RISK FACTORS INFLUENCING THE GROWTH AND SURVIVAL OF

SALMONELLA ON POULTRY PRODUCTS

by

SILVIA A. DOMINGUEZ RISCO

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Food Science

written under the direction of

Dr. Donald W. Schaffner

and approved by

_____

_____

_____

_____

New Brunswick, New Jersey

October, 2009

ABSTRACT OF THE DISSERTATION

Risk factors influencing the growth and survival of *Salmonella* on poultry products

By SILVIA A. DOMINGUEZ RISCO

Dissertation Director:
Dr. Donald W. Schaffner

Salmonella bacteria are commonly found in poultry carcasses and their presence after processing may lead to foodborne disease outbreaks. Consumption of frozen chicken products containing raw poultry has been recently identified as a risk factor for salmonellosis. Survival of the pathogen after freezing and frozen storage may lead to infection if the food product is insufficiently cooked when, for example, using a microwave oven.

In this dissertation, we have studied three scenarios that affect the risk of salmonellosis on poultry products: the effect of storage temperature on the growth of *Salmonella* on raw poultry, the survival of *Salmonella* during frozen storage in processed poultry products, and the efficacy of microwave cooking on the destruction of *Salmonella* in frozen chicken entrées. Our results show that (1) a model can be used to predict the growth of *Salmonella* on raw poultry as a function of storage temperature; (2) *Salmonella* are able to survive frozen storage on processed chicken products with structural injury as a consequence; (3) neither lipopolysaccharide nor porin defects hindered the survival of *Salmonella* during storage at -20°C; and (4) cooking of frozen entrées following the

product's label instructions using a low-wattage (500W) microwave oven resulted in non-lethal heating profiles and *Salmonella* survival.

A quantitative microbial risk model was also developed in order to assess the risk of salmonellosis associated with consumption of raw, frozen chicken products cooked in low-wattage microwave ovens. A 2005 salmonellosis outbreak in Minnesota linked to raw, frozen chicken entrees was simulated 100 times using our model. Despite certain limitations in availability of data, model predictions (5-7 reported illnesses) were in close agreement with the actual outbreak outcome (4 reported illnesses). The risk assessment model developed may provide useful quantitative data relevant for risk management initiatives, ultimately aiming at controlling the risk of salmonellosis from raw, frozen chicken products.

Acknowledgements

I would like to thank my advisor, Dr. Donald W. Schaffner, for his constant support, encouragement and trust. I sincerely appreciate and value all I learned as his student, and the great opportunities given, which allowed me to grow in both professional and personal levels.

I am grateful to Rutgers University Dining Services for funding my Graduate Assistantship, and for giving graduate students like me the opportunity to work in the Food Microbiology Risk Reduction program.

I gratefully acknowledge Dr. Jianghong Meng for providing the *Salmonella* strains used in Chapters 1, 2 and 4 of this dissertation, and Dr. Mukund Karwe for allowing me to use the research microwave oven in his laboratory.

I would like to thank my former and current labmates and colleagues: Dr. Bin Liu, Dr. Eunice Pang, Kristin Schaffner, Karla Mendoza, Wenjing Pang, Di Li, Wenchao Li, Gabriel Mootian and many others, for their advice, support and friendship.

I especially thank my friends and family in Peru for always being there for me. In particular, I thank my mother Graciela Risco de Dominguez, my brother Alberto Dominguez and my fiancé Jean-François Fortin for their love.

Dedication

I dedicate this dissertation to my parents, Dr. Pedro Dominguez and Dr. Graciela

Risco de Dominguez, who showed me the value of learning and education.

Table of Contents

List of tables

List of illustrations

General introduction and project overview

1. *Salmonella* spp.

Salmonella is one of the leading causes of foodborne illness in the United States. According to the Centers for Disease Control and Prevention (CDC) Foodborne Disease Outbreak Surveillance System, *Salmonella* was responsible for 585 outbreaks, 16,821 cases and 20 deaths in the United States between 1998 and 2002 (*75*). During this 5-year period, 50 salmonellosis outbreaks were associated with poultry. The actual number of cases however may be more than 30 times larger than that reported, considering the underreporting of milder illnesses (*86,94*). The CDC estimates that approximately 1.4 million nontyphoidal *Salmonella* infections occur in the United States every year (*86*).

Salmonella are facultative anaerobic, gram-negative rods, which belong to the *Enterobacteriaceae* family. *Salmonella* have an optimum growth temperature of 37°C, however they readily adapt to extreme conditions. Growth at temperatures as low as 5.9°C (*83*) and as high as 54°C (*41*) have been reported for specific experimental conditions. Extended survival under freezing temperatures when inoculated in non-fluid matrixes has been demonstrated in previous studies (*15,42,51,57*). Survival when exposed to heat treatment, especially in low water activity foods, is currently a matter of concern. *Salmonella* has an optimum growth pH of 6.5-7.5, however it can grow at pH values of 4.5-9.5. Poultry meat and eggs are the most common reservoir of *Salmonella*, with the characteristic hen-to-egg transovarian transmission of serovar Enteritidis posing a major difficulty in the control of this pathogen in eggs, but in recent years, the occurrence of salmonellosis outbreaks linked to fresh produce has increased. Non-

typhoidal human salmonellosis is often characterized by the onset of non-bloody diarrhea and abdominal pain 8-72 hours from ingestion of the pathogen. The mechanism by which *Salmonella* infection generates these symptoms in the human host has not yet been fully elucidated. The illness is usually self-limiting, however in extreme cases it can lead to systemic infections and chronic conditions. A *Salmonella* dose-response curve based on outbreaks data has been constructed by FAO/WHO (*48*) and indicates that even very low doses can cause illness. Extensive information on the identification, growth and survival of *Salmonella,* as well as on human salmonellosis can be found elsewhere (*33,89*).

The taxonomy of the genus *Salmonella* has been reviewed in recent years. According to the CDC (*21*), the genus *Salmonella* contains two species, *S. enterica* and *S. bongori*. Twenty serotypes are classified within *S. bongori* and 2,443 within *S. enterica*. The majority of these serotypes belong to *S. enterica* subsp. *enterica*, and strains in this group are responsible for approximately 99% of *Salmonella* infections in humans and warm-blooded animals. It is accepted that when referring to *S. enterica* subsp. *enterica* serovars Typhimurium and Kentucky – taking as example the strains used in this dissertation - the abbreviated nomenclature *S.* Typhimurium and *S.* Kentucky may be used.

2. *Salmonella* **and poultry products**

Due to the frequent occurrence of *Salmonella* in poultry carcasses (*117*) and the risk it poses, the chilling and freezing of ready-to-cook poultry is regulated with respect to processing time and temperature limits (*127*), and performance standards have been issued for the presence of *Salmonella* in poultry carcasses (*53*). In addition, frozen, breaded chicken products containing raw poultry have been recently identified as risk

factors for *Salmonella* infection (*32,76,77,77*). Within the last decade, salmonellosis outbreaks in Australia (*71*), British Columbia - Canada (*32,76,77*) and Minnesota – United States (*120*) have implicated raw, frozen chicken nuggets, strips and entrees as transmission vehicles of infection. The cooked appearance of these products makes them potentially dangerous; consumers are likely to identify them as fully cooked and only reheat them before consumption, as suggested by epidemiological investigations of recent outbreaks (*32,120*). Previous studies (*15,42,51*) and Chapter 2 of this dissertation have demonstrated the survival of *Salmonella* during frozen storage, as well as during microwave cooking at low wattages when inoculated into processed chicken products (Chapter 4). These two types of poultry products - raw, refrigerated poultry and raw, frozen chicken products - are the focus of this research project.

3. Predictive food microbiology

Predictive microbiology has been used to model the growth of pathogenic bacteria in several food products as a function of environmental variables (*28,88,101,129*). Most of the work in predictive food microbiology has studied the growth of microorganism on culture media, minimizing the influence of the food matrix as a growth medium and the potential competition posed by the background microflora of the food product. Models describing the effect of temperature in the growth of *Salmonella* have been developed for sterile chicken (*69,102*) and laboratory media (*59*). In non sterile substrates, the growth of *Salmonella* as a function of temperature has been modeled in minced beef (*79*) and ground chicken (*103*). Published studies related to the growth of *Salmonella* as a function of temperature may be compiled and analyzed providing useful data. The number of studies providing a systematic and critical analysis of the published literature, referred to

as "meta-analysis", has increased in the past years (*84*). In the first Chapter of this dissertation, we developed a model for the growth of *Salmonella* in poultry products as a function of storage temperature utilizing previously published data. These compiled data in the form of a model, when compared to existing predictive models for *Salmonella* and experimental data collected in our laboratory may lead to a better understanding of the growth of this pathogen in poultry products.

4. Freezing

Previous research has extensively studied the effect of freezing and frozen storage on bacterial viability, mostly in fluids. Rapid cooling (>50°C/h) is characterized by intracellular ice crystals formation and slow cooling (1-10 °C/h) by extracellular ice formation (*22,109*). In slow freezing, the concentration of solutes in the external environment leads to osmotic dehydration of the cell (*46,109*). In commercial freezing of food products (10-50 °C/h), the consequences of both slow and rapid freezing have been observed (*22*). During frozen storage, cells are subject to the growth of existing crystals by recrystallization and to the extended effect of the increased concentration of solutes (*10,109*). Death rates during frozen storage are more pronounced at temperatures just below the freezing point, and are usually slow below -20°C (*51,57*). The lethal effects of freezing are significantly more evident in liquids than in solids (*81, 83*) and may be enhanced by the addition of electrolytes such as NaCl to the freezing medium (*27,81*). It has also been demonstrated that stationary phase cultures are more resistant to freezing than exponential phase cultures (*82*). In general, Gram positive bacteria are more resistant to freezing than Gram negatives (*123*), however, the latter are also capable of surviving this stress.

