism in a specific medium or food at a constant temperature. A *z* value is the temperature increase required to reduce the thermal death time by 90% (Doyle and others 2001). In general, *D* values for *Salmonella* at 60°C range from 5-6 min in chicken, 5-13 min in turkey, and 3-5 min in beef. In most studies, the *z* values for *Salmonella* ranged from 5.0-6.5°C (Orta-Ramirez and Smith 2002).

Previously, the USDA established time/temperature protocols with specific endpoint temperatures for commercial thermal processes. In 1999, USDA-FSIS finalized the regulations for the production of certain meat and poultry products. The USDA-FSIS lethality performance standards for RTE products are based on *Salmonella*. Although *E. coli* O157:H7 is of greater concern, especially due to the higher number of outbreaks associated with consumption of ground meat products, it is less heat resistant than

Salmonella. In contrast, *Listeria monocytogenes* is typically more heat resistant than *Salmonella*; however, contamination occurs during post-processing steps.

The lethality performance standards for thermal processing are expressed in terms of specific decimal reductions or $\log_{10}$ reductions (USDA-FSIS 1999). The USDA requires a process to achieve a 6.5-$\log_{10}$ reduction in RTE cooked beef, roasted beef, and cooked corned beef and a 7-$\log_{10}$ reduction in RTE poultry products (USDA-FSIS 1999). A cocktail of *Salmonella* serotypes consisting of pathogenic strains that exhibit relatively high heat resistance and have been previously implicated in foodborne outbreaks is recommended in pathogen thermal inactivation studies used to validate a thermal processes (USDA-FSIS 1999).

Various factors affecting bacterial thermal resistance have been documented, including meat species, product composition (e.g., carbohydrates, fat content), water activity, pH, salt and other additives, pathogen species and strains, growth temperature, stage of bacterial growth, initial population load, heat shock, and methodology used for detection of survivors. Generally, thermal resistance of bacteria is higher in meat products than in other model systems, such as buffer solutions, peptone, and agar (Juneja and others 1995, 2001). Murphy and others (2000) reported increased thermal resistance of *Salmonella* in food products compared to laboratory media. Physical arrangement of various components within the food matrix might also cause differences in bacterial thermal resistance. When Orta-Ramirez and others (2005) and Velasquez and others (2005) studied *Salmonella* heat resistance, beef and pork (whole or ground), respectively, a significantly greater heat resistance of *Salmonella* was seen when present in the whole muscle, as opposed to ground muscle products.

Doyle and Mazzotta (2000) suggested that bacteria location in a food (surface attachment vs. interior dispersion) may also affect the resistance of *Salmonella*. Bacteria attached to muscle tissue are more heat resistant than bacteria suspended in liquid media (Murphy and others 2000). When a cocktail of *Salmonella* was inoculated and heated in meat, including beef, pork, turkey, and chicken, *D* values were significantly higher than those in chicken broth. According to Juneja and others (2001), this effect may be attributed to poor heat transfer through the heating menstrum or other factors present in the process before cells were subjected to heat injury. Murphy and others (2000) compared the D-values in meat to those in a semi-liquid medium and found that the D-values were higher in ground chicken breast than in a 0.1% peptone-agar solution using temperatures ranging from 55 to 70 °C in a controlled temperature water bath. They also demonstrated that the sample size and shape affected thermal inactivation of *Salmonella* in ground chicken breast meat.

Higher levels of fat (Juneja and others 1997, Ahmed and others 1995, Veeramuthu and others 1998, Smith and others 2001), as well as the addition of additives such as salts, lactates, and phosphates (Kotrola and Conner 1997; Maurer 2001), may enhance thermal resistance of pathogens. In a study by Juneja and others (2000), asymptotic *D* values (*D* values for large times) were determined in ground beef containing different fat levels. Increased heat resistance of an 8-strain *Salmonella* was positively correlated to the fat content. According to Ahmed and others (1995), these higher *D* values were due to the reduction of water activity because bacteria suspended in fat are more difficult to destroy than those in aqueous media. The effect of water activity itself on thermal inactivation of *Salmonella* in ground turkey was also determined. As

water activity in the meat was reduced, thermal resistance of *Salmonella* significantly increased (Carlson and others 2005).

Salt is a typical ingredient frequently added to meat products. Many studies have shown that high concentrations of salt increase thermal resistance of *Salmonella*. When heated at less than 63.5 °C, the lethality rate for *Salmonella* increased with increasing concentrations of salt and pyrophosphate. However, heat resistance of *Salmonella* was unaffected by sodium lactate (Juneja and other 2003). The effect of sublethal stress (heat-, cold-, and starvation-injured) on *Salmonella* thermal inactivation kinetics was evaluated in ground turkey (Wesche and others 2005). The results indicated that heat-shocked *Salmonella* (54 °C for 30 min) were more thermal resistance; however, no effects were observed when cells were subjected to cold shock or starvation stress.

Microscopy in food research

The microscope is a powerful tool in food science research revealing structural details beyond what the naked eye can see. Microscopic examination most often employs the use of either light microscope or the electron microscope. For most routine work, the light microscope is helpful. For special research purposes, especially in studies on internal cell structure, an advanced microscopy technique such as electron microscopy (i.e. transmission electron microscopy and scanning electron microscopy), is required in addition to the light microscope (i.e. fluorescence microscopy and confocal scanning laser microscopy) (Madigan and others 2000).

Transmission electron microscopy (TEM) has been used to study the ultrastructural changes in muscle foods because high resolution of 0.2 nm is achieved

compared to 200 nm using conventional light microscopy (Flegler and others 1993). Electromagnets function as lenses and electrons are used instead of light rays, in a high vacuum system. However, to be able to view internal cell structures, special techniques of thin sectioning are required to allow the electron beam to penetrate the sample. TEM of thin sections is appropriate for viewing inter- and intracellular structures and for measuring sarcomere length and spacing to intermyofibrillar connections (Silva and others 1992). Birkhold and Sams (1995) studied the ultrastructural changes induced by high voltage post-mortem electrical stimulation and muscle tension in broiler chickens using TEM. This technique was also used to study changes in chicken skin morphology after specific processing stages, such as scalding, picking, and chilling (Kim and others 1993). Using the same technique, Pohlman and others (1997) demonstrated microstructure differences in beef muscle subjected to ultrasound and conventional cooking treatments. Even though, high resolution is achieved using TEM, the technique requires extensive sample preparation. Light microscopy, in contrast, involves simple sample preparation and specimens can be thicker compared to electron microscopy.

Fluorescence microscopy is most often in studying the location and movement of molecules and subcellular components in the cell. This technique is used to study specimens that can be made to fluoresce. Usually, cellular components do not fluoresce themselves. Fluorescent markers are therefore introduced so that the labeled material will emit energy detectable as visible light when irradiated with the light at a specific wavelength. The use of fluorescent dyes, immunofluorescence, and tagging of proteins can be applied to allow the sample to fluoresce and be viewed under the fluorescence microscope.

In 1994, the use of green fluorescent protein (GFP) as a cell and tissue marker was developed (Chalfie and other 1994). GPF is a small protein (27 kDa) found in the jellyfish *Aequorea victoria*. This protein is fluorescent with absorption and emission peaks at 395 nm and 509 nm, respectively. Therefore, GFP-transformed organisms can be easily seen under the conventional fluorescence microscope and confocal filter sets (Cubitt and others 1995). High resolution optical techniques can be used non-invasively to monitor dynamic activities, three-dimensional arrangement, and behavior of living cells (Paddock 1999). GFP has been expressed in several bacterial pathogens including *E. coli* O157:H7 (Fratamico and others 1997), *Listeria monocytogenes* (Freitag and others 1999), and *Salmonella* (Cho and Kim 1999). Prachaiyo and McLandsborough (2000) observed no difference in behavior of GFP-transformed *E. coli* O157:H7 and parental strains. The growth characteristics and surface properties including hydrophobicity and cell surface charge of organisms were not affected by introducing GFP into the plasmid. Noah and others (2005) also observed comparable growth characteristics of GFP-transformed *Salmonella* to the parental strains. In addition, the transformed strains maintained their fluorescence following a series of culture transfers and after refrigeration (4 °C) for up to 12 days.

Other than the conventional fluorescence microscopy, the tagged protein can also be seen under confocal scanning laser microscopy (CSLM). CSLM has been used to study the location and viability of bacteria in food products. The location of *Salmonella* attached to poultry skin was determined using CSLM after staining with Pyronin Y (Kim and others 1996). Using the same technique, Takeuchi and other (2000) evaluated attachment of *S.* Typhimurium to lettuce stained with fluorescein isothiocyanate. Wong-

Liong and others (1997) stained eggshell membranes with fluorescein isothiocyanate to observe the interaction of *S.* Enteritidis with eggshell and the migration of *Salmonella* into the egg membrane.

Microscopy is a valuable tool and has been extensively applied into a wide range of scientific fields. There are a variety of microscopes and techniques available. Since new techniques are being developed, the suitable tool for individual food research must be wisely chosen in order to support the finding in the chosen medium.

CHAPTER 3

EFFECTS OF MARINATION ON *SALMONELLA* PENETRATION AND MUSCLE STRUCTURE OF TURKEY BREAST

INTRODUCTION

Deep muscle tissue from healthy animals is generally sterile indicating that the interior of intact, undamaged whole muscle should be free from microorganisms (Elmossalami and Wassef 1971). However, contamination of the meat can occur through many routes including contact with slaughtering instruments, the environment, animal hide, fecal materials, and water.

Many meat and poultry products sold in retail markets are value added by means of marination and/or mechanical tenderization. Tenderizing techniques, including blade and needle tenderization can carry bacteria from the meat surface into the interior (Phebus and others 1999; Raccach and Henrickson 1979; Boyd and others 1978). Warsow (2007) evaluated the potential for *Salmonella* penetration into vacuum tumbled and still-marinated intact whole muscle turkey breasts. Numbers of *Salmonella* increased with vacuum processing with lower numbers seen in the center of the samples. Cut tissue (Anderson and others 1992) and meat that has undergone multiple freeze-thaw cycles (Sikes and Maxcy 1980) are more prone to bacterial penetration. Such newly introduced bacteria may proliferate in the interior of the muscle tissues as the nutrients and environment permit (Raccach and Henrickson 1979), thus leading to concerns regarding subsequent thermal inactivation during cooking.

Marination is one of the value-added processes that affords convenience and variety for consumers while increasing profits for the processor by improving product

yield. To control fluid loss, salt and alkaline phosphates are commonly dissolved in the marinade. Tumbling is a process used to increase marinade uptake and promote tenderization of meat (Chen 1982, Xiong and Kupski 1999). In addition, a vacuum is often applied during tumbling to improve marinade distribution and speed of penetration into the product. However, the increased water content between muscle fibers has been reported to increase bacterial penetration (Thomas and others 1987; Sikes and Maxcy 1980).

Various hypotheses have been proposed for the mechanisms by which bacteria attach to meat surfaces. Several microscopic and sample preparation techniques are available to observe these mechanisms. Use of a marker protein (or tagging protein) attached to the bacteria of interest coupled with fluorescence microscopy is one useful method. Green fluorescence protein (GFP) is a fluorescent protein with absorption and emission peaks at 395 nm and 509 nm, respectively. Therefore, GFP-transformed microorganisms can be seen using fluorescence microscopy. GFP has been successfully expressed in several bacterial pathogens, including *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* (Fratamico and others 1997; Freitag and others 1999; Cho and Kim 1999). The GFP expressing strains have been confirmed to have behaviors that are not significantly different from parental strains (Prachaiyo and McLandsborough 2000).

Consumer trends show an increasing demand for ready-to-eat products, which include marinated whole muscle foods (Russell 2002). One concern when the marinade is absorbed into the interior of value-added meat and poultry products is the potential migration of bacterial pathogens from the meat surface into the interior. In response to

these concerns, the specific objectives for this study were to (1) evaluate the effects of marinade composition, microbial load, and contamination stage on multidirectional migration of *Salmonella* into intact whole muscle turkey during marination; (2) assess changes in turkey muscle microstructure after marination; and (3) determine the location of GFP-labeled *Salmonella* in fresh whole muscle turkey tissue after marination in an inoculated marinade.

MATERIALS AND METHODS

Multidirectional penetration of *Salmonella* into intact whole muscle turkey breast

Preparation of turkey breast samples

Fresh, whole muscle, boneless, skinless turkey breasts (approximately 1.5-2 kg) were obtained from a local grower and packer in a single large lot to eliminate lot-to-lot variability. The muscles were vacuum packaged individually in plastic bags (Cryovac Sealed Air Corp., Duncan, SC, USA), frozen at -20 °C and irradiated (~10 kGy, CFC Logistics, Quakertown, PA, USA) to eliminate the natural microflora. When the irradiated whole muscle turkey breasts were used in the study, they were assessed for background flora after thawing by diluting 1-g samples 1:5 in 0.1% peptone water followed by plating in duplicate on Petrifilm™ aerobic count plates (3M Corp., St. Paul, MN, USA) followed by incubation at 37 °C for 48 h.

Marinade preparation

Two marinade formulations were typical marinade solution containing 3.2% NaCl (EMD Chemicals Inc., Gibbstown, NJ, USA) and 0.8% phosphate solution (Butcher and Packer Supply Company, Detroit, MI, USA) calculated to target 0.4% NaCl and 0.06% phosphate in the marinated product and a high concentration marinade containing 10.2% NaCl and 4.05% phosphate calculated to target 1.25% NaCl and 0.5% phosphate in marinated product. A 520 mL aliquot of marinade was poured into glass bottles with screw caps and autoclaved for 15 min at 121 °C to ensure sterility. All autoclaved marinades were stored at room temperature (25 °C) until used. The pH of low (7.39) and

high (7.65) marinade concentrations were measured using a Corning 145 pH meter (Corning, Medfield, MA, USA).

Preparation of test organisms

The following eight serovars of *Salmonella* were obtained from Dr. V.K. Juneja (USDA-ARS, Eastern Regional Research Center, Philadelphia, PA, USA): *S.* Thompson FSIS 120 (chicken isolate), *S.* Enteriditis H3527 and H3502 (clinical isolates phage types 13A and 4, respectively), *S.* Typhimurium DT 104 H3380 (human isolate), *S.* Hadar MF60404 (turkey isolate), *S.* Copenhagen 8457 (pork isolate), *S.* Montevideo FSIS 051 (beef isolate), and *S.* Heidelberg F5038BG1 (human isolate). These serovars have shown moderate to high thermal resistance and been implicated in outbreaks (Juneja and others 2001). All serovars were stored at -80 °C in tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol.

The cultures were propagated by weekly transferring one loop of frozen culture to 9 mL of sterile TSB. The cultures were maintained by daily transfer to fresh TSB followed by incubation for 18-24 h at 37 °C, with two consecutive transfers prior to use. All strains were maintained separately and then combined on the day of experiment. After centrifugation at 6000 x *g* for 20 min at 4 °C to obtain an 8-servovar cocktail, the supernatant was decanted and the cell pellet was suspended in 500 mL of sterile marinade to give a final bacterial concentration of approximately 10^8 or 10^4 CFU/mL.

Exposure to inoculated marinade

Frozen turkey breast was thawed at 4 °C for 2 days prior to the experiment. Whole turkey breasts were transferred to a sterile stomacher bag (Fisher Scientific, Pittsburgh, PA, USA), and inoculated marinade was added at 14% w/w. The bag was tied, placed in another bag to ensure sealing, and tied again before placing into a laboratory-scale vacuum tumbler. After vacuum (100 kPa) was drawn, the tumbler vessel was rotated for 20 min at 4 °C.

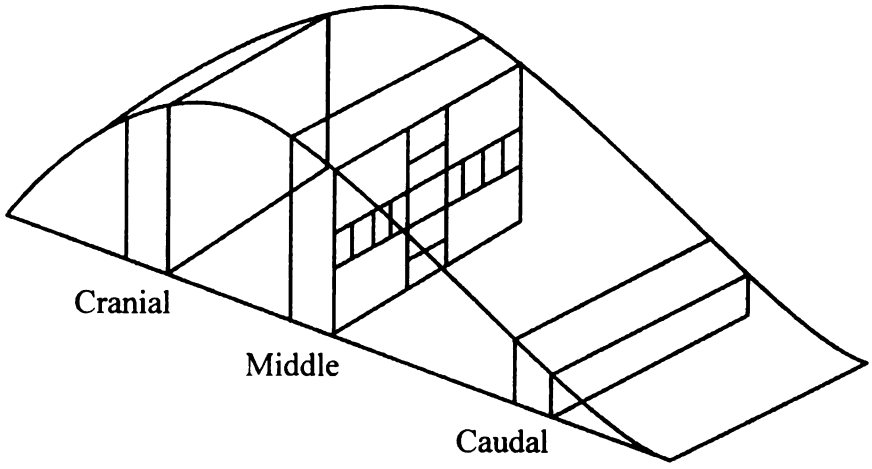
Alternatively to simulate contamination during processing, 20 mL of inoculated marinade was added drop wise onto a sterile tray, after which the turkey was placed on the contaminated surface and immediately transferred to a sterile bag. After adding sterile marinade at 14% w/w, the bag was tied, double bagged, and vacuum tumbled for 20 min. To simulate contamination after marination, breast muscle was tumbled with sterile marinade (added at 14% w/w), removed from double bags after tumbling, and placed with the cut surface and the opposite side down on a tray contaminated with 20 mL of inoculated marinade to simulate a contaminated food contact surface.

Sampling and recovery

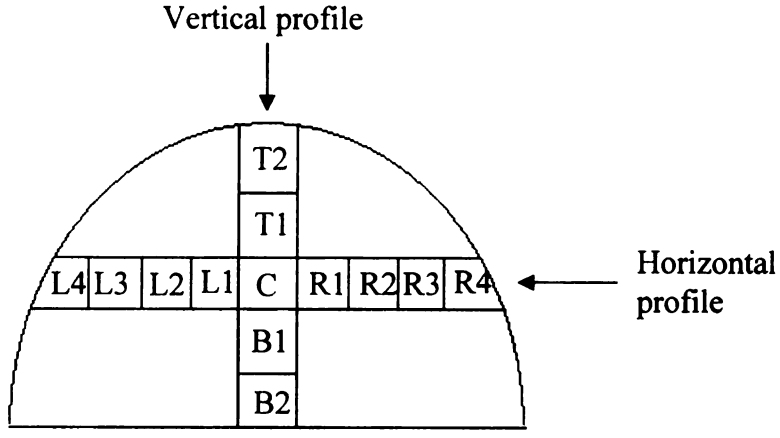
Three 1 cm-thick slices, defined as cranial, middle, and caudal, were removed from turkey breast muscle (Figure 3.1-a). The middle slice was taken at the thickest part of the breast. Cranial and caudal slices were sectioned at approximately 5 and 10 cm, respectively, measured from both ends. Then 1-cm cubes were excised from those slices as illustrated in Figure 3.1b. Horizontal cubes were labeled L4, L3, L2, L1, R1, R2, R3, and R4, whereas vertical cubes were labeled T1, T2, B1, and B2. The sampling plan

design was used to generate a quantitative 3-D *Salmonella* migration pattern. *Salmonella* was recovered by serially diluting the cubes in 0.1% peptone water, plating in duplicate on Petrifilm™ aerobic plates and incubating at 37 °C for 48 h before counting. The minimum detection level was 10 CFU/g due to the actual bacterial count on 1:10 dilution plating.

A self-sterilizing electrosurgical unit (ESU) (Surgistat™ II-20, Valleylab™, Boulder, CO, USA) with a setting at 120 W coagulation and 40 W in a pure cut mode was used for al dissections as reported by Warsow and others (2003). An advantage of this innovative ESU protocol over traditional excision methods, confirmed by a preliminary study, is that bacterial transfer from the outer to inner surfaces of the sample is eliminated by the tissue cauterizing blade. However, the heat generated by the ESU may inactivate some surface and near-surface organisms, resulting in a slight underestimation of microbial penetration during marination. Thus, bacterial counts from two excision methods (ESU vs. traditional) were compared to ensure that heat generated by ESU did not destroy the target pathogen. Warsow (2003) demonstrated that although the ESU maintained a sterile cutting surface, the instrument did not supply sufficient heat to significantly affect bacterial counts within the sample.



(a)



(b)

Figure 3.1 Sampling protocol (a) 3 slices, cranial, middle, and caudal, excised from marinated whole muscle turkey breast (b) 1-cm cubes dissected from the slices. Segments labeled R1, R3, L1, L3, T1, and B1 were dissected for cranial and caudal slices because cubes T2, B2, R4, L4 did not exist since these pieces are smaller and segments labeled C, R2, R4, L2, L4, T2, and B2 were dissected for middle slice.

Data analysis

Each treatment, from microbial load, marinade concentration, and contamination stage treatments, was replicated three times. The *Salmonella* migration pattern was determined by plotting the logarithm of the surviving cells vs. cube identification. Analysis of Variance (ANOVA) was run to evaluate the effect of treatment factors and sample location on the migration of *Salmonella*. Tukey-Kramer's test (α=0.05) was performed to compare means. Statistical analyses were performed using JMP statistical package (Version 3.2.2, SAS Institute, Inc., Cary, NC, USA).

Microstructure of marinated whole muscle turkey breast

Preparation of turkey breast samples

Irradiated frozen turkey breasts (boneless and skinless, approximately 1.5-2 kg) were thawed 48 h at 4 °C. A Warner-Bratzler hand-coring device (1.27-cm diameter, G.R. Electrical Mfg. Co., Manhattan, KS, USA) was used to aseptically remove cores from the top of the turkey breast (2-3 cores/treatment) (Warsow and others 2008). Cores were submerged into the two marinades (3.2% NaCl + 0.8% phosphate solution and 10.2% NaCl + 4.05% phosphate) for 20 min at 4 °C with the remaining cores serving as untreated controls, placed in Petri dishes, and sectioned, using a sterile surgical blade into smaller pieces measuring approximately 2 x 4 x 2 mm (W x L x H). The treated samples were then placed into small vials (5-6 pieces/treatment) in preparation for microscopic examination.

Sample preparation for bright-field microscopy (BFM)

Fixation:

Treated samples were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde and 2.0% (v/v) paraformaldehyde and stored at 4 °C for 5 d. Samples were then rinsed in cacodylate buffer (0.1 M, pH 7.4) and post-fixed in 0.1 M cacodylate buffer containing 2% (v/v) osmium tetroxide to preserve the lipids and maintain the three-dimensional structure. After 24 h of dehydration, the sections were subjected to three 15-min rinses in cacodylate buffer and dehydrated using 30 to 100% acetone.

Infiltration and embedding:

Samples were infiltrated with Poly/Bed 812 resin and acetone at ratios of 1:3, 2:2, and 3:1, followed by the pure resin. Samples were rotated for each step at least for 2 h and up to 1 d. To provide support during ultramicrotomy and retain spatial organization of the specimen sections, samples were placed in a mold, embedded in Poly/Bed 812 resin, and polymerized in an oven at 60 °C for 24 h.

Thick sectioning and staining:

Thick sections were used to determine how the tissue samples were affected by different salt and phosphate concentrations in the marinade solutions. After trimming blocks surfaces with a razor blade to expose the tissue, the embedded tissue was sectioned with a MTX ultramicrotome (Boeckeler Instruments, Tucson, AZ, USA) to 500-1000 nm using a glass knife. These thick sections (5-6 pieces) were mounted on

glass slides and stained with Epoxy Tissue Stain (ready-to-use Toluidine Blue and Basic Fuchsin, Electron Microscopy Science, Hatfield, PA, USA) on a hot plate for approximately 20-30 s. Observation of the stained sections was done under a confocal laser scanning microscope (Zeiss LSM5 Pascal, Thornwood, NY, USA) using a 633 nm laser to obtain a transmitted image.

Image analysis:

Digital images of muscle structure obtained from bright-field microscopy were processed using Matlab Image Processing Toolbox. The threshold value was calculated by using the "Otsu method", a function available in the Toolbox. Subsequently this threshold was used to determine "white" and "black" pixels, i.e., when the values are below or above the threshold, respectively. "White" space was converted into percentage of extracellular muscle tissue space by dividing the number of "white" pixels by the total number of pixels in the image. The extracellular spaces of treated samples (TC and HC) were then compared to the untreated samples (control). Five images per treatment were taken digitally. Significance was determined at the 95% confidence level for all mean comparisons. In addition, the muscle cell diameters from each treatment were averaged and compared to that of the control sample. Only muscle cells that were shown in full structure were determined for diameters using a ruler.

Sample preparation for transmission electron microscopy (TEM)

TEM sample preparation involved the following two extra steps from that of BFM.

Thin sectioning:

After re-trimming the blocks to reduce the sectioning area and improve thin section quality due to compression and wrinkle, sections were obtained with an MTX ultramicrotome using a diamond knife (90-110 nm thickness, 0.7 mm/sec cutting speed, DDK, Delaware Diamond Knives, USA) and collected on 300-mesh copper grids.

Positive staining:

Uranyl acetate (2% (w/v) in 50% ethanol) and lead citrate (Reynolds formulation) were used as positive stains. Grids were placed section side down on a drop of uranyl acetate (1 drop/grid) placed on a piece of Parafilm and covered with a Petri dish. Grids were incubated for 10 min, rinsed with distilled water, and allowed to air dry. On another piece of Parafilm, sodium hydroxide pellets were placed inside a Petri dish to create a CO_2-free environment. Grids were placed section side down on the lead citrate (1 drop/grid) and incubated for 15 min before being rinsed with distilled water, dried, and viewed under a transmission electron microscope (JEOL 100CX, Japan) at an accelerating voltage of 100 kV.

***Salmonella* location in fresh whole muscle turkey breast tissue**

Preparation of fresh whole muscle turkey breast tissue

Fresh, commercially processed, whole muscle turkey breast (skinless and boneless) was obtained from a local grower and packer and used immediately. To visualize a cross section, turkey breast tissue was excised to measure approximately 1 cm x 1 cm x 0.3 mm (W x L x H) using a scalpel.

Preparation of green fluorescent protein (GFP)-*Salmonella* inoculum

GFP- labeled *Salmonella (S.* Typhimurium DT104 ATCC 700408/ISSAGFP) was obtained from Dr. John Sofos, Department of Animal Science, Colorado State University (Fort Collins, CO, USA) and stored frozen at -80 °C in TSB containing 10% (vol/vol) glycerol.

The culture was propagated by transferring one loop of frozen culture to 9 mL of sterile TSB supplemented with yeast extract (TSB-YE). The cultures were maintained by daily transfer (100 µL) to fresh TSB-YE followed by incubation for 18-24 h at 37 °C, with a minimum of two consecutive transfers prior to use.