Previous research has studied the survival of *Salmonella* in meat and poultry products - mostly unprocessed- during frozen storage, with results ranging from complete to minimal survival, with varying degrees of injury. Briefly, Georgala and Hurst (*57*), Foster and Mead (*51*), Dykes and Moorhead (*43*) and Barrell (*15*) demonstrated almost complete survival of *Salmonella* in meat products during frozen storage, with varying degrees of structural injury. However, freeze-induced metabolic injury in *Salmonella* has also been reported (*122*). Escartin *et al.* (*47*) reported drastic reductions of *Salmonella* population in naturally-contaminated raw pork during frozen storage, conflicting with the aforementioned studies.

Due to the variability in results reported in the published literature regarding the survival of *Salmonella* in meat and poultry products during frozen storage, and in response to the recent association of frozen chicken products with salmonellosis outbreaks (*77*), we investigated the survival of *Salmonella* during frozen storage when inoculated in chicken nuggets and strips. As described in Chapter 2 of this dissertation, we conducted frozen storage experiments at -20°C, aiming at obtaining results relevant to food processing conditions.

The results reported in Chapter 2 of this dissertation indicate that *Salmonella* can indeed survive frozen storage, with structural injury as a consequence. Extensive evidence points at damaged outer membranes as the major effect of freezing bacteria (*81*), as suggested by leakage of cellular components into the growth medium, penetration of surfactants, dyes and enzymes into the cell (*11,27,109*) and sensitization to antibiotics (*110*) and bacteriocins (*20*). Sublethally damaged foodborne pathogens

however are able to rapidly repair the injury once environmental conditions become favorable and regain their capacity to cause disease (*22,63,108*).

The outer membrane of Gram-negative species is formed by lipopolysaccharides (LPS), phospholipids, porins, the enterobacterial common antigen, and the capsular polysaccharide M antigen (*96*). LPS, in particular the length of its polysaccharide chain, has been previously implicated in the response of *Salmonella* to freezing, however the experimental conditions of previous studies were not relevant to food production (*18,20*). In addition, research has shown that LPS mutants exhibit less protein content than wild type strains (*3,19,119*). These outer membrane proteins are predominantly porins, transmembrane proteins that form non-specific channels that allow the passage of small hydrophilic molecules into the cell (*96*). The space that should be occupied by proteins is filled in with phospholipids in LPS-defective strains (*96,119*). Thus, in Chapter 3 of this dissertation we studied the effect of LPS and porin mutations separately in the survival of *Salmonella* during frozen storage, at relevant food processing conditions.

5. Microwave cooking

In response to the recently recognized risk of *Salmonella* infection associated with the improper cooking of processed chicken products that appear to be ready-to-eat but contain raw poultry, microwave cooking has been referred to as "not advisable" for these products (*92*). In addition, FSIS (*56*) has announced that the label of such products must discourage the use of a microwave oven for cooking purposes. The uneven heating profile typical of microwave cooking and the variability in equipment characteristics, particularly wattage, make it difficult to define standardized microwave oven cooking instructions (*116*). The occurrence of two outbreaks in 2008 (*7,8*) linked to undercooking

of raw, frozen chicken entrees however suggests that consumers will continue to use microwave ovens for cooking these products, in most cases without the use of a food thermometer.

The generation of temperature gradients within food products cooked in microwave ovens is well documented, particularly in multi component foods (*62,65,66,132*). From a food safety point of view, the occurrence of "cold spots" within a food product during cooking is of concern; failure to achieve safe final cooking temperatures may lead to pathogen survival. Several factors related to the food product and equipment characteristics may influence the heating rates achieved in different parts of the product. Among these, microwave oven power has been identified as a major variable determining heating profiles and thus bacterial survival (*62*). The challenges posed by microwave cooking for the development of standardized cooking instructions represent also a food safety concern.

In Chapter 4 of this dissertation, we assessed the effect of microwave cooking at different wattages in the survival of *Salmonella*, when inoculated in a frozen entrée containing raw poultry cooked following the label instructions. Our objective was to generate experimental data related to epidemiological investigations (*32,120*) which have recognized microwave cooking of chicken products containing raw poultry as an unsafe practice, as well as for risk assessment purposes (see Chapter 5).

6. Quantitative microbial risk assessment

Quantitative microbial risk assessment (QMRA) is a methodology used to organize and analyze relevant data in order to estimate the public health consequences associated with microbiological risk (*37*). The main outcome of QMRA is traditionally

defined as the estimated probability of illness from the consumption of the food product under study (*37*). Monte Carlo simulation is currently the most widely used technique for conducting microbial risk assessments. This methodology uses a stochastic approach, where key factors in the model are represented by distributions and a set of output values in the form of a distribution is generated as a result of multiple iterations (*37*). Because high-risk scenarios often arise from outlying data points rather than average results (*130*), Monte Carlo simulation has the potential to provide a more realistic estimation of risk compared to a strictly deterministic approach. In addition, taking into account the variability described by a frequency distribution produces a more realistic assessment of risk than one based on a sole discrete value, such as the mean or worst case, at each step modeled (*23*). QMRA is typically described as consisting of four stages: hazard identification, hazard characterization, exposure assessment and risk characterization.

This methodology was applied in Chapter 5 of this dissertation, where a risk assessment model for salmonellosis associated with consumption of raw, frozen chicken products cooked in a microwave oven is presented in. The proposed risk assessment may represent a valuable tool for the evaluation of different strategies aiming at controlling the risk posed by raw, frozen chicken products as well as creating awareness of this risk among consumers.

7. Overall project overview

Assessing the microbiological consequences, specifically *Salmonella* growth and survival, of different time-temperature scenarios during the processing, storage and consumer cooking of raw poultry products may provide valuable information for more

accurate risk assessments and ultimately, for controlling the risk of salmonellosis associated with these products.

Several factors may influence the risk of salmonellosis associated with poultry products. The objective of this study was to investigate three major factors, relevant to the food industry and consumers' practices. First, we modeled the growth of *Salmonella* on raw poultry as a function of temperature. Secondly, we studied the survival of *Salmonella* under frozen storage in processed poultry products. Thirdly, we assessed the lethality of *Salmonella* in frozen chicken entrees when heated using a microwave oven. In addition, a risk assessment for salmonellosis associated with consumption of raw, frozen chicken products cooked in a microwave oven was developed using our experimental data as well as published data.

The results of this dissertation demonstrate that a systematic analysis of previously published data may be utilized to generate new valuable information, in our case a predictive growth model for *Salmonella* on poultry, thus significantly reducing the costs associated with generation of new data. Validation of the model against experimental data was conducted, and we concluded that its application may be considered fail-safe for temperatures below 28°C. The effect of frozen storage at conditions relevant to the food industry (-20°C) in the survival of *Salmonella* inoculated in a chicken matrix proved to be mild, causing only structural injury in a portion of the population. It was confirmed that selective media does not provide an adequate quantification of the entire bacterial population in a sample subject to frozen storage. Furthermore, an analysis of the effect of lipopolysaccharide (LPS) and outer membrane protein mutations (Omp) indicated that severe defects in LPS did not affect survival of

Salmonella under the conditions tested, and that OmpC but not OmpD may favor survival of *Salmonella* during storage at -20°C. In addition, in response to recent outbreaks implicating raw, frozen chicken products as risk factor for salmonellosis, we investigated the effect of different oven wattages when cooking these products with respect to final temperatures achieved and survival of *Salmonella.* Our results confirm the findings of epidemiological investigations, which identified microwave cooking as a consumer practice leading to infection, particularly low wattage ovens. These results as well as relevant previously published data were included in a risk assessment model for salmonellosis associated with consumption of raw, frozen chicken products cooked in a microwave oven. The proposed model was successfully validated against the reported outcomes of a 2005 salmonellosis outbreak in Minnesota, and may constitute a valuable tool for evaluating risk mitigation strategies for raw, frozen chicken products.

Chapter 1: Modeling the growth of *Salmonella* in raw poultry stored under aerobic conditions[1]

1.1. Abstract

The presence of *Salmonella* in raw poultry is a well-recognized risk factor for foodborne illness. The objective of this study was to develop and validate a mathematical model that predicts the growth of *Salmonella* in raw poultry stored under aerobic conditions over a variety of temperatures.

One hundred and twelve *Salmonella* growth rates were extracted from 12 previously published studies. These growth rates were used to develop a square-root model relating the growth rate of *Salmonella* to storage temperature. Model predictions were compared to growth rate measurements collected in our laboratory for four poultry-specific *Salmonella* strains (two antibiotic-resistant and two antibiotic-susceptible strains) inoculated onto raw chicken tenderloins. Chicken was inoculated at two levels (10^3 CFU/cm^2 and ≤ 10 CFU/cm^2) and incubated at temperatures ranging from 10 to 37°C. Visual inspection of the data, bias and accuracy factors, and comparison with two other published models were used to analyze the performance of the new model.

Neither antibiotic resistance nor inoculum size (to the extent of the data collected in this study) affected *Salmonella* growth rates. Also, the presence of spoilage micro flora did not appear to slow the growth of *Salmonella*. Compared with the two other published models, our model provided intermediate predicted growth rates. Our model predicted slightly faster growth rates than those observed in inoculated chicken in the

temperature range of 10-28°C, but slightly slower growth rates than those observed between 30°C and 37 °C. Slightly negative bias factors were obtained in every case (-5 to -3%), however application of the model may be considered fail-safe for storage temperatures below 28°C.