Preparation of GFP-*Salmonella* inoculated marinade

After centrifuging the TSB-YE culture at 6000 *x g* for 20 min at 4 °C, the cell pellet was suspended in 10 mL of marinade containing 3.2% NaCl and 0.8% phosphate. Subsequently, turkey samples were spot-inoculated on the upper side with 5 µL of marinade and incubated for 1 h at 4 °C to allow migration of *Salmonella.*

Cryostat sampling of inoculated sample block

Inoculated meat was placed in a mold at room temperature. An embedding medium (Tissue-Tek® OCT compound, Sakura Finetek USA Inc., Torrance, CA, USA) for frozen tissue specimens was used to support the tissue in the specimen block. After immersing the mold into liquid nitrogen for 5 min, the frozen tissue samples were transferred to a -20 °C cryostat chamber (Leica CM3050, Leica Instruments GmbH, Wetzlar, Germany) and allowed to equilibrate for at least 2 h. The sample was

temperature equilibrated to that of the cryostat chamber. The frozen tissue was then mounted onto the specimen disc using OCT compound before being returned to the cryostat chamber. After the OCT hardened (approximately 20 min), the specimen disc was attached to the chuck holder and sectioned from the uninoculated side toward the inoculated surface to a thickness of 8 μm using disposable Teflon® coated microtome blades (DuraEdge Low Profile Blades, Source Medical Products, Lake Forest, IL, USA). The sections were transferred by contacting a dry microscope slide and mounted with PermaFluor mounting medium (Thermo Sandon, Pittsburgh, PA, USA) and a cover slip. The location of bacterial cells in turkey breast tissue was determined by fluorescent microscopy (Leica DMLB, Leica, Wetzlar, Germany) at 400X and 1000X magnification.

RESULTS AND DISCUSSION

Multidirectional penetration of *Salmonella* into intact whole muscle turkey breast

The overall results of this study demonstrated bacterial penetration into whole muscle turkey breast (Figures 3.2 to 3.8). Earlier research with bacteria penetration was limited to individual serovar's proteolytic properties (Gill and Penney 1977, 1982; Gupta and others 1983; Sike and Maxcy 1980). Proteolytic enzymes indigenous to the bacteria were hypothesized to break down the connective tissue (endomysium) between muscle fibers and consequently facilitate penetration into tissue. However, protease production secreted by the bacteria does not occur until the meat starts to deteriorate (Gill and Penney 1977). Therefore, this circumstance does not apply because uncontaminated meat was used and no time was allowed for proteolytic enzymes to be produced and secreted by *Salmonella* serovars.

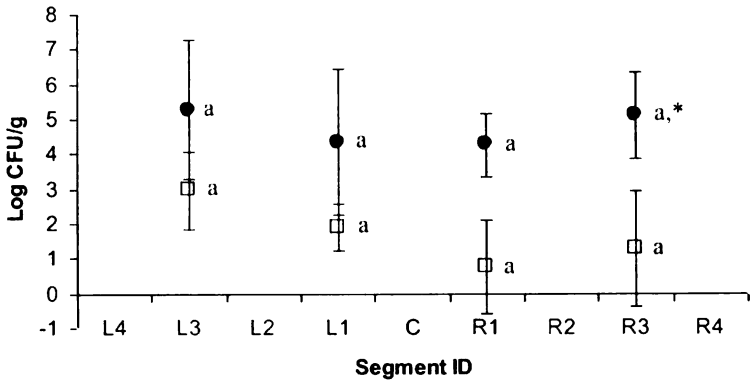
Salmonella distribution as a result of migration was significantly ($P<0.0001$) impacted by microbial load and excised cube location (or cube ID) (Figures 3.2 and 3.3). However, no interactions ($P=0.7786$) were seen between microbial load and cube location. With the higher microbial load (10^8 CFU/mL as opposed to 10^4 CFU/mL), an increase in bacterial number was observed within the turkey breast. Figures 3.2 a, b, and c illustrate the penetration pattern in the cranial, middle, and caudal slices, respectively. The highest *Salmonella* counts were generally observed in the cubes L4 and R4, immediately below the inoculated outside surface (Figures 3.2 b and 3.3). Cube C at center yielded fewer salmonellae ($P\leq0.05$) compared to cubes R4 and L4. As clearly seen in the middle slices (Figures 3.2 b) *Salmonella* penetrated toward the center of the turkey

breast. The cranial and caudal slices showed no significant movement of *Salmonella* towards the middle section with numbers of *Salmonella* seen horizontally (Figures 3.2 a and c). This may be due to the structure, thickness variation, and orientation of the turkey breast at both ends. Populations of *Salmonella*, Figure 3.4, recovered from cubes T2 and B2 next to the inoculated surface were significantly ($P \leq 0.05$) greater compared to cube C at the center. Both inoculum levels showed a decrease in *Salmonella* from the outside surface toward the center.

Figure 3.2 Horizontal profile of *Salmonella* in (a) cranial slice (b) middle slice, and (c) caudal slices of whole muscle turkey breast, subjected to 10^4 CFU/mL (open square) and 10^8 CFU/mL (solid circle) inoculum levels. Segment ID corresponds to the sampling diagram as illustrated in Figure 3.1 in materials and methods section. Each data point is an average of three replications with standard deviation.

* and $^{a-c}$ indicate significant difference ($P \leq 0.05$) between and within treatment levels, respectively.

(a)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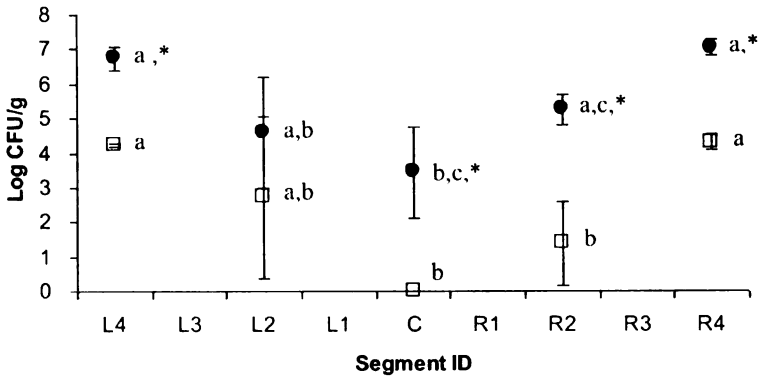
(b)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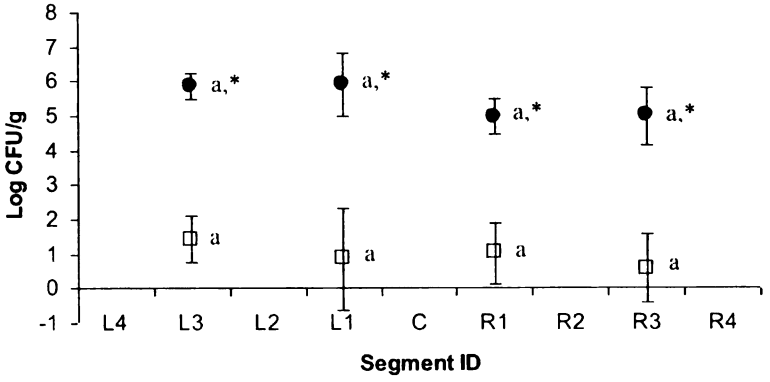
(c)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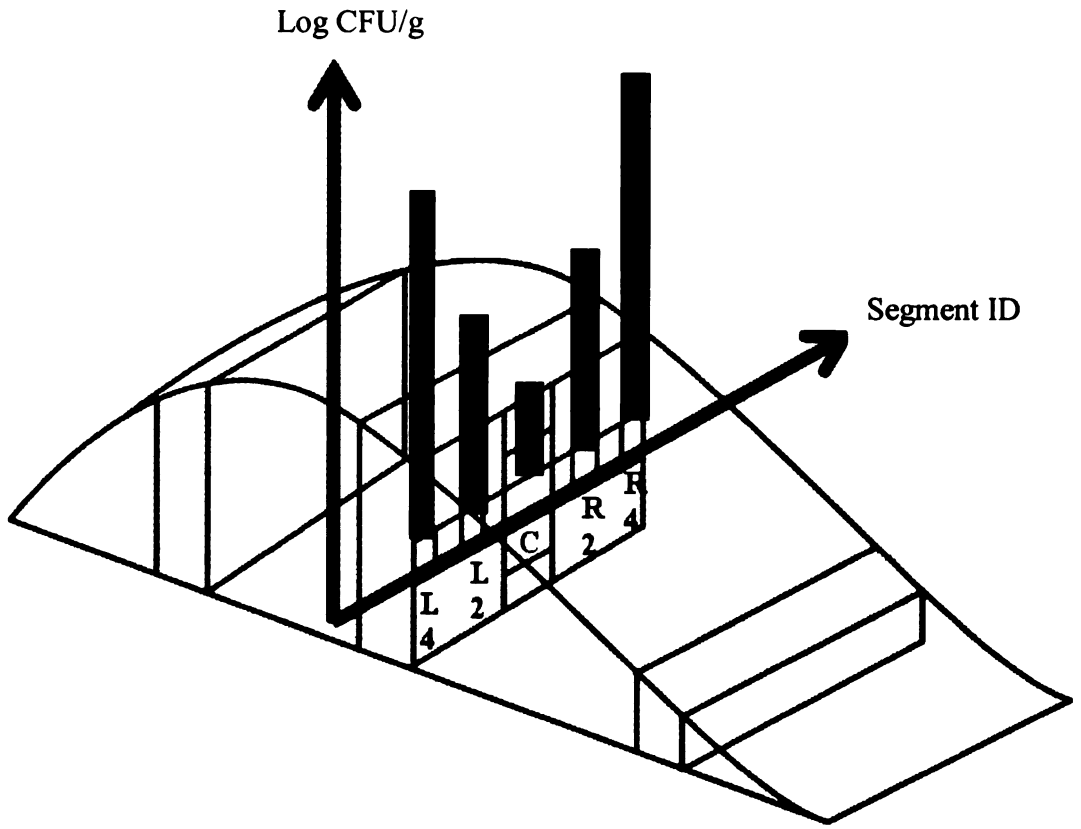


Figure 3.3 *Salmonella* penetration diagram in middle slice of turkey breast showing the highest population in the cubes immediately below inoculated surface.

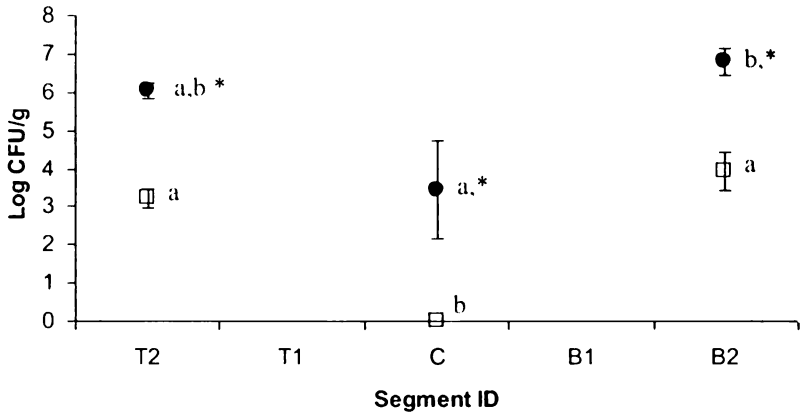


Figure 3.4 Vertical profile of *Salmonella* in the middle slice of whole muscle turkey breast, subjected to 10^4 CFU/mL (open square) and 10^8 CFU/mL (solid circle) inoculum levels. Segment ID corresponds to the sampling diagram as illustrated in Figure 3.1 in materials and methods section. Each data point is an average of three replications with standard deviation.

* and $^{a-b}$ indicate significant difference ($P \leq 0.05$) between and within treatment levels, respectively.

Inoculum level previously contributed to microbial penetration (Kim and Doores 1993). In this study, penetration was highest for marinade inoculated at 10^8 CFU/mL. This high bacterial load was needed to quantify the extent of penetration (Butler and others 1979).

Muscle fiber orientation also impacts the depth of penetration with Gill and Penney (1982) showing that penetration is more extensive when the inoculum is applied vertical to the meat fibers as opposed to horizontal. In addition, bacteria not only move downward from the point of inoculation (as affected by gravity) but also ascend from the point of inoculation (Sikes and Maxcy 1980). Although, fiber orientation was not considered in this study, a penetration gradient in multiple directions was seen toward the middle portion of turkey breast.

In addition to inoculum level and fiber orientation, bacterial motility can also impact penetration with organisms moving between the endomysial sheath and associated muscle fibers (Thomas and others 1987). Flagellated bacteria more readily attach to surfaces compared to nonmotile bacteria (Butler and others 1979; Gill and Penney 1982). While *Salmonella* is motile, this motility is not the only factor facilitating the migration of the organism into the meat. Incubation temperature is also important when determining extent of penetration (Gill and Penney 1977, 1982). As the temperature increases, the distance bacteria penetrate increases. Gill and Penney found that motile proteolytic strain of *S.* Typhimurium migrated further into beef muscle at 37 °C as opposed to 20 °C. However, in order to mimic industry conditions, all experiments were performed at 4 °C.

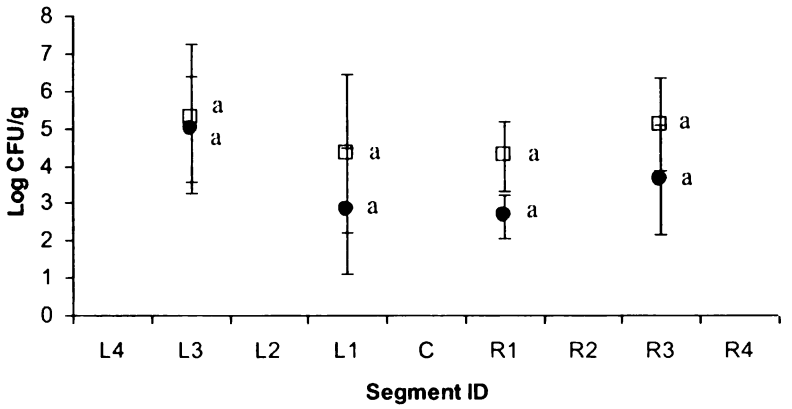
Multidirectional penetration of *Salmonella* serovars into whole muscle turkey breast, subjected to typical (TC: 3.2% salt and 0.8% phosphate) and high (HC: 10.2% salt

and 4.05% phosphate) marinades was also examined as shown in Figures 3.5 and 3.6. Marinade concentrations did not significantly (P=0.096) affect *Salmonella* migration into whole muscle, while cube location showed a significant (P<0.0001) effect. The interaction between these two factors was not significant (P=0.8189). Fewer salmonellae were observed near the center cube within middle slices for both marinade concentrations (Figure 3.5 b). There was no difference in *Salmonella* recovery among cubes from cranial and caudal slices (Figures 3.5 a and c). Figure 3.6 illustrates a vertical sectioning profile of *Salmonella* in the middle slice of the turkey breast. Significantly (P≤0.05) higher numbers of salmonellae were recovered in the segments immediately below the surface (T2 and B2) compared to the C cube.

Figure 3.5 Horizontal profile of *Salmonella* in (a) cranial slice (b) middle slice, and (c) caudal slices of whole muscle turkey breast, subjected to marinade solutions containing 3.2% salt and 0.8% phosphate (open square) and 10.2% salt and 4.05% phosphate (solid circle). Segment ID corresponds to the sampling diagram as illustrated in Figure 3.1. Each data point is an average of three replications with standard deviation.

$^{a-c}$ indicate significant difference ($P \leq 0.05$) within treatment levels. There were no significant ($P > 0.05$) difference between treatment levels.

(a)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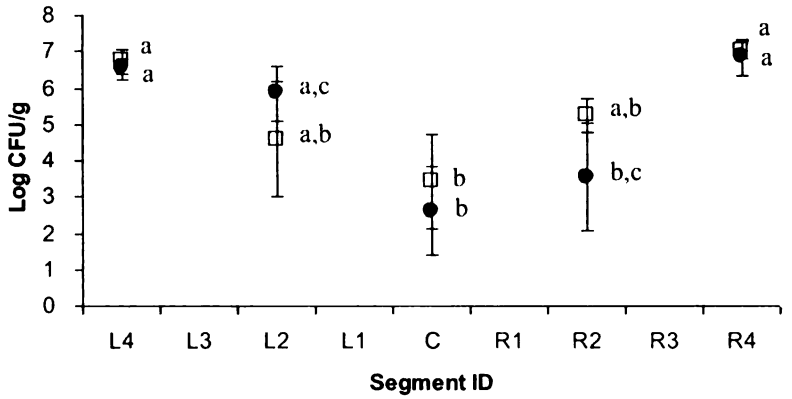
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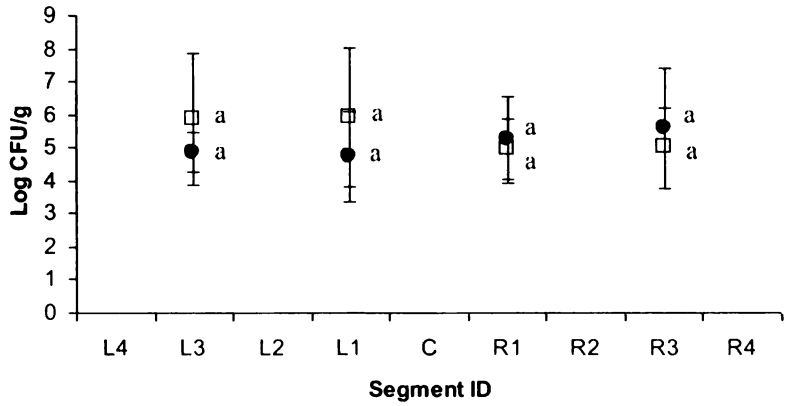
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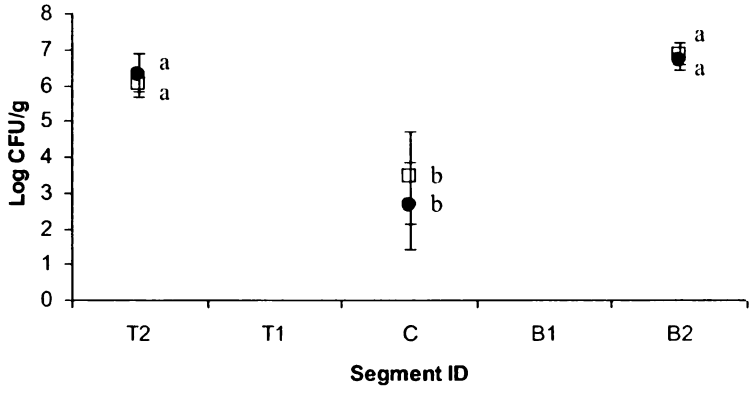


Figure 3.6 Vertical profile of *Salmonella* in the middle slice of whole muscle turkey breast, subjected to marinade solutions containing 3.2% salt and 0.8% phosphate (open square) and 10.2% salt and 4.05% phosphate (solid circle). Segment ID corresponds to the sampling diagram as illustrated in Figure 3.1. Each data point is an average of three replications with standard deviation.

[a-b] indicate significant difference ($P \leq 0.05$) within treatment levels. There were no significant ($P > 0.05$) difference between treatment levels.

Non-meat ingredients such as salt and polyphosphate are commonly used to enhance muscle tenderness (Lyon and Hamm 1986) and are usually incorporated into marinades during tenderization. Tumbling turkey breast muscle with these non-meat ingredients reduces fluid water loss during cooking and improves meat quality (Froning and Sackett 1985, Maki and Froning 1987). Alkaline pyrophosphates act as a fluidizing agent in muscle, dissociating actin and myosin, changing ionic strength and pH, which leads to increased water uptake (Xiong and Kupski 1999). High water content was shown to increase the rate of bacterial penetration, presumably because water uptake by muscle increases the inter-fiber distance of muscle and thereby decreasing bacterial resistance to movement within the tissue. In addition, increased inter-fiber gaps from high water content in muscle may contribute to increase microorganism penetration rate (Thomas and others 1987). However, Sikes and Maxcy (1980) have found that the addition of sodium tripolyphosphate to comminuted pork reduced the penetration depth. In our study, no significant increase or decrease in *Salmonella* penetration was seen using HC as opposed to TC marinade. This may be because bacterial migration occurred before water was absorbed by the individual muscle cells.

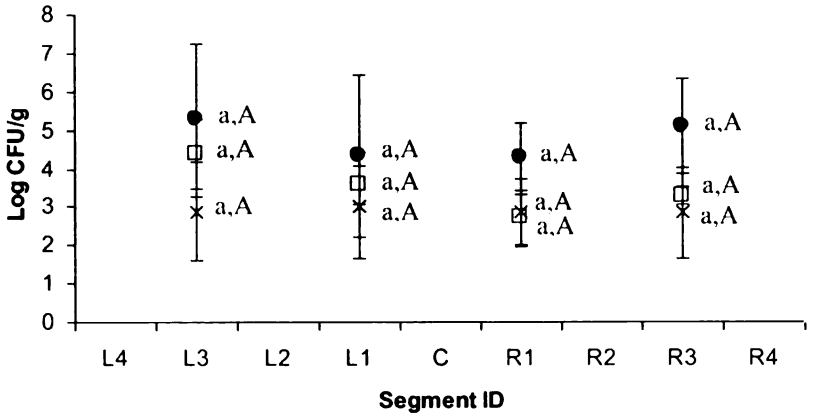
The points of *Salmonella* exposure; before, during, and after marination, were also assessed. The point at which contamination occurred significantly ($P<0.0001$) affected the extent of *Salmonella* penetration (Figures 3.7 and 3.8). There were no interactions ($P=0.4939$) observed. Migration of *Salmonella* was observed in samples subjected to these treatments. For all treatments examined (Figures 3.7 b and 3.8), *Salmonella* counts in the middle slice significantly decreased ($P\leq0.05$) as the depth below the inoculated surface increased. However, the cranial and caudal slices (Figures 3.7 a

and c) did not show any specific penetration pattern. Greater recovery was observed when the turkey was vacuum tumbled in contaminated marinade. Less *Salmonella* migration into marinated turkey breast was observed in pre- and post- marinade treatments. This may be due to the lower *Salmonella* levels, as opposed to during marination when the pathogen was suspended in the marinade. The C segment from the middle slice had the lowest bacterial count among all cubes.

Figure 3.7 Horizontal profile of *Salmonella* in (a) cranial slice (b) middle slice, and (c) caudal slices of whole muscle turkey breast, subjected to contamination at pre- (open square), during (solid circle), and post- (cross) vacuum tumbling process. Segment ID corresponds to the sampling diagram as illustrated in Figure 3.1. Each data point is an average of three replications with standard deviation.

$^{A-B}$ and $^{a-d}$ indicate significant difference ($P \leq 0.05$) between and within treatment factors, respectively.

(a)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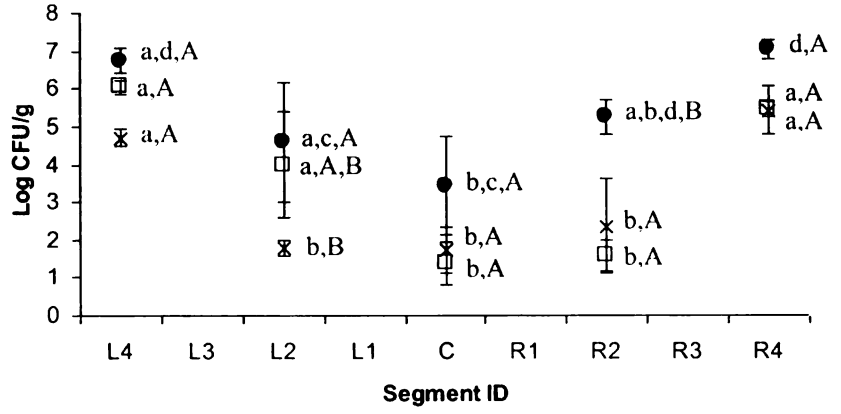
Segment ID

(b)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b,A
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a,b,d,B
b,A
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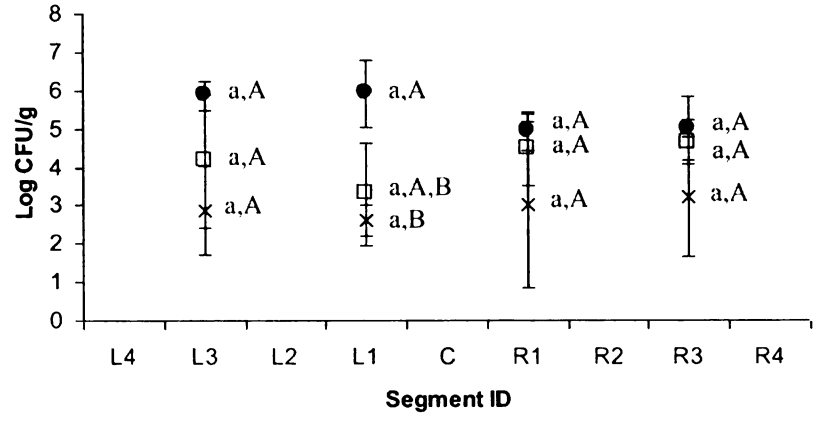
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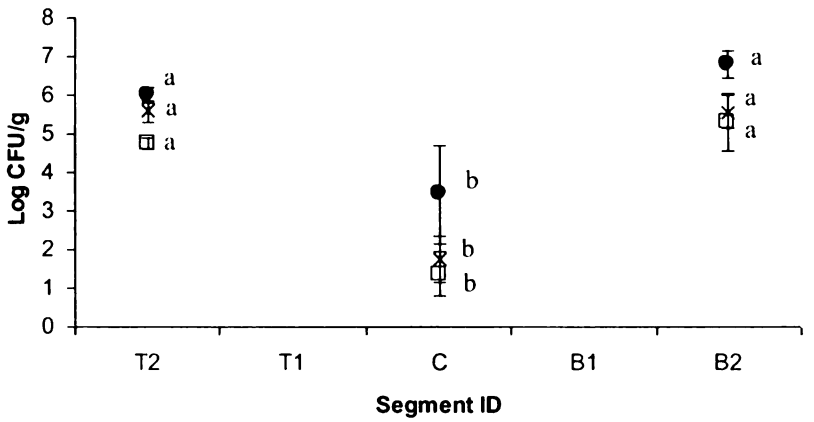


Figure 3.8 Vertical profile of *Salmonella* in the middle slice of whole muscle turkey breast, subjected to contamination at pre- (open square), during (solid circle), and post- (cross) vacuum tumbling process. Segment ID corresponds to the sampling diagram as illustrated in Figure 3.1. Each data point is an average of three replications with standard deviation.

[a-b] indicate significant difference ($P \leq 0.05$) within treatment factors. There was no significant difference between treatment factors.

Tenderization is widely used in the meat industry to enhance both marketability and profits. Mechanical tenderization breaks down the connective tissue and fragments the myofibrillar structure leading to increased tenderness (Boyd and other 1978). These physical actions carry bacteria from the outer surface of intact muscle into the interior portion and allow for potential proliferation (Raccach and Henrickson 1999).

Significant bacterial penetration has also been observed without any invasive procedures that would enhance mechanical damage to muscle tissue. Gupta and others (1983) demonstrated that *S.* Typhimurium penetrated at least 3 cm into poultry muscle at atmospheric pressures after 20 h of incubation at 37°C. In addition, Warsow and others (2008) evaluated *Salmonella* migration into intact whole muscle turkey breasts during a still marination process. *Salmonella* counts significantly increased in vacuum-marinated samples (3 cm into the turkey breast) and decreased with depth below the inoculated surface. The results from Warsow's study are similar to the results in this study indicating that pathogens can penetrate into the interior of turkey breast without using an invasive marination process involving blade tenderization or needle injection. However, this study, which presents novel information about *Salmonella* penetration, assessed the effects of marinade ingredients (salt and phosphate), microbial load, and contamination stage (pre-, during, and post-marination). In addition, multiple directional penetration was studied, as opposed to a single directional penetration, using improved sampling methods.