1.2. Introduction

Salmonella are commonly present on raw poultry carcasses (*117*) and their presence may lead to outbreaks of foodborne illness when the product is undercooked, or when cross-contamination occurs. The chilling and freezing of ready-to-cook poultry is regulated with respect to processing time and temperature limits (*127*), and performance standards have also been issued for the presence of *Salmonella* in poultry carcasses (*53*). Predicting the expected change in *Salmonella* concentration during different time-temperature scenarios which may occur during the processing and storage of poultry carcasses may provide valuable information to both processors and regulatory officials seeking to manage risk.

Several predictive models describing pathogen growth on different foods as well as in laboratory media have been published. Models describing the effect of temperature on the growth of *Salmonella* have been developed for laboratory media (*59*). Predictive models for *Salmonella* have been developed in sterile meat and poultry products (*36,69,98, 102*). Models have also been published describing the growth of *Salmonella* (*79,103*) in raw meat and poultry pieces.

Published studies describing the growth of *Salmonella* as a function of temperature may contain useful data, even if modeling was not the explicit objective of the investigators. The number of studies providing a systematic and critical analysis of

the published literature, referred to as "meta-analysis", has increased in recent years, especially those relating to food safety (*84*). In our study, we create a model for the growth of *Salmonella* in poultry products as a function of storage temperature utilizing previously published data. This model is then compared to existing predictive models for *Salmonella* and to experimental data collected in our laboratory, with the intent of developing a better understanding of the growth of this pathogen in poultry products.

1.3. Materials and methods

Literature search. A literature search was conducted and twelve studies were identified as sources for *Salmonella* growth rates at different temperatures (*2,4,50,59,60,74,78,79,83,101,121,125*). Studies presenting *Salmonella* growth data on meat and poultry products, as well studies presenting growth data for poultry-associated *Salmonella* strains on laboratory media were selected. Objectives, methods and data presentation formats varied widely among the studies. Growth parameters were extracted directly from tables or growth curves (by superimposing a regression line over the exponential phase of growth); calculated from generation time values (Growth Rate = Ln(2) /Generation Time); or from specific growth rate values (Growth Rate = 1/Ln(10)*Specific Growth Rate).

Growth rate corrections. Variations in methodologies and systematic deviations had to be corrected for certain studies. It was deduced from the data presented that Gibson *et al*. (*59*) and Mackey and Kerridge (*79,79*), used Log(2) instead of Ln(2) in the equation Growth Rate = Ln(2)/Generation Time. Also, systematic deviations were detected in the growth rates calculated from the Mackey *et al*. (*78*) and Smith (*121,121*) generation times when compared to those from the other ten studies

(*2,4,50,59,60,74,79,83,101,125*). Baranyi (personal communication) has also noticed

such systematic deviations during the creation of the ComBase (*6*) database, where a

large number of observations were extracted from the literature and compared. Where

needed, a correction factor of Ln(2)/Log(2) was applied to reported generation times

(*59,78,79,121*), before being converted to growth rate values.

Modeling. The data were modeled using a square-root or Ratkowsky equation

(*107*) relating the square-root of the bacterial growth rate and storage temperature (T):

$$\sqrt{Growth\ rate} = b \bullet (T - T_0) \quad \textbf{Equation 1.1}$$

Although purely empirical, the Ratkowsky equation, and variations of it, has been

used with good results to model microbial growth in many foods

(*35,39,70,85,91,95,128,129,131*). This equation has been shown to be suitable for

modeling bacteria growth rate between minimum and optimum growth temperatures

(*105*); thus only growth rates for temperatures between 5.9 and 37°C were modeled in

this study.

The model presented in this study is a simple linear equation relating growth rate

and temperature. The lag phase was not modeled since the validation growth curves

obtained in the present study (see below) did not show a clearly defined lag phase at

temperatures from 10 to 37°C. This observation is consistent with those made by Oscar

(*103*) who also noted that the lag phase was not apparent in growth curves of *Salmonella*

Typhimurium in non-sterile ground chicken breast meat.

Sample preparation. Chilled raw chicken breast tenderloins were purchased from local stores and transported to the laboratory within 15 minutes. They were then either tested immediately or stored frozen for no more than one week. If frozen, chicken pieces were thawed using a microwave before being prepared for testing. The microwave treatment was mild, and was only applied to render the chicken pieces easier to handle and prepare for the experiments. The microwave was set to "defrost" and the time was dependent on the weight of the chicken pieces being thawed. In instances when microwaving caused an overheating or cooking of the tenderloins on the edges of the tray, these were discarded, and only the ones that remained completely raw, but slightly thawed, were used.

No significant difference was detected in the growth curves obtained from immediately analyzed versus frozen and thawed samples (data not shown). No growth in total plate count agar with 0.01g/L tetracycline and in XLT4 was found for controls of uninoculated tenderloins, indicating bacterial counts below the detection limit of 10 CFU/cm^2 and total aerobic counts for the uninoculated controls averaged 10^2 CFU/cm^2 (data not shown).

Inoculum preparation. Tenderloins were co-inoculated with antibiotic-resistant and susceptible *Salmonella* strains (Table 1.1) isolated from conventional and organic chicken samples (*31*) and kindly provided by Dr. Jianghong Meng, University of Maryland. *Salmonella* strains were preserved frozen (-70 ºC) in Brain Heart Infusion Broth (Difco Laboratories, Detroit, MI) with 15% glycerol (Fisher Scientific, Pittsburgh, PA). Each non antibiotic-resistant strain was grown to stationary phase in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) for 18 hours at 37°C. Each antibiotic-resistant

strain was grown to stationary phase in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) with 0.01g/L of tetracycline for 18 hours at 37°C. After incubation, a cocktail of strains was prepared by combining 1ml of each culture. The cocktail ($\sim10^8$ CFU/ml) was diluted to a final concentration of $\sim10^5$ CFU/ml in a 500ml solution of 0.1% peptone water (Difco Laboratories, Detroit, MI) in a 7x12" sterile stomacher bag (Fisherbrand, sterile stomacher bag, Pittsburgh, PA). No more than 10 tenderloins were immersed for 1 minute in the inoculation solution. A final inoculum level of $\sim10^3$ CFU/cm^2 was targeted.

In a previous study from our lab (*39*), we determined that *Pseudomonas* spp., (the major constituent of the background microflora on chicken tenderloins), was present at an average initial population of $\sim10^2$ CFU/cm^2, lower than the initial population used in both this study and that of Juneja *et al*. (*69*). Therefore, to further investigate the effect of *Salmonella* starting population and the effect of the natural background microflora on its growth rate, we conducted a smaller set of experiments at lower *Salmonella* inoculum level (≤10 CFU/cm^2). For lower inoculum experiments (targeted ~10 CFU/cm^2) the same procedure was followed but the *Salmonella* cocktail was diluted to $\sim10^2$ CFU/ml in the 500ml solution of 0.1% peptone water. Initial *Salmonella* concentrations for the low inoculum samples could not be determined because they were, on average, below the detection limit (10 CFU/cm^2), data not shown.

After inoculation, the tenderloins were drained and placed individually in a 5x7"sterile stomacher bag (Fisherbrand, sterile stomacher bag, Pittsburgh, PA) with an absorbent pad (4.5"x7" super absorbent tray pad, International Tray Pads & Packaging Inc., Aberdeen, N.C.) and incubated in a water bath set at a constant holding temperature. The absorbent pad was included to simulate conditions at retail. Samples were then

tested with a frequency dependent on the holding temperature. For each time point, a sample was taken from the water bath, the absorbent pad was removed, and the tenderloin, still in the sterile bag, was rinsed with 100 ml 0.1% peptone water (Difco Laboratories, Detroit, MI) for 30 seconds (*67*). Rinsing rather than homogenizing was applied considering the samples were surface-inoculated. The average surface area of single tenderloins was estimated from pieces tested as ~ 100 cm^2. The rinse solution, or a serial dilution of the rinse solution, was plated in duplicate both on Plate Count Agar (Difco Laboratories, Detroit, MI) with 0.01g/L of tetracycline (Acros Organics, Pittsburgh, PA), and on XLT4 Agar (Difco Laboratories, Detroit, MI). Colonies were counted after incubation at 37°C for 24 hours. Growth curves were obtained for temperatures 10, 12, 15, 18, 20, 23, 25, 30, 33, 35 and 37°C for the ~10^3 CFU/cm^2 inoculum, and at 10, 15, 20, 25, 30 and 35°C for the ≤10 CFU/cm^2 inoculum. DMFit (Institute of Food Research, Norwich, UK) was used to model growth from experimental observations fitting the data to the Baranyi (*13*) equation. This model was used to fit the growth of *Salmonella* as a function of time, based on it's ease of use, wide acceptability to the modeling community, and the observation of Juneja *et al*. (*69*) who concluded that the growth data derived from the Baranyi equation provided a better fit than that obtained from the Gompertz equation when used to develop a secondary growth model for *Salmonella.*

Model evaluation. The indices of bias (Bf) and accuracy (Af) factors proposed by Baranyi, *et al*. (*14*) were used to analyze the performance of the model presented in this study. The bias factor measures the average relative deviation of predicted and observed values, and the accuracy factor is a measurement of how close on average

predictions are to observations. Visual inspection of the data (i.e., a plot of the logarithm

of predicted against experimental growth rate values and the residuals plots) was also

used to evaluate the model performance.

1.4. Results

Published growth rates. As noted above, a correction factor of Ln(2)/Log(2)

was applied to some generation times (*59,78,79,121*) to eliminate systematic deviations

and only then were the corrected generation times used to calculate growth rates (Figure

1.1). Although an overall trend is evident, a wide range of growth rate values can be

observed for each specific temperature (Figure 1.1), which can be attributed to the

variability in strains, methods and growth substrates among studies.

Model development. A summary of relevant details from the twelve previously

published studies used to develop a model is shown in Table 1.2. While experimental

objectives, methods, growth substrates and data presentation formats varied among

studies, the overall consistency in the data encouraged us to use it for modeling purposes

(see Figure 1.1 and Figure 1.2).