Marination is a traditional process used to tenderize and improve meat flavor (Richardson and Mead 1999). This process involves liquid with dissolved non-meat ingredients that are incorporated into the products before cooking. Many commercially produced meat products are marinated by still marination, tumbling, blending or

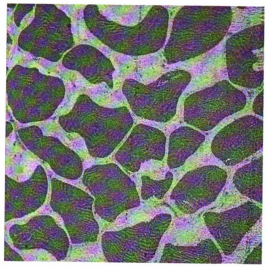
mechanical injection. Overall, this study showed that contamination of the marinade solution and/or the poultry surfaces resulted in bacterial penetration into the interior of the product during vacuum tumbling. Consequently, while marination improves meat product quality and yield, such products may pose greater health hazard if an internal cooking temperature of 71 °C or other microbial intervention techniques are not used.

Microstructure of marinated whole muscle turkey breast

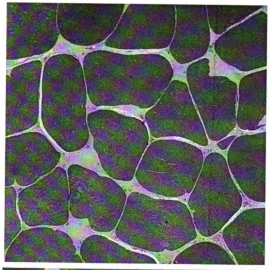
To obtain an overall view of the muscle fiber, bright-field microscopy images of muscle bundle cross sections were taken from thick sections. Figure 3.8 shows a series of transverse sections of turkey breast subjected to various treatments, including control (turkey breast tissue with no additional water), TC, and HC marinades. The images roughly indicate the difference in extracellular space between muscle fibers. In the absence of salt and phosphate (Figure 3.9 a), the extracellular spaces were wider with the muscle fibers, showing a sharp polygonal shape. After non-meat ingredient addition (Figures 3.9 b and c), muscle fiber expansion or swelling was evident along with a significant ($P \leq 0.05$) increase in cell diameter compared to the control. However, no significant differences were seen between muscle cell diameters for samples submerged in TC and HC ($P > 0.05$). When salt and alkaline phosphates were added to the marinade solution, the circumference of the myofibers tended to smooth out resulting in a more rounded fiber structure. In addition, a decrease in extracellular space was observed with the presence of non-meat ingredients. The changes in tissue organization occurred as a result of water uptake, which caused these fibers to swell and expand within the connective tissue framework.

Figure 3.9 Bright-field microscopy images of transverse sections of turkey breast whole muscles subjected to (a) control with no water, salt, and phosphate, (b) 3.2% NaCl and 0.8% alkaline phosphate, and (c) 10.2% NaCl and 4.05% alkaline phosphate. Magnification 200X; bars indicate 50 μm.

(a)



(b)



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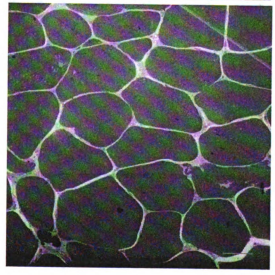


The Matlab software program was used to calculate muscle tissue pore space represented by "white" pixels (Table 3.1). The extracellular space in turkey muscle samples treated with salt and phosphate, TC and HC, was less than the control ($P \leq 0.05$). In addition, increasing 3.2% salt and 0.8% phosphate to 10.2% salt and 4.05% phosphate in marinade solution significantly ($P<0.05$) decreased the extracellular area of muscle cells.

Table 3.1 Percentage of "white" pixels or muscle tissue pore space as determined using Matlab software program.

Treatment	White area (%)*
Control	26.14 ± 6.5^{a}
Typical marinate concentration (3.2% salt and 0.8% phosphate)	13.62 ± 2.9^{b}
High marinade concentration (10.2% salt and 4.05% phosphate)	7.57 ± 0.5^{c}

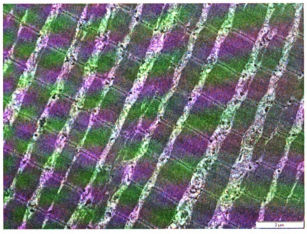
$^{a\text{-}b}$ indicate significant difference ($P \leq 0.05$) of white area percentage between treatments.

* Each value is an average of 5 representative images ± standard deviation.

Marinade solutions containing inorganic phosphates and NaCl are used to enhance the quality of poultry and other muscle foods. Such ingredients improve water holding capacity, reduce shrinkage and cooking loss, and increase product tenderness (Froning and Sackett 1985). Most previous reports have focused on the effects of salt and phosphate on water holding capacity. Several studies have shown that a combination of alkaline pyrophosphates and/or tripolyphosphates with NaCl improve water holding capacity in meat and poultry products as compared to NaCl alone (Shults and others 1972; Wierbicki and others 1976). However, Xiong and Kupski (1999) reported that a concentration of 8% salt tended to diminish the water-binding effects of phosphate. In this study TEM was used to observe the ultrastructural changes that occur in turkey breast tissue after marination. Figure 3.10 illustrates representative micrographs of turkey samples subjected to TC and HC marinades as well as the control (no water, salt, and phosphate added). The TEM images of longitudinal sections show less interspace between myofibrils as well as larger myofibrils (width) as the concentration of salt and phosphate increased. These changes in muscle fiber size may be due to the contribution of salt and phosphate incorporated in the marinade. In addition, as shown in Figure 3.10 c, increasing the salt and phosphate concentration to 10.2% and 4.05%, respectively, eliminated the striated pattern in muscle structure. This observation may be due to the high salt concentration in marinade solution that not only causes muscle fibers to swell and dissociate but also solubilizes and extracts the myofibrillar protein within the basic myofibril structure.

Figure 3.10 Transmission electron micrographs of longitudinal sections of whole muscle turkey breast subjected to (a) control with no water, salt, and phosphate and (b) typical concentration marinade with 3.2% NaCl and 0.8% alkaline phosphate, and (c) high concentration marination with 10.2% NaCl and 4.05% alkaline phosphate. Magnification 10,000X; bars indicate 2 μm.

(a)



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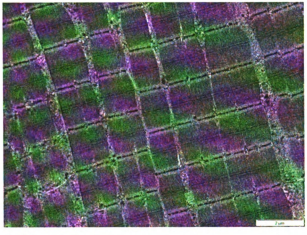

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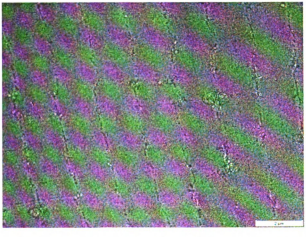


The water holding capacity of meat usually increases at pH values above the isoelectric point of myofibrillar protein. At the molecular level, alkaline phosphates break down crosslinks between myofibrillar proteins (actomyosin), increase the number of negatively charged sites on proteins available for water binding, and extend the interfibrillar spaces in which water is immobilized in the myofibril lattices. This action consequently contributes to myofibrillar expansion (Offer and Trinick 1983). Release of monophosphates from phosphate compounds is associated with additional changes in muscle pH. Li and others (2001) reported that meat samples treated with pyrophosphate and tripolyphosphate had increased levels of monophosphates and therefore increased water absorption.

The muscle structure also changes in response to salt concentration. Muscle that was immersed in a 3% salt solution exhibited swollen of fibers (as the diameter increases), which shrank at salt concentrations ≥ 6% (Bocker and others 2005). The microstructural changes depend on existing NaCl in solution and the degree of swelling or dehydration of the myofibrils. Changes at the molecular level correspond to the protein secondary structure. The NaCl concentrations above 2% create an ionic strength sufficiently high to solubilize chicken myofibrillar proteins (Liu and Xiong 1997).

Overall, this study demonstrated the effect of salt and alkaline phosphate on the microstructure of turkey breast meat by increasing the muscle fiber size, which has not been previously reported in the literature. Although, the application of salt and alkaline phosphate has been widely used in the industry to improve the quality and palatability of products, the ionic characteristics of the two ingredients are not the only factors that contribute to changes of muscle microstructure due to water absorption. Post mortem

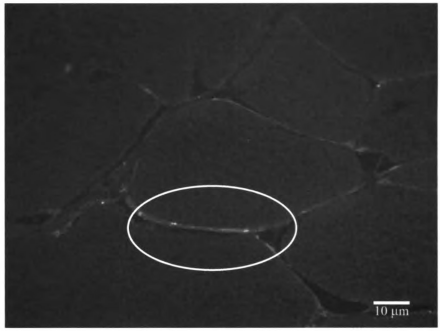
status of the meat, type of muscle and fibers, and changes in the aqueous pH surrounding the muscle fibers to a more alkaline condition also affect the extent of swelling due to increased water binding to the muscle fibers (Offer and others 1989; Egelandsdal and others 1995).

Salmonella location in fresh whole muscle turkey breast tissue

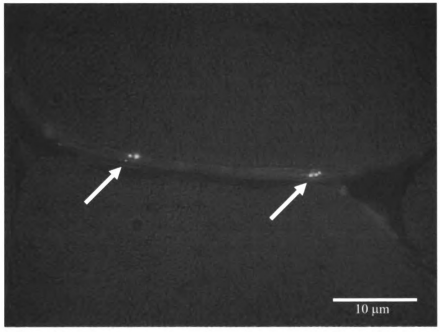
Wet-mount preparations of GFP-labeled *Salmonella* were examined using fluorescence microscopy. In order to obtain an overall view of bacterial penetration/attachment, the fluorescence images were superimposed over the transmitted images of muscle fibers at the corresponding areas. Figure 3.11 illustrates some representative images of 8-μm thickness transverse sections of muscle tissue. The images indicate that *Salmonella* cells are likely to locate between turkey muscle fibers and muscle bundles and reside near endomysial and perimysial connective tissue.

Figure 3.11 Enhanced fluorescence images of GFP-labeled *Salmonella* location in turkey breast transverse sections taken at (a) 400X magnification; (b) enlargement of circle area in (a) taken at 1000X magnification. Bars indicate10 μm and arrows point to GFP-*Salmonella*, which has penetrated the muscle. Images in this dissertation are presented in color.

(a)



(b)



GFP-transformed *Salmonella* is visibly green and fluoresces when exposed to long wave UV light, which can be detected by fluorescence microscopy. One limitation is that the GFP-transformed *Salmonella* might have different characteristics when compared to the parental strain. However, Prachaiyo and McLandsborough (2000) observed no difference in behavior of GFP-transformed *E. coli* and the parental strains. The surface properties, including hydrophobicity and cell surface charge of the organisms, were not affected by introducing a GFP-containing plasmid. In addition, Noah and others (2005) indicated the ability of GFP-transformed *Salmonella* to maintain their fluorescence following 8-15 transfers and during refrigerated (4 °C) storage for up to 12 d. Therefore, the GFP-transformed *Salmonella* in this study should behave similarly to the parental strain.

The fluorescence images were also taken longitudinally to the muscle fibers (not shown). However, the specific location of *Salmonella* was questioned when they were found near muscle fibers. Whether they were positioned inside or on top of the fiber could not be visually determined. The location of *Salmonella* could consequently be easily misinterpreted. Therefore, cross sectional areas were examined. Gill and Penney (1977) indicated that salmonellae are likely to penetrate the tissue between muscle fibers. This may be due to changes in poultry muscle post-slaughter, where spaces between muscle fibers are created by radial shrinkage of the muscle fibers that allow bacteria to enter the deeper layers of the muscle tissue (Thomas and others 1987). Collagen fibers were suggested as one point of attachment for *S.* Typhimurium (Kim and Doores 1993; Woody and others 2000). In addition, microorganisms are also present within the

sarcolemma (or surface) of individual muscle fibers or within crevices of raw meat muscle (Prachaiyo and McLandsborough 2000; Lorca and others 2002).

The mechanism by which bacteria attach to meat surfaces involves two stages. The primary stage is a weak adhesion due to physical forces and the second is a firmer adhesion by fimbriae and pili, involving formation of hydrogen or ionic bonds and a formation of extracellular polysaccharides (or extracellular polymers) (Firstenberg-Eden 1981; Lillard 1989). Dickson and Koohmaraie (1989) indicated that cell surface charges and hydrophobicity influenced bacterial attachment. A net negative charge on the bacterial cell wall aides in adhesion to positively charged collagen fibers. However, the magnitude of attachment varies from strain to strain based upon the net negative charge of the strain.

Changes in the microtopography of tissue caused by various processing procedures also contribute to bacterial attachment. Water and saline alter the muscle integrity by causing individual fibers to separate (Thomas and others 1987). Slow freezing and thawing also disrupt the integrity of the sarcolemma. Slow freezing draws intrafiber water across the sarcolemma, which results in the formation of large ice crystals external to the muscle fiber (up to 100 μm cross-sectional size) that can tear the membrane (Sikes and Maxcy 1980; Do and others 2004). These conditions contribute to structural changes and damage in the intercellular space and consequently serve as ports of entry for bacteria, thus allowing attachment at those crevices. Because of these concerns, fresh turkey breast muscle (post-rigor) that has never been frozen was used in this study to avoid the ice crystal damages from the freeze-thaw process.

The use of green fluorescence protein-transformed *Salmonella* coupled with the fluorescence microscope successfully demonstrated the target pathogen in supporting connective tissue and crevices within the muscle. This information has not been previously reported.

CHAPTER 4

SUMMARY AND OVERALL CONCLUSIONS

Marination is one of the processing methods used to increase product yield and palatability. These results suggest that *Salmonella* may be able to enter and survive in value-added marinated products. The degree of bacterial penetration into whole muscle turkey breast was associated with microbial load and time of exposure. Cube location within the middle slice had a significant effect on *Salmonella* penetration into muscle. When comparing treatment levels within each factor, inoculum level had the largest impact but vacuum tumbling also increased bacterial penetration. Marinade concentration had little effect on *Salmonella* penetration. This may be due to migration that occurred before the muscle cells absorbed water.

The photomicrographs obtained from this study indicate that the muscle fiber expanded and decreased in extracellular space after subjecting turkey breast muscle to marinades containing salt and phosphate. Marinades diffused into the fibers and myofibril matrices. TEM was used to observe the effect of marinade composition on muscle tissue. The swelling of turkey myofibrils indicated moisture absorption during marination. These findings support that non-meat ingredients, especially salt and alkaline phosphate, contribute significantly to marinade penetration and absorption in muscle fibers. *Salmonella* present in inoculated marinade solution migrate into the interior of whole muscle turkey breast and locate in the extracellular space. The organisms tend to be associated with or located near endomysial and perimysial connective tissue.

Microbial contamination is associated not only with the marination step during meat processing but also with slaughtering equipment, processing environment, animal hide, and gastrointestinal contents. Water and subsequent handling procedures also play important roles. Meat processing that lead to changes in microtopography may increase the opportunity for pathogens to reside and attach firmly to meat and poultry products. Therefore, postmortem hygiene during processing is of extreme importance. If the process encourages or permits the movement of surface bacteria deep into the beef muscle, normal cooking time/temperatures may not eliminate microorganisms or pathogens protected within the tissue, subsequently creating a microbial safety hazard. In addition, improper storage temperatures and atmospheres may allow the introduced bacteria to survive and proliferate in these products. Once they have penetrated into meat products, these bacteria may attach to a preferential area or location and fail to be inactivated during thermal processing. Therefore, thermal processing with proper cooking time and temperature is important to assure product safety for consumers.

CHAPTER 5

FUTURE RESEARCH

This study demonstrated that bacterial multidirectional penetration into intact whole muscle turkey breast can occur during marination and depends on the microbial load and time of exposure. Although unlikely, penetration may also be related to marinade composition, since only salt and phosphate were studied and other non-meat ingredients may influence penetration. Recommended future research in this area includes:

(1) development of a 3-dimensional profile of pathogen level in sample that will be useful to parameterize mathematical models in order to verify and improve the safety of marinated whole muscle poultry products.

(2) evaluation of other treatment factors (i.e. marination time, other non-meat ingredients, partial vacuum treatment, non-meat intervention techniques such as sodium lactate, packaging and storage time after marination) and incorporation of these factors into the mathematical model developed in this dissertation that will aid in the understanding of bacterial penetration in ready-to-eat poultry products.

(3) development of an extensive model by including information of other microorganisms and meat species.

Vacuum tumbling is generally known to tenderize meat and distribute the marinade throughout the product. This method of marination facilitates the penetration of *Salmonella* the interior of intact whole muscle turkey breasts. Various tenderization and marination techniques are available for commercial use; therefore, evaluation of the

effect of other tenderization techniques (i.e. still marination, needle and blade tenderization) on bacterial multidirectional penetration would be useful to generate additional migration mapping since internalized bacteria might be distributed differently as a result of other value-added processes.

Microbiological data showed that *Salmonella* could survive in marinade solutions containing salt and alkaline phosphate stored at 4 °C for up to 2 months. Concern arises when whole muscle products have been vacuum tumbled with contaminated marinades, as they might pose a risk due to survival pathogen in the product if the cooking is not sufficient. Therefore, the determination of microbial survival or multiplication in marinated whole muscle products stored in refrigerated retail cases should be further evaluated.

Bright-field and transmission electron microscopy images revealed the effect of both salt and alkaline phosphates on muscle structure. Further studies are needed to determine the effect of individual non-meat ingredients at different concentrations and marination times on muscle structure changes, as this will assist food technologists in incorporating appropriate non-meat ingredient to attain desired product attributes.

Results from this study also demonstrated that the location of *Salmonella* in extracellular areas (between muscle fibers and bundles) with the pathogen tending to be associated with supporting connective tissues. The effect of tenderization techniques on muscle structure (creating tears to the membrane and causing mechanical disruption of muscle tissue integrity and therefore creating space for potential contaminating pathogen to reside) should also be studied to assist in achieving sufficient internal temperatures to inactivate pathogens that may be embedded and protected within the interior of whole

muscle products. Bacterial attachment is a complex phenomenon involving several factors (such as microtopography of the attachment area and physicochemical properties of the exposed surface). These properties should also be considered to understand the mechanism of *Salmonella* adhesion.

Thermal inactivation work was done only on small size cores, rather than on full sized turkey breasts or breast roasts. Small core samples are necessary to run a simple isothermal inactivation process and to estimate the inactivation parameters. Applying these estimated thermal inactivation parameters to industrial cooking processes for whole turkey breast might be another focus area for future projects. Results suggest that internalization of *Salmonella* in whole muscle turkey breast leads to enhanced thermal resistance. Additional studies are needed to elucidate the mechanisms by which bacterial pathogens are protected in whole muscle compared to ground muscle when subjected to thermal inactivation. Future research should include product physical state (whole muscle or ground muscle) when developing predictive thermal inactivation models for different cooking processes.

APPENDICES

APPENDIX A

THERMAL INACTIVATION OF *SALMONELLA* IN WHOLE MUSCLE AND GROUND TURKEY BREAST

ABSTRACT

Previous research has demonstrated the potential for *Salmonella* penetration into whole muscle turkey breast when processed with inoculated marinade. Moreover, when internalized, this organism may exhibit enhanced thermal resistance. In this study, the effect of turkey physical structure (ground and whole muscle) on the thermal resistance of *Salmonella* was evaluated. Irradiated whole and ground turkey breasts were exposed to a salt and phosphate marinade containing 8 serovars of *Salmonella* (~10^8 CFU/mL) for 20 min. Samples were subjected to isothermal heating treatments (55, 60, and 62.5 °C) in triplicate for predetermined times after which surviving cells were enumerated. Core temperatures were recorded to determine the thermal lag time (time to reach the target temperatures).

The differences in *Salmonella* counts before and after the thermal lag time were not significantly different ($P>0.05$). The first-order kinetic rate constants (k) for whole muscle were approximately 50% lower than those for ground muscle, of same composition, at each temperature ($P<0.05$), indicating that the *Salmonella* inactivation rate was greater ($P<0.05$) in ground than in whole muscle samples. These results suggest that internalization of *Salmonella* in whole muscle products leads to enhanced thermal resistance. Therefore, the cooking times and temperatures currently recommended for

pathogen inactivation, especially for marinated value-added meat products, may need to be reevaluated.

INTRODUCTION

Salmonella continues to be one of the most common foodborne pathogens implicated in gastroenteritis cases in humans and continues to be a major public health concern to the food industry. Salmonellosis can be fatal and the cost associated with infection can be very high (Mattick and others 2001). Many outbreaks of salmonellosis have resulted from the consumption of contaminated meat, eggs, or dairy products. Poultry and poultry products remain one of the leading sources of *Salmonella* infection in humans (Tietjen and Fung 1995).

Microorganisms that contaminate meat surfaces during processing may migrate into the products where they may survive and/or propagate (Raccach and Henrickson 1979). In addition, these microorganisms may exhibit enhanced thermal resistance during cooking or thermal processing (Orta-Ramirez and others 2005). Bacterial thermal resistance can be affected by many intrinsic and environmental parameters including meat species, muscle type, product composition (e.g., carbohydrates, fat content), suspending medium, water activity, pH, and salt. Generally, thermal resistance of bacteria is higher in meat products than in other model systems, such as buffer solutions, peptone, and agar (Juneja and others 1995, 2001). Vegetative bacteria are more susceptible to heat in foods with higher water activities typically because of the better heat transfer capability. Increasing the fat content in the substrate decreases moisture content and therefore, accounts for increased pathogen survivability because of poor heat penetration through the heating menstruum (Ahmed and others 1995; Juneja and Eblen 2000). Non-meat additives, such as salts, lactates, and phosphates, may also enhance thermal

resistance of pathogens (Kotrola and Conner 1997, Maurer 2001). Doyle and Mazzotta (2000) suggested that bacterial location in the food system (surface attachment vs. interior dispersion) may also affect the resistance of *Salmonella*. Physical arrangement of various components within the food matrix might also cause differences in bacterial thermal resistance. *Salmonella* was significantly more heat resistant when present in whole muscle beef and pork as opposed to ground products (Orta-Ramirez and others 2005; Velasquez and others 2005). In addition, the cooking/heating conditions and methods (Murphy and others 2001), and bacterial recovery methodologies (Wesche and others 2005) also dictate the results obtained from thermal inactivation experiments.

Under commercial conditions, thermal processing with proper cooking time and temperature is necessary for both product quality and safety. Recently, mathematical models have been used to validate various thermal processes for better food safety assurance (Leaper and Richardson 1999). Therefore, a knowledge of thermal death (inactivation) kinetics is essential to eliminate or reduce target organisms. Several factors other than processing time and temperature also influence thermal death rates of bacteria. It is important that an adequate model that includes the impact of product and process variables on the inactivation kinetics of microbial contaminants be developed. However, the validity of current thermal inactivation data from meat models needs to be reexamined for marinated products along with thermal resistance data for whole and ground muscle products to ensure product safety. Because of this concern, the objective of this study was to determine the effects of turkey physical structure, whole muscle and ground product, on the thermal resistance of *Salmonella*.

MATERIALS AND METHODS

Preparation of turkey breast samples

Fresh, whole muscle, boneless, skinless turkey breasts were obtained from a local packer within 12 h of slaughter and chilled in a single large lot to eliminate lot-to-lot variability. On the receiving day, turkey breasts were cored using a hand-coring device to create cylinder-shaped whole muscle samples measuring 1.2 cm in diameter and 5-7 cm in length. Ground turkey samples were obtained by grinding whole muscle turkey breasts twice through a 4-mm diameter plate using a Kitchen Aid grinder (Model k5-A, Hobart, Troy, OH, USA). Subsequently, whole and ground muscle samples were vacuum packaged in double plastic bags (Cryovac Sealed Air Corp., Duncan, SC, USA), frozen at -20°C and transported on dry ice to CFC Logistics (Quakertown, PA, USA) for irradiation. All samples were irradiated with approximately 10kGy to eliminate indigenous microflora. To determine microbial populations after irradiation, 1-g samples from whole muscle turkey breast were diluting 1:5 in 0.1% sterile peptone water (Difco, Becton Dickinson, Sparks, MD, USA), homogenized in a masticator (Model 0410, IUL Instruments USA, Inc. Cincinnati, OH, USA), plated in duplicate on Petrifilm™ aerobic count plates (3M Corp., St. Paul, MN, USA), and incubated at 37 °C for 48 h.

Moisture, fat, and protein levels were determined in triplicate using AOAC methods 950.46B, 991.36, and 981.1, respectively (AOAC 1996). To determine the pH, 10 g of turkey was homogenized in 90 mL of distilled water using a Polytron homogenizer (Model PT 10/35, Brinkmann Instruments, Westbury, NJ, USA) at speed

setting 3 and measured directly using a pH meter (Model 145, Corning, Medfield, MA, USA).

Preparation of marinade solution

The marinade solution contained 96% water (filtered and deionized), 3.2% NaCl (EMD Chemicals Inc., Gibbstown, NJ, USA) and 0.8% phosphate (50% food grade liquid potassium phosphates from Butcher and Packer Supply Company, Detroit, MI, USA). A 520 mL-aliquot of the marinade was poured into 650 mL-glass bottles with screw caps and autoclaved for 15 min at 121 °C to ensure sterility. The marinades were stored at room temperature (25°C) until used.

Preparation of test organisms

The following eight strains of *Salmonella* were obtained from Dr. V.K. Juneja (USDA-ARS, Eastern Regional Research Center, Philidelphia, PA, USA): *S.* Thompson FSIS 120 (chicken isolate), *S.* Enteriditis H3527 and H3502 (clinical isolates phage types 13A and 4, respectively), *S.* Typhimurium DT 104 H3380 (human isolate), *S.* Hadar MF60404 (turkey isolate), *S.* Copenhagen 8457 (pork isolate), *S.* Montevideo FSIS 051 (beef isolate), and *S.* Heidelberg F5038BG1 (human isolate). These serovars have shown moderate to high thermal resistance and been implicated in outbreaks (Juneja and others 2001). All serovars were stored frozen at -80 °C in a tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol.

Prior to use, each serovar of *Salmonella* was propagated weekly by transferring one loop of frozen culture to a 9-mL TSB tube. The cultures were maintained separately

by daily transfer to fresh TSB followed by incubation for 18-24 h at 37 °C, with a minimum of two consecutive transfers to obtain cells in late lag phase (Smith and others 2002).

Preparation of inoculated marinade

On the day of experiment, the 8 serovars of *Salmonella* were transferred to a centrifuge bottle and centrifuged at 6000 *x g* for 20 min at 4 °C, the supernatant was decanted off and the pellet was resuspended in 500 mL of sterile marinade to give a target *Salmonella* cocktail concentration of approximately 10^8 CFU/mL. The concentration was confirmed by platting on Petrifilm™. The final concentration was confirmed by serial dilution in 0.1% peptone water followed by duplicate plating on aerobic Petrifilm™ count plates and incubating at 37 °C for 24 h before enumeration.

Exposure to inoculated marinade

Frozen whole muscle cores and ground muscle were thawed overnight at 4 °C. The core samples were transferred to sterile Whirl Pak™ bags (Nasco, Fort Atkinson, WI, USA) and *Salmonella*-inoculated marinade was added to cover the samples. Core weights were recorded before and after a 20 min of marination at 4°C to determine marinade uptake. The resulting average marinade uptake, 0.14 g marinade/g turkey, was used to determine the amount of marinade needed for ground turkey samples. Ground turkey was manually mixed in a sterile plastic container with inoculated marinade that was aseptically added dropwise. Uniformity of inoculation was verified by randomly plating inoculated unheated whole muscle and ground turkey. Samples were diluted with 0.1%

peptone water and plated on aerobic Petrifilm™ count plates. Inoculated whole and ground muscle samples were subsequently packed into sterile brass tubes (1.27 cm diameter and 10 cm length), sealed with sterile rubber stoppers, and both ends of the tubes were wrapped with Teflon tape. All tubes were stored at 4°C before subjected to the thermal treatment (within 2 h).