A square-root or Ratkowsky equation (*107*) was used to model the data between

5.9 and 37°C (Figure 1.2). This equation, in its basic form, was chosen in order to

provide a simple estimation of growth within the minimum and optimum growth

temperatures for the pathogen. *Salmonella* have been reported to grow at temperatures as

low as 5.9°C on laboratory media (*83*) and the optimum growth temperature for

Salmonella is known to be 37°C (*117*). *Salmonella* growth rate values at temperatures

between 5.9°C and 37°C were selected from the literature to develop the model. No

published growth rates at temperatures less than 5.9ºC were found in the literature, while

growth rates at temperatures greater than 37ºC were specifically excluded. The

Ratkowsky equation in its basic form is not designed to describe growth rates at

temperatures greater than the optimum, and these temperatures are unlikely to occur

during the production and handling of raw poultry. Fitting the data to the Ratkowsky

equation (Eq.1.1) resulted in a straight line relating the square-root of the growth rate and

temperature (Figure 1.2, Eq. 1.2), with good correlation between the model and the

observations used to generate it ($R^2 \sim 0.95$).

$$\sqrt{Growth\ rate} = 0.027 \bullet (T - 4.122) \quad \textbf{Equation 1.2}$$

Model comparison with experimental data and other models. Forty-eight

growth rate measurements collected in our laboratory for raw chicken tenderloins

inoculated at $\sim 10^3$ CFU/cm^2 for both antibiotic-resistant and susceptible *Salmonella*

strains (Table 1.1) at twelve temperatures from 10 to 37°C are shown on Table 1.3. R^2

coefficients between 0.883 and 0.999, and standard errors between 0.937 and 0.069, were

obtained when fitting the experimental data to the Baranyi equation. Initial concentrations

for the inoculated samples averaged 3.4 log CFU/cm^2 ($\pm$ 0.24 log CFU/cm^2) for

antibiotic-resistant *Salmonella* strains and 3.2 log CFU/cm^2 ($\pm$ 0.35 CFU/cm^2) for

susceptible strains. The optimum temperature for growth of *Salmonella* in chicken

tenderloins was between 33 and 35°C.

A comparison of predicted growth rates at different temperatures by the models of

Juneja *et al*. (*69*), Oscar (*103*) and the model developed in this study are shown in Figure

1.3. In their publication, Juneja *et al*. (*69*) developed 3 models for the effect of

temperature on growth rate. Each of these secondary models corresponded to growth rates estimated from 3 different equations (Gompertz, logistic and Baranyi). For consistency, in our study we only considered the Juneja *et al.* model (*69*) for growth rates estimated from the Baranyi equation. The model developed in our study is in close agreement with that of Juneja *et al.* (*69*) (Figure 1.3).

Figure 1.4 shows that the model tends to slightly over predict growth rate at temperatures < 26 °C, and slightly under predict at temperatures > 28 °C, which is also supported by the *Salmonella* growth rates from a smaller second set of observations for raw tenderloins inoculated at concentrations ≤10 CFU/cm^2 and incubated at six temperatures from 10 to 35°C (Table 1.4). R^2 coefficients between 0.756 and 0.999, and standard errors between 1.098 and 0.032, were obtained when fitting this lower-inoculum experimental data to the Baranyi equation. Although this data set consisted of a limited number of observations, consistent with the observations seen in the higher inoculum level experiments (Table 1.3, Figure 1.4), the model predictions were slightly faster than the observations for temperatures 10-30°C, but slower for 35°C (Table 1.4). Both antibiotic-resistant and susceptible *Salmonella* at initial populations of ~10^3 and ≤10 CFU/cm^2 showed similar behavior when growing on the surface of raw chicken with background microflora (Figure 1.4 and 1.5).

Model evaluation. The bias and accuracy factors proposed by Baranyi *et al.* (*14*) were used to evaluate the performance of the model. The accuracy factor measures how close predictions are to observations and the bias factor indicates whether the model, on average, over- or under-predicts (*112*). The bias and accuracy factors expressed as % Discrepancy and % Bias (Table 1.5) show that there is a 5% discrepancy and no bias

between the model predictions and the literature data used in its generation. For the 10^3

CFU/cm^2 inoculum data set, a 17% discrepancy was calculated between the model

predictions and the experimental data collected for both antibiotic-resistant and

susceptible *Salmonella.* The negative percent bias obtained when comparing the model

predictions to the experimental data (-5% and -3% for antibiotic-resistant and susceptible

strains, respectively) are indicative of a "fail-dangerous" model (*14*), predicting slower

growth rates than those observed. Plots of observations vs. predictions may be used to

reveal systematic deviations that are not revealed by the bias factor (*14,112*). The results

shown in Figure 1.5 are consistent with the bias factor, revealing several experimental

data points above the equivalence line, representing those observed growth rates that are

faster than the model predictions. Positive residuals (not shown) were also calculated for

these data points, as evidence of faster observed growth rates than those predicted. This

under-prediction zone corresponds to growth rates between 28 and 35°C (Figure 1.4).

For the ≤10 CFU/cm^2 inoculum data set, similar results were obtained. Faster

experimental growth rates than those predicted can be seen in Table 1.4 for 35°C,

identified also by the negative bias factor (Table 1.5) and in Figure 1.5 as points above

the equivalence line.

1.5. Discussion

Other models - Juneja *et al*. These researchers (*69*) developed a model for

Salmonella growth on poultry, fitting the data to the Baranyi equation as primary model,

and extended the Ratkowsky equation ($R^2 = 0.999$) as secondary model. The growth

medium used, however, was irradiated ground chicken; therefore the effect of the

competitive microflora naturally present on raw poultry was not considered when

modeling the *Salmonella* growth. Thomas and Wimpenny (*124*) showed that background microflora may impair the growth of *Salmonella* in culture media at 20°C.

Other models – Oscar. This researcher (*103*) incorporated the effect of competitive microflora when modeling the growth of a low bacterial population of a multiply antibiotic resistant *Salmonella* Typhimurium DT104 on raw ground chicken samples. By utilizing a low initial inoculum level ($10^{0.6}$ CFU/g) and measuring growth on non-sterile chicken samples, Oscar's model (*103*) attempts to more closely mimic reality. However, only one strain (*Salmonella* Typhimurium DT104) – apparently linked to a raw milk outbreak (*30*) was used inoculate the raw ground chicken. Also, a logistic with delay function was used to model growth rate as a function of temperature, indicating that at lower temperatures (between 10 and 15.6°C) growth rate values remain constant, an unusual behavior not consistent with previous observations (*59,69,78,79,121*).

The predicted growth rates from Oscar (*103*) are lower than those predicted by our model. These slower growth rates might be the result of background microflora competing with the single strain being measured (*Salmonella* Typhimurium DT104), a characteristic of this particular strain when growing on raw chicken, or a result of effects produced when chicken was ground. Also, Oscar (*103*) modeled the growth of *Salmonella* Typhimurium DT104 starting with a smaller concentration ($10^{0.6}$ CFU/g) than did Juneja *et al*. (*69*). Previous studies have observed an effect of inoculum size on growth rate (*32,34*). Others, like Mackey and Kerridge (*79*) concluded the opposite for *Salmonella* growing on not-sterile minced beef, and the data collected as part of the

validation component of this study (Table 1.3 and 1.4) show no consistent differences with inoculum size.

Current model. Our study is unique: by validating our model against surface growth of *Salmonella* on whole raw poultry pieces, we have provided a realistic validation. The R^2 coefficients of the published models that utilized the Ratkowsky equation (0.986, 0.98 and 0.999 for (*79*), (*121*) and (*69*) respectively), are slightly higher that that for the model presented in our study (0.95). Given that our model was developed using growth data collected on different substrates using a variety of microbiological media this would be expected. A similar result was obtained in our previous *Pseudomonas* study (*39*).

Bias and accuracy. Neither Juneja *et al*. (*69*) nor Oscar (*104*) used the indices proposed by Baranyi *et al*. (*14*) to evaluate model performance. Due to the consistency in equations used and the potential correlation between both data sets, we more thoroughly compared our results with those of Juneja *et al*. (*69*). We estimated percent bias and percent discrepancy values for Juneja *et al*'s model (*69*) using the data presented graphically in their study, obtaining values of ~0% bias and ~8% discrepancy. Compared to our experimental data set, the Juneja *et al*. (*69*) model gave a 2% bias and 15% discrepancy. Thus, the data collected in our study seems to be in close agreement with the predictions of the model by Juneja *et al*. (*69*).

The equivalent results obtained with ≤ 10 CFU/cm^2 and ~10^3CFU/cm^2 inoculum levels suggest that the growth characteristics of *Salmonella* in raw poultry are not affected by its initial population, within the range tested. We did encounter difficulties in measuring the growth of *Salmonella* on XLT4 for the ≤ 10 CFU/cm^2 initial population

data set at 10°C, due to the high interference of non-*Salmonella* colonies able to grow on this agar. At this temperature, *Salmonella* starting with a low initial population seemed to be outgrown by the spoilage background microflora, probably *Pseudomonas* which, in previous studies (*39*), was shown to be present in retail raw poultry pieces at ~10^2 CFU/cm^2.

1.6. Conclusions

The published *Salmonella* growth rates collected show a reasonably high correlation with temperature when fitted to the Ratkowsky equation ($R^2 = 0.95$), although validation data also collected as part of this study does indicate that the model gives fail-dangerous predictions > 28°C. The growth rate observations collected in this study for antibiotic-resistant and susceptible *Salmonella* at both high (10^3 CFU/cm^2) and low ($\leq$10 CFU/cm^2) inoculum levels showed a high degree of similarity to one another. It should be noticed however that the low inoculum data set ($\leq$10 CFU/cm^2) is not exhaustive and only intends to support the main inoculum data set (10^3 CFU/cm^2). The model presented here gives growth rate predictions that are intermediate to those from previously published *Salmonella* models develop using sterile chicken (*69*) and using a single strain of *Salmonella* in raw chicken (*104*).