Thermal inactivation

Brass tubes containing whole and ground samples were placed into a temperature controlled water bath (NESLAB Instruments Inc., Newington, NH, USA) and heated isothermally at 55, 60, and 62.5°C. Internal temperature was monitored using a 1-mm type T thermocouple (Omega Engineering, Stamford, CT, USA) inserted into the geometric center of a turkey sample and connected to a DuaLogR data logger (model 91100-50, Cole Parmer Instrument Company, Vernon Hills, IL, USA). The thermal lag time, or time required for the internal temperature to reach within 0.5°C of target temperature, was recorded. After the internal temperature reached the target temperature, the first tube was removed from the water bath and directly placed in an ice-water bath to stop additional heat processing until enumeration. For whole muscle, samples heated at 55, 60, and 62.5 °C, tubes were removed every 5 min, 45 s, and 7 s, respectively. For ground muscle, samples heated at 55, 60, and 62.5 °C, tubes were removed every 4 min, 30 s, and 7 s, respectively. All whole and ground muscle experiments were performed in triplicate.

Sampling and enumeration

After heating, samples were aseptically removed from the brass tubes, placed in sterile Whirl Pak™ bags, diluted 1:10 in 0.1% peptone water, and homogenized for 3 min in a masticator (Model 0410, IUL Instruments, Inc., Cincinnati, OH, USA). Appropriate serial dilutions were plated on Petrifilm™ aerobic plates in duplicate. *Salmonella* survivors were enumerated after 48 h of incubation at 37 °C. The minimum detection level was 10 CFU/g.

Data analysis

Salmonella survivor curves were generated for whole muscle and ground turkey by plotting the logarithm of the survival ratio vs. holding time at each temperature. Analysis of variance (ANOVA) was used to determine the effects of muscle structure (whole vs. ground), heating temperature, and holding time on *Salmonella* survival. Mean values were compared using Tukey-Kramer's test (α=0.05). Thermal inactivation rate constants (k, min^{-1}) were determined for first-order kinetic response by plotting the natural log of the *Salmonella* survivors vs. time. An Arrhenius-type equation ($k = \beta_0 e^{-\beta_1/T}$) was parameterized by plotting Ln(k) vs. (1/T). ANOVA was run to evaluate the effects of temperature and grinding on k. Statistical analyses were performed using JMP (Version 3.2.2, SAS Institute, Inc., Cary, NC, USA).

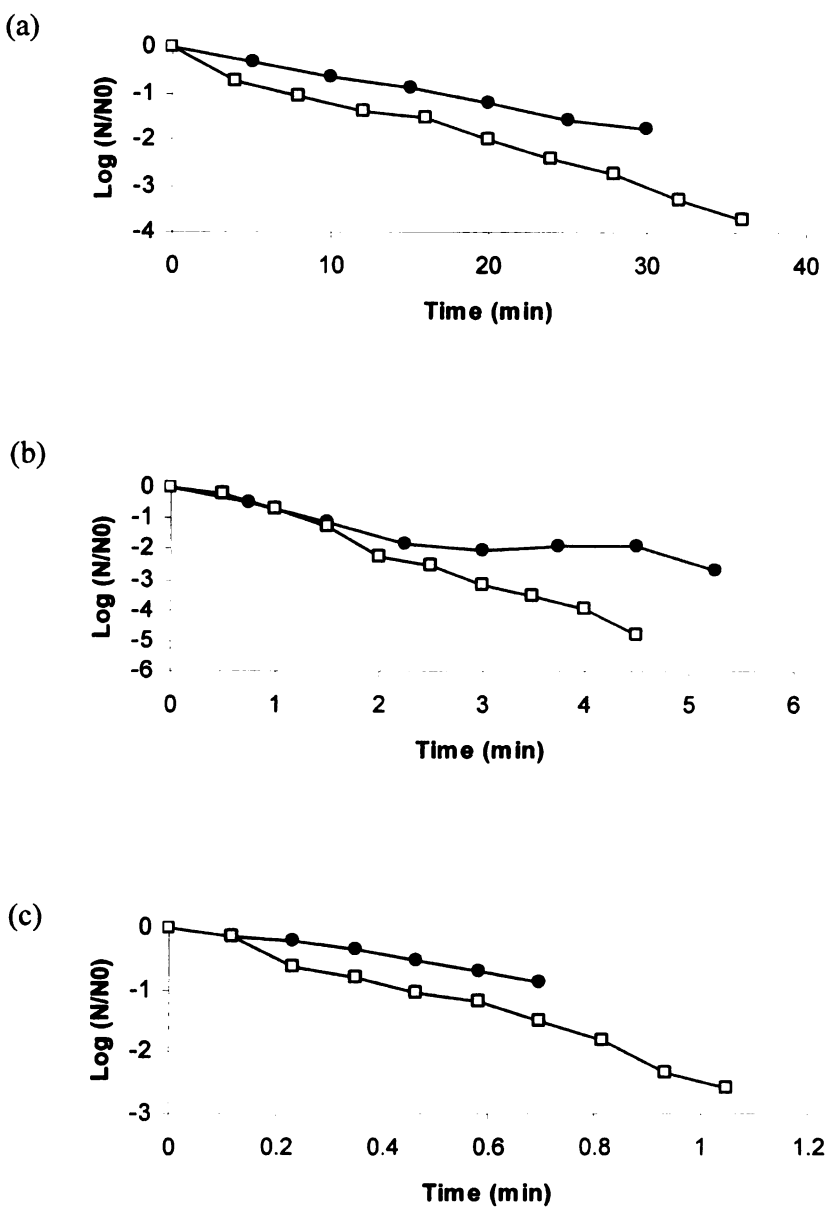


Figure A.1 Survival curves for *Salmonella* in whole (solid circle) and ground (open square) turkey muscle subjected to isothermal heating of; (a) 55, (b) 60, and (c) 62.5 °C. Each point is an average of 3 observations.

RESULTS AND DISCUSSION

Raw whole and ground turkey muscle contained 72.5 ± 0.2% moisture, 0.95 ± 0.56% fat, and 26.7 ± 0.59% protein. After receiving 10kGy irradiation, whole muscle turkey was randomly plated using Petrifilm™ to determine background flora. The control had no *Salmonella* initially present in the turkey samples.

Figure A.1 shows the survivor curves generated by plotting the logarithm of the survivors (Log N/N_0) versus the heating time at each isothermal temperature. N/N_0 is the fraction of the survivors, where N and N_0 are the Salmonella count at a specific holding time and at time zero, respectively. Time zero is the time immediately after thermal lag time; the time required for the internal temperature to reach within 0.5°C of the target temperature. As expected, thermal inactivation was affected by heating temperature and time. As heating temperature and holding time periods increased, a decrease in *Salmonella* counts was observed. At all isothermal heating temperatures, both ground and whole muscle samples exhibited log-linear decline in the number of survivors as heating time increased. No lag periods, shouldering, or tailing were evident in any of the *Salmonella* survivor curves suggesting a homogeneous response to heat resistance and heat transfer through the turkey (Juneja and others 2001). However, some survival curves in the literature exhibit shouldering, which may be caused by poor heat transfer through the heating medium (Murphy and others 2000; Juneja and others 2001).

ANOVA was used to assess the effect of heating time, holding temperature, and type of meat (whole and ground) on *Salmonella* survival. The *P* values are presented in Table A.1. The analysis indicates that all of the factors and 2-way interactions affected the survival of *Salmonella* ($P \leq 0.05$). Table A.2 shows the initial *Salmonella* counts in inoculated samples before heating and initial counts at time zero (after thermal lag time). The results indicated no differences ($P>0.05$) in initial *Salmonella* counts between muscle types, which means they started with a comparable microbial load. In addition, the come-up times for both types of turkey meat were not significantly different ($P>0.05$). Several factors including initial cell numbers (Smith and others 2001), bacterial strain and growth phase (Heddleson and other 1991), heating medium composition (Ahmed and others 1995; Lorca and others 2003, sample size and shape (Smith and others 2001), ice crystal formation (Smith and others 2001; Doyle and Cliver 1990), and additives (Murphy and others 2000), can account for increased thermal stability of pathogens. However, the factors mentioned above were experimentally controlled in this study.

Table A.1 *P* values from ANOVA for thermal inactivation of *Salmonella* spp. in whole and ground turkey breast muscle.

Test factors	*P* values
Grinding	0.009
Temperature	0.0072
Time	<0.0001
Grinding*Temperature	0.0034
Grinding*Time	0.0019
Temperature*Time	<0.0001

Table A.2 *Salmonella* numbers in inoculated unheated and heated turkey breast samples after thermal lag time at 55, 60, and 62.5 °C.

Heating temperature	*Salmonella* survival count* (Log CFU/g)			
	Unheated sample		Heated sample at time zero	
	Whole muscle	Ground muscle	Whole muscle	Ground muscle
55	7.38 ± 0.1^{a}	7.28 ± 0.1^{a}	7.09 ± 0.1^{d}	7.43 ± 0.6^{d}
60	6.72 ± 0.7^{b}	7.29 ± 0.1^{b}	6.48 ± 0.2^{e}	6.85 ± 0.1^{e}
62.5	7.30 ± 0.1^{c}	7.27 ± 0.0^{c}	6.62 ± 0.1^{f}	5.33 ± 1.2^{f}

* *Salmonella* numbers shown are the means of three replications and expressed as mean ± standard deviation.
[a-f] indicate significant difference ($P \leq 0.05$) of mean *Salmonella* survival number between muscle types within heating temperature.

Assuming log-linear inactivation kinetics, the first-order kinetic rate constant or thermal inactivation rate constant (k, $time^{-1}$) was obtained from linear regression of Ln N/N0 = -kt; where t is the heating time. Within the type of muscle, the calculated k values increased as the heating temperature increased (Table A.3). These findings are supported by research done by Orta-Ramirez and others (1997), Veeramuthu and others (1998), and Murphy and others (2000). Veeramuthu and others (1998) and Orta-Ramirez and others (1997) who evaluated thermal inactivation of S. Senftenberg in turkey thigh meat at 55-65 °C and ground beef at 53-68 °C, respectively. They reported increased kinetic constants with increased heating temperatures. In the study performed by Murphy and others (2000) which determined thermal kinetics in chicken breast meat at 55-70 °C the kinetic rate constants for *Salmonella* at 55, 60 and 62.5 °C were 0.08, 0.39, and 0.92, respectively, which are approximately 40-70% lower than ours. The difference in bacterial thermal resistance is likely from variation in *Salmonella* serovars and heating medium composition.

Table A.3 First-order thermal inactivation rate constants (k, min^{-1}) determined from linear regression of *Salmonella* survivor data (Ln N/N0 = -kt) for whole and ground turkey breast muscle.

Muscle type	k values*		
	55 °C	60 °C	62.5 °C
Whole muscle	0.14 ± 0.01[a]	1.25 ± 0.16[a]	3.43 ± 1.09[a]
Ground muscle	0.23 ± 0.05[b]	2.54 ± 0.04[b]	6.25 ± 0.62[b]

* k values shown are the means of three replications and expressed as mean ± standard deviation.
[a,b] indicate significant difference ($P \leq 0.05$) of mean k values within similar heating temperature.

When the kinetic rate constants between muscle structure were compared, k values for whole muscle were approximately 50% lower ($P<0.05$) than those for ground muscle at all heating process (Table A.3). In these experiments, both muscle types contained equivalent raw meat composition, bacterial counts before and after thermal lag time, and were subjected to the same thermal process. As a result, the only difference accounting for higher *Salmonella* heat resistance in whole muscle was due to its muscle structure. *Salmonella* seemed to be protected from heat penetration within intact whole muscle as opposed to ground turkey. The grinding process affects microstructure and physical characteristics of the product. The organized tissue structure of whole muscle will be damaged during grinding, thus creating larger inter-spaces within the muscle tissue and a more porous structure. This porous structure is an important factor that influences heat transfer within the meat (Ngadi and others 2001). Moreover, these structural changes might expel sarcoplasmic fluid from muscle fibers, resulting in increased water availability between muscle cells for heat transfer.

These data will make a positive contribution to food safety for manufacturers whose processes involve a heat treatment step. Understanding these variations is necessary in order to design adequate thermal inactivation to eliminate *Salmonella* in thermally processed foods. However, others factors (such as meat composition, thermal history of bacteria, heating medium and condition, and additional additives) also need to be considered when developing a thermal inactivation model in order to verify processing adequacy for meat and poultry products.

CONCLUSIONS

In this chapter, *Salmonella* was inoculated into whole and ground muscle and the thermal resistance was determined. The thermal inactivation rate constants or k values were affected by heating time and temperature. Results suggest that internalization of *Salmonella* in whole muscle turkey breast leads to enhanced thermal resistance. These findings should assist the food industry in designing Hazard Analysis Critical Control Point (HACCP) plans to effectively eliminate the pathogen in thermally processed turkey, including whole and ground muscle. The cooking times and temperatures currently recommended for pathogen inactivation may need significant adjustment to cover all aspects and parameters as the products and processes change. Accountability for product physical state will reinforce regulatory standards. If more detailed predictive model is developed, ready-to-eat poultry processors will be able to better meet lethality performance standards, and ensure the safety and quality of their products.

APPENDIX B

SALMONELLA VIABILITY IN SALT AND PHOSPHATE MARINADES AS AFFECTED BY STORAGE TEMPERATURE

ABSTRACT

The effect of salt and phosphate concentration and storage temperature on *Salmonella* viability in marinade was investigated. An eight-strain *Salmonella* cocktail (~ 8 log CFU/mL) was inoculated into two different marinade formulations; "typical" and "high" concentrations, which contained 3.2% NaCl and 0.8% phosphate, and 10.2% salt and 4.05% phosphate, respectively. The marinade solutions were stored at 4 and 37 °C and sampled for surviving *Salmonella* for up to 63 d.