We have developed a model relating the growth of *Salmonella* on poultry to temperature, based upon previously published data, using an approach we have used previously to model *Pseudomonas* spoilage of chicken (*39*). Meta-analysis was applied (*84*) and published data including more than four decades of research was utilized to develop the model. While previously published data are often used to validate predictive models (*88,112*), one advantage of the approach used here is that the judicious use of

existing data can significantly reducing the costs associated with the generation of new data (*113*).

1.7. Tables

Table 1.1: *Salmonella* strains[a] used

S. enterica serovar	Resistant to
Kentucky	Streptomycin and tetracycline
Typhimurium	Amoxicillin/clavulanic acid, ampicillin, cephalothin, ceftiofur, cefoxitin, streptomycin and tetracycline
Kentucky	Antibiotic-susceptible
Typhimurium	Antibiotic-susceptible

a. Provided by Dr. Jianghong Meng, University of Maryland (*31*)

Table 1.2: Published studies used as *Salmonella* growth data sources

Reference	N[b]	Growth substrate	Sterile?
Alford and Palumbo 1969	5	Ground pork	N
Angelotti *et al.* 1961	4	Chicken a la King	Y
Farrell and Upton 1978	1	Bacon	N
Gibson *et al.* 1988[a]	13	Tryptic soy broth/Pork slurry	Y/N
Goepfert and Kim 1975	2	Raw ground beef	N
Li and Torres 1993	8	Media	Y
Mackey and Kerridge 1988[a]	12	Minced beef	N
Mackey *et al.* 1980[a]	22	Beef surface	N
Matches and Liston 1968	6	Tripticase soy agar	Y
Oscar 2002	42	Chicken burgers	Y
Smith 1985[a]	7	Sheep meat	N
Tiwari and Maxcy 1972	3	Raw ground beef	N

a. A correction factor of Ln(2)/Log(2) was applied to the generation times presented in these studies before using them to calculate growth rates
b. Number of data points extracted from the publication

Table 1.3: Predicted and experimental antibiotic-resistant and susceptible *Salmonella*
(initial population ~10^3CFU/cm^2) growth rates on raw poultry at temperatures 10 to 37ºC

| Temperature (ºC) | *Salmonella* growth rates (ΔlogCFU/hr) | | |
| | Predicted | Experimental | |
		Antibiotic-resistant	Antibiotic-susceptible
10	0.0252	0.0098	0.0081
		0.0101	0.0126
12	0.0452	0.0196	0.0248
		0.0291	0.0360
15	0.0862	0.0422	0.0578
		0.0560	0.0572
18	0.1403	0.0748	0.0944
		0.1696	0.1978
20	0.1837	0.1999	0.0992
		0.1450	0.1037
23	0.2597	0.1877	0.1985
		0.1979	0.2288
25	0.3177	0.3491	0.2859
		0.2995	0.3012
28	0.4155	0.5499	0.3579
		0.7201	0.5755
30	0.4880	0.5826	0.3939
		0.6408	0.5228
33	0.6078	0.7586	0.9523
		1.0970	1.0640
35	0.6949	1.0690	1.0700
		0.7198	0.8821
37	0.7878	0.6362	0.6078
		0.7819	0.7678

Table 1.4: Predicted and experimental antibiotic-resistant and susceptible *Salmonella*

(initial population ≤10 CFU/cm^2) growth rates on raw poultry at temperatures 10 to 35ºC

Temperature (ºC)	*Salmonella* growth rates (ΔlogCFU/hr)		
		Experimental	
	Predicted	Antibiotic-resistant	Antibiotic-susceptible
10	0.0252	0.0147	ND[a]
15	0.0862	0.0457	0.0364
20	0.1837	0.1059	0.1102
25	0.3177	0.1584	0.0957
30	0.4880	0.5955	0.3118
35	0.6949	1.0130	1.4950

a. Not Determined. *Salmonella* growth could not be determined at 10°C because of high interference from non-*Salmonella* able to grow on XLT4 media

Table 1.5: Evaluation of the model predictions against published growth rates used to create the model and experimental antibiotic-resistant and susceptible *Salmonella* growth rates using % Discrepancy and % Bias

Model predictions	% Discrepancy	% Bias
vs. Published	5	0
Initial Population ~ 10^{3}CFU/cm^2:		
vs. Antibiotic-resistant *Salmonella*	17	-5
vs. Antibiotic-susceptible *Salmonella*	17	-3
Initial Population ≤ 10 CFU/cm^2:		
vs. Antibiotic-resistant *Salmonella*	38	-4
vs. Antibiotic-susceptible *Salmonella*	46	-5

1.8. Figures

Figure 1.1: *Salmonella* growth rates at different temperatures collected from published data.
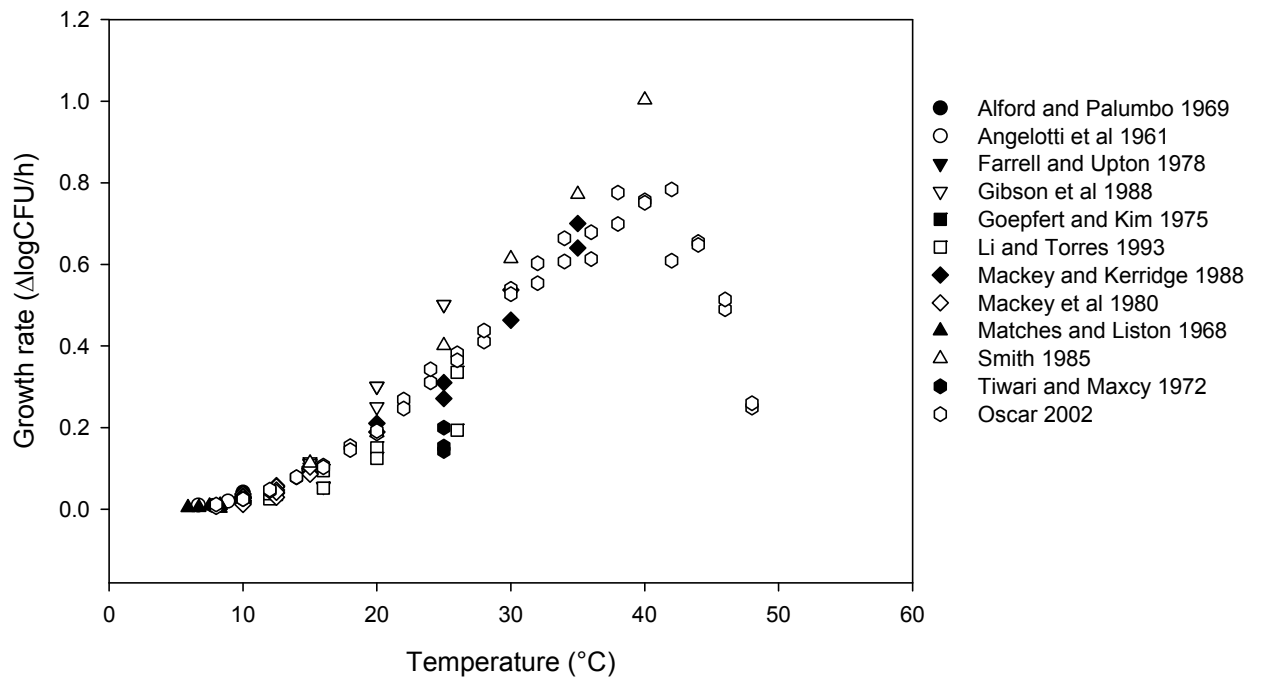
Figure 1.2: Ratkowsky equation relating published *Salmonella* growth rates and temperature.
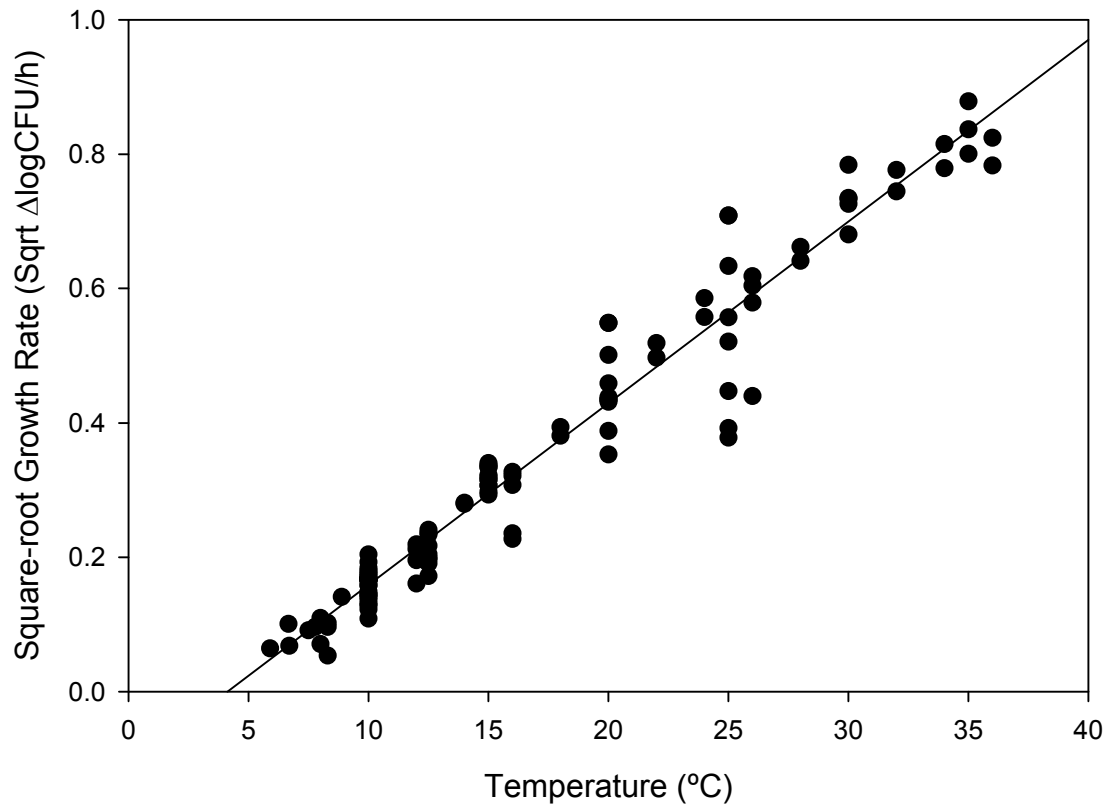
Figure 1.3: Predicted growth rates by the models of Juneja *et al.* (*69*), Oscar (*103*) and the

model developed in this study [a]
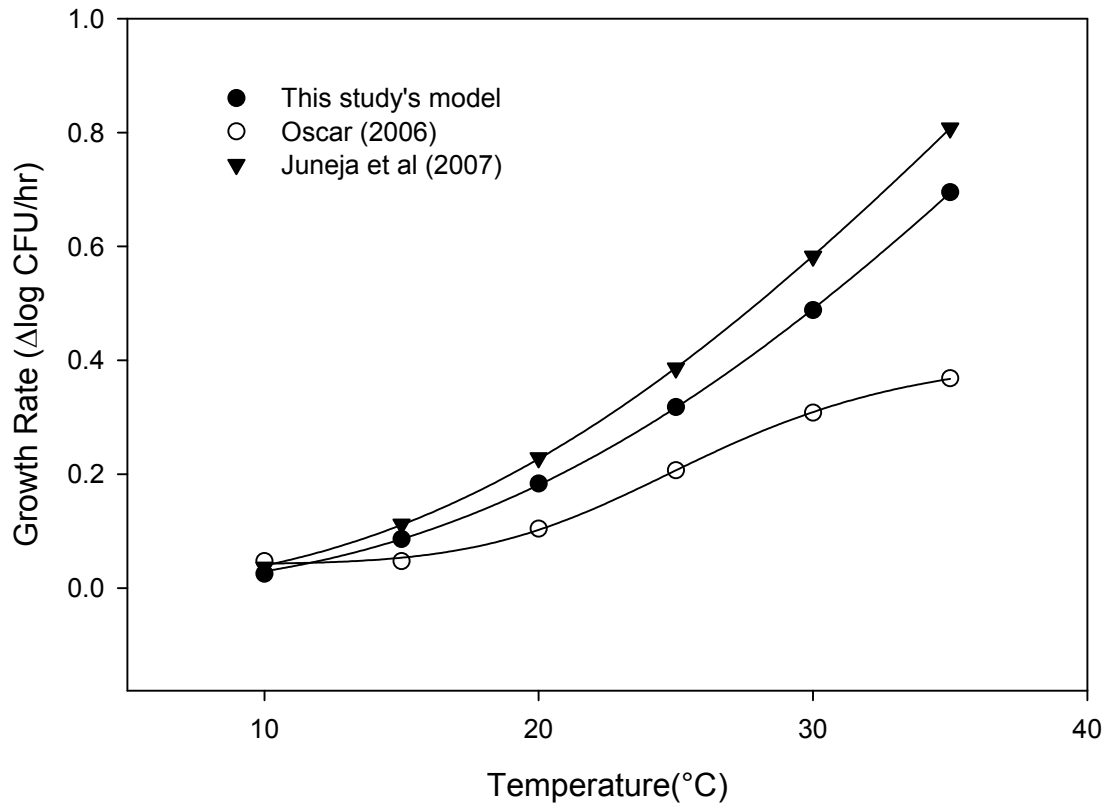
a Growth rates estimated from Baranyi model. Specific growth rates were transformed to growth rates multiplying by 0.43

Figure 1.4: Square-root of observed growth rates for antibiotic-resistant and susceptible

Salmonella (initial population ~10^3CFU/cm^2) at temperatures 10 to 37ºC
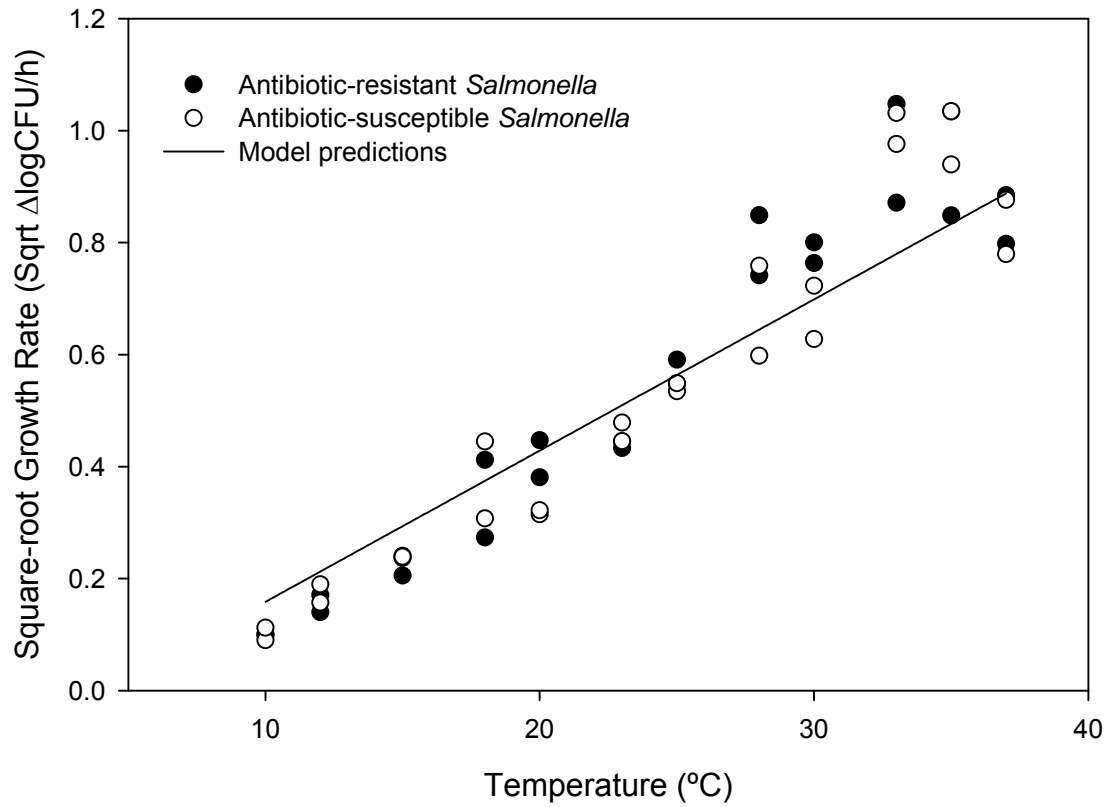
Figure 1.5: Logarithm of predicted against observed (*Salmonella* initial population ~10^3CFU/cm^2) growth rate values
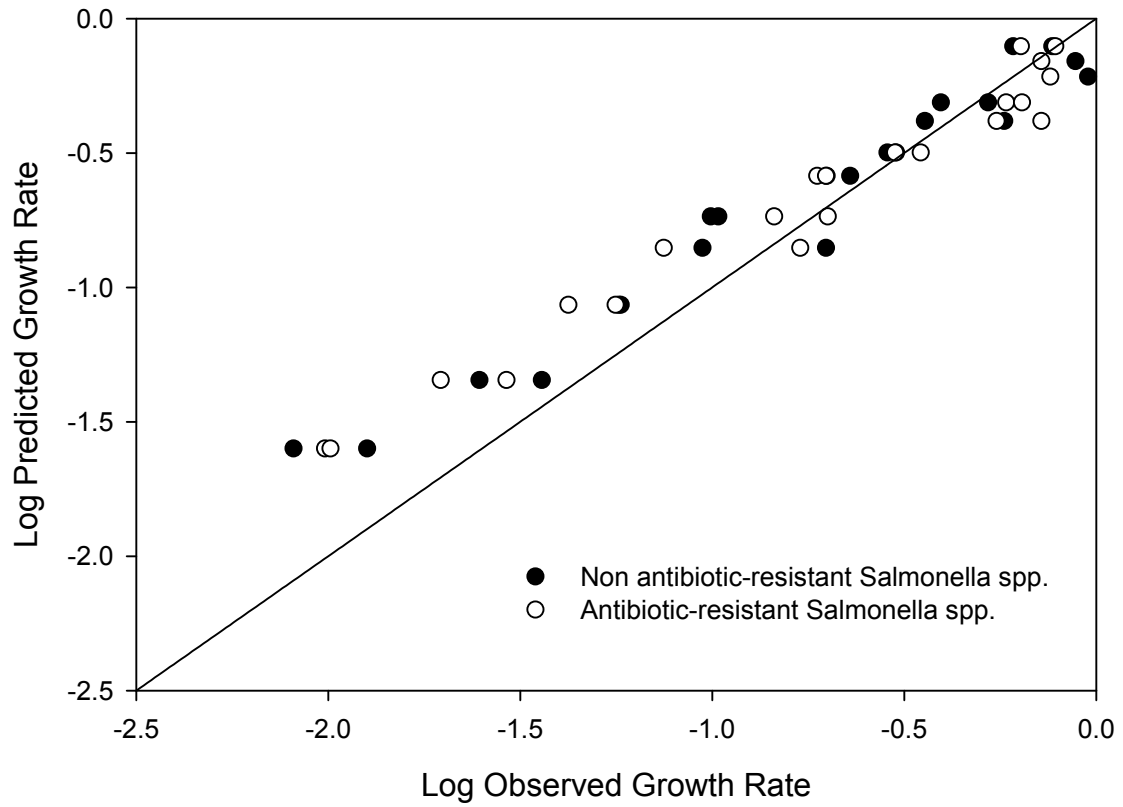
Chapter 2: The survival of *Salmonella* in processed chicken products during frozen storage[2]

2.1. Abstract

Frozen chicken products have been identified recently as a cause of salmonellosis. At least eight salmonellosis outbreaks from 1998-2008 have implicated undercooked frozen chicken nuggets, strips and entrees as infection vehicles. Thus, the presence of *Salmonella* in frozen products may pose an infection risk if the product is improperly cooked. The objective of this study was to assess the survivability of *Salmonella* during frozen storage (-20°C) when inoculated in processed chicken products.