The *Salmonella* population in a typical marinade solution held at 37 °C decreased 2.85 logs compared to 0.8 log after 62 days at 4 °C. After 63 days, a reduction of 1.4 logs was observed with the high concentration marinade stored at 4 °C. In contrast, rapid death of *Salmonella* was observed when marinade containing high salt and phosphate was kept at 37 °C. Consequently, *Salmonella* can potentially persist in marinades during long-term storage at 4 °C.

INTRODUCTION

Tenderness and juiciness are the two sensory attributes that consumers use to evaluate meat products to determine satisfaction (Smith and Acton 2001). To enhance these factors, the meat industry injects non-meat ingredients into meat products, which can work synergistically to enhance their functionality. Water, salt and phosphate are often incorporated into marinade solutions to achieve this purpose.

Several food processing procedures may introduce unexpected pathogens into the finished meat product. One of those steps is the preparation of brine solutions that may be contaminated with pathogenic organisms. Yet, marination procedures are used to reduce and prevent the growth of spoilage organisms. Marinades can support the survival of selected known pathogens. Perko-Makela (2000) studied the survival rate of seven *Campylobacter jejuni* strains in a marinade sauce and demonstrated that a commercial marination procedure did not eliminate this microorganism.

Factors affecting microbial growth in foods include water activity (a_w), pH, and temperature. Minimum growth conditions for *Salmonella* are: 6.5 °C, pH 4.5, and a_w >0.95 (FDA-CFSAN 1992). Values that deviate from these conditions will affect the growth and survival of *Salmonella*. These bacteria tolerate many stressful conditions and can survive sub-optimal conditions for a period of time. While the original pH of a food may prevent growth of *Salmonella*, a shift in pH by other microbes may permit bacterial growth. Water activity varies with the change of temperature and decreases with the addition of solute. Mattick and others (2000) reported low a_w values when supplementing growth media with several humectants that affected the survival of *Salmonella* Enteritidis

and Typhimurium. The survival of *Salmonella* at a specific temperature depends on the specific *Salmonella* serovar, their growth phase in the media, their physical conditions, food composition or testing media, and competing microflora (Doyle and Mazzotta, 2000). Airoldi and Zottola (1988) found that *Salmonella* Typhimurium was viable at low temperatures for extended incubation times. Moreover, the temperature associated with the propagation period affected the growth of organisms in the first several days.

Since *Salmonella* is recognized as a significant foodborne pathogen that has been involved in major outbreaks, the ability of this organism to survive in marinade solutions is a safety concern. The objective of this study was to monitor survival of *Salmonella* in marinade solution containing different salt and phosphate concentrations stored at 4 and 37 °C for up to 63 days.

MATERIALS AND METHODS

Marinade preparation

Two marinades were prepared: a typical marinade containing 96% water (filtered and deionized), 3.2% NaCl (EMD Chemicals Inc., Gibbstown, NJ, USA), and 0.8% phosphate (50% food grade liquid potassium phosphates from Butcher and Packer Supply Company, Detroit, MI, USA) and a high concentration marinade containing 10.2% NaCl and 4.05% phosphate. A 520 mL-aliquot of each marinade was poured into glass bottles with screw caps and autoclaved for 15 min at 121 °C to ensure sterility. The pH of the marinade was measured using Corning 340 pH meter (Corning, Suffolk, UK).

Preparation of test organisms

The following eight strains of *Salmonella* were obtained from Dr. V.K. Juneja (USDA-ARS, Eastern Regional Research Center, Philidelphia, PA, USA): *S.* Thompson FSIS 120 (chicken isolate), *S.* Enteriditis H3527 and H3502 (clinical isolates phage types 13A and 4, respectively), *S.* Typhimurium DT 104 H3380 (human isolate), *S.* Hadar MF60404 (turkey isolate), *S.* Copenhagen 8457 (pork isolate), *S.* Montevideo FSIS 051 (beef isolate), and *S.* Heidelberg F5038BG1 (human isolate). These serovars have shown moderate to high thermal resistance and been implicated in outbreaks (Juneja and others 2001). All serovars were stored frozen at -80 °C in a tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD, USA) containing 20% glycerol.

Each strain of *Salmonella* was propagated by transferring one loop of frozen culture to a 9-mL TSB tube. The cultures were maintained by daily transfer to fresh TSB

followed by incubation for 18-24 h at 37 °C, with a minimum of two consecutive transfers prior to use.

Preparation of inoculated marinade

On the day of the experiment, the eight serovars of *Salmonella* were combined in a centrifuge bottle and centrifuged at 6000 *x g* for 20 min at 4°C, the supernatant was decanted off and the cells were washed with 50 mL of sterile peptone water, pelleted by centrifugation and resuspended in 500 mL of sterile marinade to give a final *Salmonella* cocktail containing ~10^8 CFU/mL. Three bottles of each marinade concentration were capped and stored at 4°C and 37°C for subsequent microbial analyses.

Sampling

Salmonella populations were determined by serially diluting aliquots in sterile 0.1% peptone water, plating in duplicate on Petrifilm™ aerobic plates and incubating at 37°C for 48 h. All marinades were sampled for *Salmonella* for up to 63 d or until population decreased below the detection limit of 10 CFU/mL.

Data analysis

All treatments for each marinade concentration and temperature combination were conducted in triplicate on every sampling day. Numbers of *Salmonella* were plotted as log CFU/mL against incubation time on a semi-log scale.

RESULTS AND DISCUSSION

Salmonella populations in marinades stored at 4 and 37 °C illustrated in Figure B 1. Initial *Salmonella* populations in the inoculated marinade ranged from 7.53 to 8.23 log CFU/mL with numbers of *Salmonella* decreasing as storage time progressed. Viability of *Salmonella* was highest in the typical marinade stored at 4 °C. Exposure to high salt and phosphate concentrations and a temperature of 37 °C dramatically reduced the survival of *Salmonella* with populations decreasing to less than 10 CFU/mL after 5 days.

Regardless of storage temperature, the high concentration marinade was more detrimental to survival compared to the typical marinade. The more rapid decline of *Salmonella* in the high concentration marinade may relate to the increased levels of solutes that decrease the water activity to the point where the organism are no longer able to survive. The minimum a_w for survival of *Salmonella* is reportedly 0.95 (FDA 1992). Mattick and others (2000) reported the optimal growth of *Salmonella* strains at a_w of 0.99. High salt concentrations are associated with low water activity. A 3.2 % salt concentration is equivalent to an a_w of 0.98, which would allow greater survival of *Salmonella* in marinade. In contrast, the high concentration marinade containing 10.2% salt had a survival, which limits a_w to approximately 0.92. Fletcher and Csonka (1998) reported that the addition of solutes (i.e. salt and sugar) can raise the upper limit growth temperature and viability of microorganism at lethal temperature. Their results showed that addition of 0.9-1.7% of NaCl could enhance the survival of *S.* Typhimurium at 50 °C.

Within the same concentration of salt and phosphate in the marinade, lower storage temperature maintained higher viability of *Salmonella*. For the typical marinade solution, only a 0.8-log reduction was observed at 4 °C compared to a 2.8-log reduction at 37 °C. In the marinade solution containing high salt and phosphate, the *Salmonella* population decreased 1.4 logs during refrigerated storage, as opposed to about 8 logs when the marinade was stored at 37 °C. Previous work by Warsow (2008) who examined the survival of a *Salmonella* cocktail in a specific marinade supports our findings that the microorganism survived longer at 4 °C. Warsow's marinade solution contained 7% salt and 3% phosphate. This study revealed greater viability of *Salmonella* when stored at 4 °C compared to 37 °C. Airoldi and Zottola (1988) examined the growth and survival of *S.* Typhimurium in nutrient deficient media incubated at low temperature. They found that *Salmonella* could survive in 0.1% peptone water for more than 1 year at 7 °C.

Mattick and others (2000) demonstrated similar results to our study, describing improved survival in the presence of high salt concentrations at lower temperatures. At a low a_w, a rapid decrease in *Salmonella* survival was observed at 37 °C compared to 21 °C. It was concluded that survival does not depend solely on water activity in growing media but also on a combination of growth condition factors. This same research group also studied long-term survival of *Salmonella* strains at low water activity. *Salmonella* survived at low a_w for extended periods; however, an 8% NaCl (~ a_w 0.95) was bactericidal. In addition, pH was an important factor influencing the survival of *Salmonella* at low a_w when exposed to heat (Mattick and others 2001). Rather than having typical short cell morphology, filamentous phenotypes have been found in organisms that are under stress conditions. Mattick and others (2000) demonstrated that,

in response to sub-optimal growth conditions, filaments were observed when *S.* Typhimurium was incubated at both 21 and 37 °C in tryptic soy broth (TSB) supplemented with NaCl. With NaCl at a_w values of 0.95-0.98 (approximately 3.5-8 % NaCl), the filaments appeared to be longer and more numerous. They explained that the formation of filaments was due to the low water activity that may affect the regulation of cell division genes. However, rehydration with fresh TSB containing no salt revealed rapid filamentous division into large numbers of viable daughter typical cells.

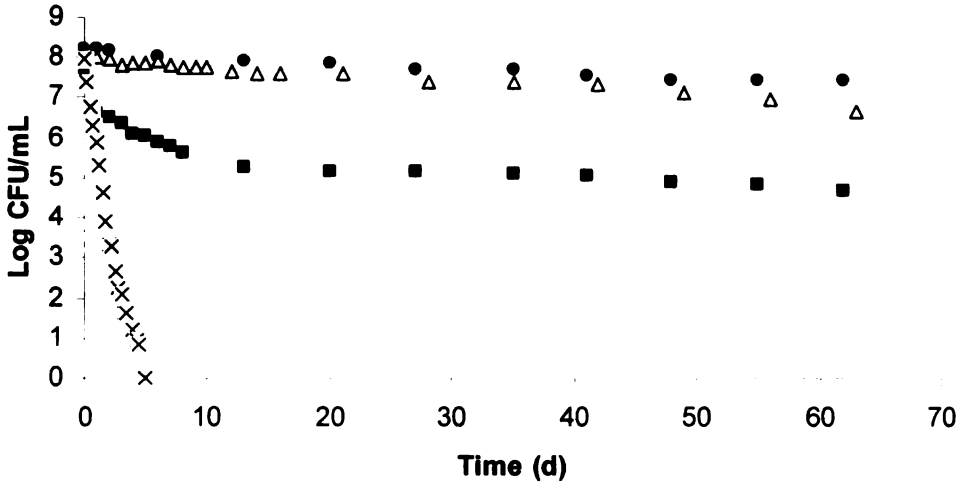


Figure B.1 Viability of an 8-strain *Salmonella* cocktail in typical marinade containing 3.2% salt and 0.8% phosphate maintained at 4 °C (solid circle) and 37 °C (solid square) and high concentration marinade containing 10.2% salt and 4.05% phosphate stored at 4 °C (open triangle) and 37 °C (cross). Each point is an average of 3 observations.

CONCLUSIONS

Most foods contain sufficient nutrients to support microbial growth. Several factors support, prevent, or limit the growth of organisms in foods. Various solutes are incorporated into foods in order to reduce the water activity and maintain a reasonable safety margin before growth of microorganisms can occur. In addition to water activity of the culture medium, other endogenous and exogenous factors also affect the survival of *Salmonella*. The interplay of factors will significantly determine whether a specific microorganism will survive or grow in a given food. A synergism or antagonism might occur altering the survival of a particular microbe from one product to another. Therefore bacterial survival prediction can only be achieved through experimentation.

This study assessed the viability of *Salmonella* in a marinade solution containing different salt and phosphate concentrations that was stored at 4 and 37 °C. *Salmonella* survived sub-optimal growth conditions for extended periods of time at refrigeration temperature. The properties of marinade did impact the survival of *Salmonella*. Highest survival was observed when both marinades (3.2% salt + 0.8% phosphate and 10.2 % salt + 4.05% phosphate) were kept at 4 °C. A combination of high storage temperature and high salt and phosphate concentration in marinade was most detrimental to the survival. However, extended survival was seen in the high concentration marinade at 4 C°.

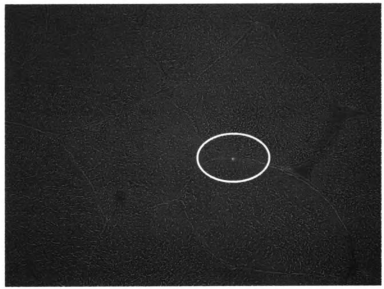
One goal of industrial marination procedures is to rapidly achieve marinade equilibrium in the products. Consequently, if a *Salmonella* contaminated marinade is incorporated into meat, it is possible that *Salmonella* could survive during refrigerated

storage of marinated poultry products. Therefore, it is critical that these marinated products be adequately cooked to eliminate any potentially internalized salmonellae.

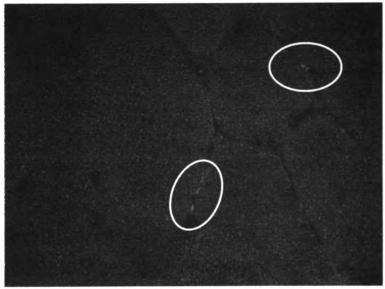
APPENDIX C

ADDITIONAL MICROSCOPY IMAGES

(1)



(2)



(3)

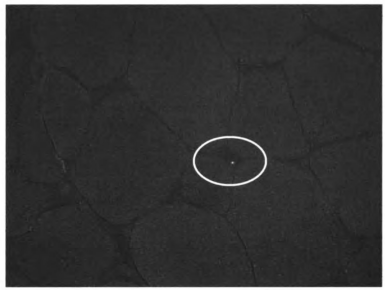


Figure C.1-3 Enhanced fluorescence images of GFP-labeled *Salmonella* location (circle areas) in turkey breast transverse sections taken at 400X magnification. Images in this dissertation are presented in color.

APPENDIX D

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