Four *Salmonella* strains originally isolated from poultry were inoculated into frozen chicken nuggets (fully cooked) and frozen chicken strips (containing raw poultry) at initial populations of 10^4-10^5 CFU/g. Survival was assessed during storage at -20°C for 16 weeks by measuring bacterial growth on minimal, selective and nonselective agars.

Results indicate that cell concentrations measured in nonselective (PCA and PCA-Tet) and minimal (M9) agars remained relatively constant during the entire -20°C storage period studied (16 weeks) for both chicken nuggets and strips. However, cell concentrations were significantly ($p<0.05$) lower when measured in selective agar (XLT4) during the 16 weeks of frozen storage for both chicken nuggets and strips, suggesting that these cells were structurally injured.

The data presented in this study indicates that *Salmonella* can survive frozen storage when inoculated in frozen, processed chicken products, and confirms that

---

microbial counts on selective agar are not representative of the total population of samples subject to freezing.

2.2. Introduction

Frozen, breaded chicken products containing raw poultry have been recently identified as risk factors for *Salmonella* infection (*32,76*). Salmonellosis outbreaks in Australia (*71*), British Columbia (*32,76*) and Minnesota (*120*) have implicated raw, frozen chicken nuggets, strips and entrees as transmission vehicles of infection. The cooked appearance of frozen breaded chicken products containing raw poultry makes them potentially dangerous; consumers are likely to identify them as fully cooked and only reheat them before consumption, as suggested by epidemiological investigations of recent outbreaks (*32,120*).

Previous research has studied the survival of *Salmonella* in meat and poultry products - mostly unprocessed- during frozen storage, with results ranging from complete to minimal survival, with varying degrees of injury. Georgala and Hurst (*57*) reported survival of *Salmonella* Typhimurium during 90 days of storage at -20°C when inoculated in comminuted beef. Foster and Mead (*51*) measured almost 100% survival of *Salmonella* (*S.* Agona, *S.* Cerro, *S.* Haardt, *S.* Livingstone and *S.* Typhimurium) in non-sterile minced chicken breast during 100 days at -20°C, and approximately 10% in non-sterile minced chicken leg. Barrell (*15*) demonstrated that *Salmonella* Typhimurium U285 was able to survive frozen storage (-18 to -20°C) when inoculated in cooked minced beef; structurally injured but not metabolically injured cells were detected. However, freeze-induced metabolic injury in *Salmonella* has been previously reported (*122*). Metabolic and structural injury differ only on the degree of cell damage; all injured

cells have their permeability barriers damaged, but in metabolically injured cells, functional components related to their metabolic activities are affected as well (*108*). Escartin *et al.* (*47*) reported drastic reductions, as large as 3-logs, of *Salmonella* population in naturally-contaminated raw pork during 42 and 78 weeks of storage at -15°C; structural injury was not assessed. Dykes and Moorhead (*43*) found no significant variation in *Salmonella* population (*S.* Brandenberg, *S.* Dublin and *S.* Typhimurium) during nine months of storage at -18°C when inoculated in beef trimmings; structural injury was assessed but not detected.

Due to the variability in results reported in the published literature regarding the survival of *Salmonella* in different meat and poultry products during frozen storage, and in response to the recent association of frozen chicken products with salmonellosis outbreaks, the objective of this study was to assess the survival of *Salmonella* during frozen storage when inoculated in chicken nuggets and strips.

2.3. Materials and Methods

Processed chicken products. Two kinds of frozen products were used: cooked chicken nuggets (~17g each nugget) and raw chicken strips (~25g each strip). The cooked nuggets were described in the product's label as: **"**Breast nuggets. Fully cooked. Breaded nugget-shaped chicken breast patties with rib meat", and the chicken strips as: "Crunchy chicken strips. Contains uncooked poultry. Breaded strips-shaped chicken patties." Chicken meat, water, sodium phosphates and salt are listed as ingredients in both products, with variations in other components.

Bacterial strains. Two strains of *Salmonella* Kentucky and two strains of *Salmonella* Typhimurium isolated from chicken (*31*) with and without antibiotic resistance (Table 1.1) were used.

Inoculation procedures. Cells were grown to stationary phase on Tryptic Soy Broth (Difco Laboratories, Detroit, MI) or Tryptic Soy Broth (Difco Laboratories, Detroit, MI) with 0.01g/L of tetracycline for 18 hours at 37°C. Stationary-phase cultures were used in order to realistically represent the state of natural contaminants of meat. After incubation, all four strains were combined in one inoculation cocktail. The day before inoculation, the frozen chicken products were thawed overnight at 4°C. A total of 0.2 ml of the *Salmonella* cocktail was inoculated using a syringe in two spots (0.1 ml each) of the thawed chicken nuggets, and 0.3ml (0.15ml in each of two spots) in the thawed chicken strips. A set of samples was tested immediately for initial population determination. All other samples were placed on their original package and stored in a laboratory freezer at -20°C immediately after inoculation. An inoculation level of ~10^5 CFU/g was targeted for both products. Inoculation spots were located on the center and edge of the products, at a depth equivalent to half their width (total width ~1cm for both nuggets and strips).

Frozen storage. A laboratory freezer set to -20°C (± 1°C) was used to store samples during frozen storage. Frozen poultry products are commonly stored at temperatures close to -20°C (*11*). Temperature inside the freezer was monitored at every sample collection point using a mercury thermometer in glycerol. Freezing rate was determined for two separate sets of samples (nuggets and strips). Thawed samples (initial

temperature $3 \pm 1°C$) were placed in the freezer and temperatures were monitored hourly using a calibrated infrared thermometer.

Microbiological analysis. Duplicate samples were analyzed for each set (i.e., cooked nuggets and raw strips) at frequencies ranging from weekly to every three weeks depending on the stage of the study (Figures 2.1 and 2.2). Frozen samples were stomached, serially diluted, plated on minimal, selective and nonselective agars and incubated at 37°C for 24 hours. Metabolically-injured cells are not be able to produce colonies on minimal agar, and structurally-injured cells in selective agar; metabolically and structurally-injured cells are able to produce colonies on nonselective agar (*109*) (*108*) (*15*). Controls for not-inoculated nuggets and strips were determined. The detection limit of the method is 50 CFU/g.

Microbiological media. Nonselective agars: Plate Count Agar (Difco Laboratories, Detroit, MI) - referred to as PCA in this document - and Plate Count Agar (Difco Laboratories, Detroit, MI) with 0.01g/L of tetracycline (Acros Organics, Pittsburgh, PA) - referred to as PCA-Tet in this document. Selective agar: XLT4 Agar (Difco Laboratories, Detroit, MI). Minimal agar - referred to as M9 agar in this document: 200ml of M9 minimal salts 5X (Difco Laboratories, Detroit, MI), 800ml distilled water and 15g agar powder (Fisher Scientific, Pittsburgh, PA) were mixed and autoclaved (121°C x 20min). When cooled to room temperature, 2ml of autoclaved 1M $MgSO_4$ solution, 0.1 ml of autoclaved 1M $CaCl_2$ solution and 20ml of filter-sterilized (0.45μm syringe filter) 20% glucose solution were added.

Statistical analysis. The software SPSS for Windows version 16.0 (SPSS Inc., Chicago IL) was used in the statistical analysis of the data.

2.4. Results

As determined on PCA, PCA-Tet and M9 agar, cell concentrations of $\sim 10^5$ CFU/g were achieved for both nuggets and strips sample sets (Table 2.1) after inoculation. Mean cell concentrations measured immediately after inoculation (week 0) were compared - for the nuggets and strips data sets separately - using ANOVA ($p < 0.05$) and Duncan multiple range test as post-hoc test. Results indicate that cell counts immediately after inoculation on XLT4 agar for both nuggets and strips (Table 2.1) were significantly different ($p < 0.05$) than those on PCA, PCA-Tet and M9 agar. Decreased bacterial counts on XLT4 agar may be a result of stress associated with microbial growth on selective media, and have been previously reported in a similar study (*15*). No growth was observed on XLT4, PCA, PCA-Tet or M9 agar for controls of not-inoculated chicken nuggets (fully cooked). For controls of not-inoculated chicken strips (containing raw poultry), no growth was observed on XLT4 or PCA-Tet but bacterial counts were detected on PCA and M9 agar at levels of 10^2-10^3 CFU/g.

After inoculation, samples were subject to frozen storage ($-20 \pm 1°C$). For both nuggets and strips, the average freezing rate was 10.5°C/h until reaching the freezing point (about -12°C), and 7°C/h after it, until reaching the final storage temperature of 20°C. The total freezing process was completed in 4-5 hours for samples starting at $\sim 3°C$. During the storage period studied (16 weeks), for both nuggets and strips sets, a relatively constant population level of $\sim 10^5$ CFU/g was measured when monitoring growth on PCA, PCA-Tet and M9 agar (Figures 2.1 and 2.2). Mean cell counts averaged over the 16 weeks studied measured on these four agars were compared - for the nuggets and strips data sets separately - using ANOVA ($p < 0.05$) and Duncan multiple range test

(Table 2.2). Mean cell counts on XLT4 agar were identified as significantly different from counts on PCA, PCA-Tet and M9 agar in both nuggets and strips data sets, and were the lowest measured of all four agars (3.2 ± 0.5 and 3.1 ± 0.6 CFU/g for nuggets and strips, respectively). Mean cell counts on PCA, PCA-Tet and M9 agar were considered equivalent ($p<0.05$) from weeks 0 to 16 in the chicken strips data set. For the nuggets data set, significant differences were identified between counts on PCA, PCA-Tet and M9 agar. These statistical differences however are considered to be of no practical significance, as can be deducted from Figure 2.1.

In addition, mean cell counts measured on each agar over time (weeks 0 to 16) were compared - for the nuggets and strips data sets separately - using ANOVA ($p<0.05$) and Duncan multiple range test (statistical analysis not shown). Cell counts on XLT4 agar measured immediately after inoculation (week 0) were identified as significantly different from counts during the frozen storage period (weeks 1-16) in both nuggets and strips data sets. As seen on Figures 2.1 and 2.2, after an initial decrease during the first week of frozen storage (~1 log), counts on XLT4 agar remained consistently lower than those on PCA, PCA-Tet and M9 agar during the 16 weeks of frozen storage studied for both nuggets and strips sets. For the strips data set, statistical analysis of cell counts on XLT4 over time also revealed significant differences between counts from weeks 1 to 16, supporting the trend seen on Figure 2.2 of slight decrease followed by recovery after week 10. For PCA, PCA-Tet and M9 in both nuggets and strips data sets, averaged cell counts for every time point measured throughout the storage period were either found to be equivalent or different, based on apparent statistically significant differences but with no clear chronological trend, as can be deducted from Figures 2.1 and 2.2.

2.5. Discussion

The results presented in this study demonstrate that *Salmonella* can readily survive frozen storage (-20°C ± 1°C), for at least 16 weeks, when inoculated in raw and cooked frozen, breaded chicken products, with structural injury as a consequence. These results are in close agreement with those of Barrell (*15*) in cooked minced beef; however Barrell (*15*) reports a slight trend of decreasing population (~0.5 log over 10 weeks of frozen storage). In our study, cell concentrations measured in complete and minimal agars remained relatively constant from week 0 to 16. Also, Barrell (*15*) observed a less pronounced difference between counts on selective and nonselective agars suggesting a lower proportion of structurally injured cells. Georgala and Hurst (*57*) reported a rapid decrease in *Salmonella* population during initial freezing when inoculated in comminuted beef, followed by constant levels during frozen storage. A similar decrease in population during initial freezing was also observed in our study when monitoring cell concentration in selective agar. Georgala and Hurst (*57*) do not specify however which kind of microbiological media was used to measure *Salmonella* populations. High survival of *Salmonella* during frozen storage was also observed by Foster and Mead (*51*) in minced chicken breast, however survival in minced chicken leg was lower. These results were attributed to the different pH of breast (5.8) and leg (6.4) samples. The pH of the samples used in our study was determined experimentally as ~6.3 for the chicken strips and ~6.6 for the chicken nuggets, suggesting predominance of chicken leg meat. Foster and Mead (*51*) also found that polyphosphates had a negative effect on the survival of *Salmonella* during frozen storage when inoculated in both chicken breast and leg. Chicken nuggets

and strips used in our study report sodium phosphates in the ingredients list, the concentration however is unknown.

Our results conflict with those of Dykes and Moorhead (*43*), which report complete survival of *Salmonella* in beef trimmings during frozen storage but no significant difference (p<0.05) between counts on selective and nonselective media, indicating absence of structural injury. These results were attributed by Dykes and Moorhead to the high fat content of the samples used (10%) and to the fact that the pathogen was surface-inoculated and/or to factors intrinsic to the *Salmonella* serotypes used. The fat content of the samples used in our study, as deducted from information on the labels, were 19.7% and 19.6% for the chicken strips and nuggets respectively, and may have contributed to protecting the cells during freezing. The results presented in our study also conflict with those of Escartin *et al.* (*47*), which indicate a significant decrease in *Salmonella* and total aerobes population in raw pork during frozen storage. Although the use of naturally-contaminated pork may suggest high variability in the study of Escartin *et al.* (*47*), a clear decreasing trend was observed in three different trials of 22, 42 and 78 weeks of frozen storage. The freezing rate of pork samples was not reported in their study; however the fact that samples were frozen and stored at -15 ± 2°C may have negatively affected the survival of *Salmonella*. Previous research has demonstrated that slow freezing (1-10 °C/h) - characterized by extracellular ice formation and osmotic dehydration of the cell - is more detrimental to the cell than rapid freezing (>50°C/h) - characterized by intracellular ice formation (*11,22,46,109*). Also, death rates during frozen storage are more pronounced at temperatures just below the freezing point, and are usually slow below -20°C (*51,57*) In our study, the freezing rate of chicken nuggets and

strips qualifies as slow freezing (7 to 10.5°C/h), however no death or severe damage to the cells was observed, suggesting a protective effect of the chicken matrix.

A recent study conducted in Canada by Bucher *et al*. (*25*) identified *S*. Heidelberg as the most commonly isolated serovar from chicken nuggets and strips. *S*. Heidelberg has been previously implicated in a salmonellosis outbreak in Canada associated with chicken nuggets and strips (*32,76*). The second most commonly isolated serovar in the study of Bucher *et al.* (*25*) was *S*. Kentucky, and it was reported that both *S*. Heidelberg and *S*. Kentucky are also the most commonly isolated serovars from chicken in Canada. *S*. Heidelberg, Typhimurium and Enteritidis have been identified in recent salmonellosis outbreaks associated with frozen chicken products in the United States (*120*). The *Salmonella* strains used in our study – *S*. Typhimurium and *S*. Kentucky (Table 1.1) - were previously isolated from poultry (*31*). It is expected that *Salmonella* naturally found in raw poultry, the main ingredient of chicken nuggets and strips, may also be found in the final product. However, differential sensitivity to freezing, if any, may result in increased prevalence of certain serovars in the frozen product. The variability in results reported in the literature regarding the survival of *Salmonella* during frozen storage may be partially attributed to the variability in serovars used, however factors related to the meat/poultry product and storage temperature, among others, may have influenced the results as well.

2.6. Conclusions

This study - and those of Barrell (*15*), Georgala and Hurst (*57*), Foster and Mead (*51*) and Dykes and Moorhead (*43*) - reports a high survival rate of *Salmonella* during frozen storage. Similar results have been obtained for *Campylobacter* spp. in meat packs

(*58*) and broilers. It has been hypothesized that the meat matrix acts as a hydrocolloid, binding free water and thus reducing the harmful effect of freezing on bacterial cells (*106*). In addition, it can be inferred that storage at -20°C represents a mild stress for *Salmonella*, particularly when inoculated in a meat matrix, and is only capable of temporarily injuring bacterial cells. Thus, in our study we report no death or metabolic injury of *Salmonella* during 16 weeks of frozen storage, only structural injury. Though not only concerning food processing conditions, extensive evidence points at damaged outer membranes as the major effect of freezing bacteria (*80*), as suggested by leakage of cellular components into the growth medium, penetration of surfactants, dyes and enzymes into the cell (*11,20,27,109,110*) and sensitization to antibiotics (*110*) and bacteriocins (*20*). Sublethally damaged foodborne pathogens are able to rapidly repair the injury once environmental conditions become favorable and regain their capacity to cause disease (*22,108*). Failure to detect these injured cells, when using selective microbiological media for example, may represent a significant risk of foodborne illness. As early anticipated by Speck and Ray (*123*) , frozen foods contaminated with pathogenic bacteria can be an unsuspected health hazard for the consumer. This risk is currently of higher concern - particularly for frozen, breaded chicken products containing raw poultry - due to the protective effect of the chicken matrix for bacterial survival during freezing, as well as the likelihood of undercooking by the consumers.

2.7. Tables

Table 2.1: Average concentration (log CFU/g ± standard deviation) of cells immediately

after inoculation (week 0) in chicken nuggets and strips [a]

Agar	Nuggets	Strips
XLT4	4.2 ± 0.20^a	4.1 ± 0.30^A
PCA	5.3 ± 0.30^b	5.2 ± 0.10^B
PCA-Tet	4.9 ± 0.02^b	4.8 ± 0.03^B
M9	5.0 ± 0.10^b	4.9 ± 0.03^B

a. Nuggets and strips data sets analyzed separately. Within columns, means with different letters are considered significantly different (p<0.05).

Table 2.2: Average concentration (log CFU/g ± standard deviation) of cells in

Salmonella-inoculated chicken nuggets and strips during frozen storage (weeks 0-16) [a]

Agar	Nuggets	Strips
XLT4	3.2 ± 0.5^a	3.1 ± 0.6^A
PCA	$5.1 \pm 0.3^{b,c}$	5.2 ± 0.2^B
PCA-Tet	4.8 ± 0.3^b	5.0 ± 0.2^B
M9	5.1 ± 0.2^c	5.2 ± 0.3^B

a. Nuggets and strips data sets analyzed separately. Within columns, means with different letters are considered significantly different (p<0.05).

2.8. Figures

Figure 2.1: Concentration of cells in *Salmonella*-inoculated chicken nuggets during frozen storage as measured in different agars: ● (XLT4); ○ (TPC + tetracycline); ▼(TPC); and ▽ (M9 Minimal agar)
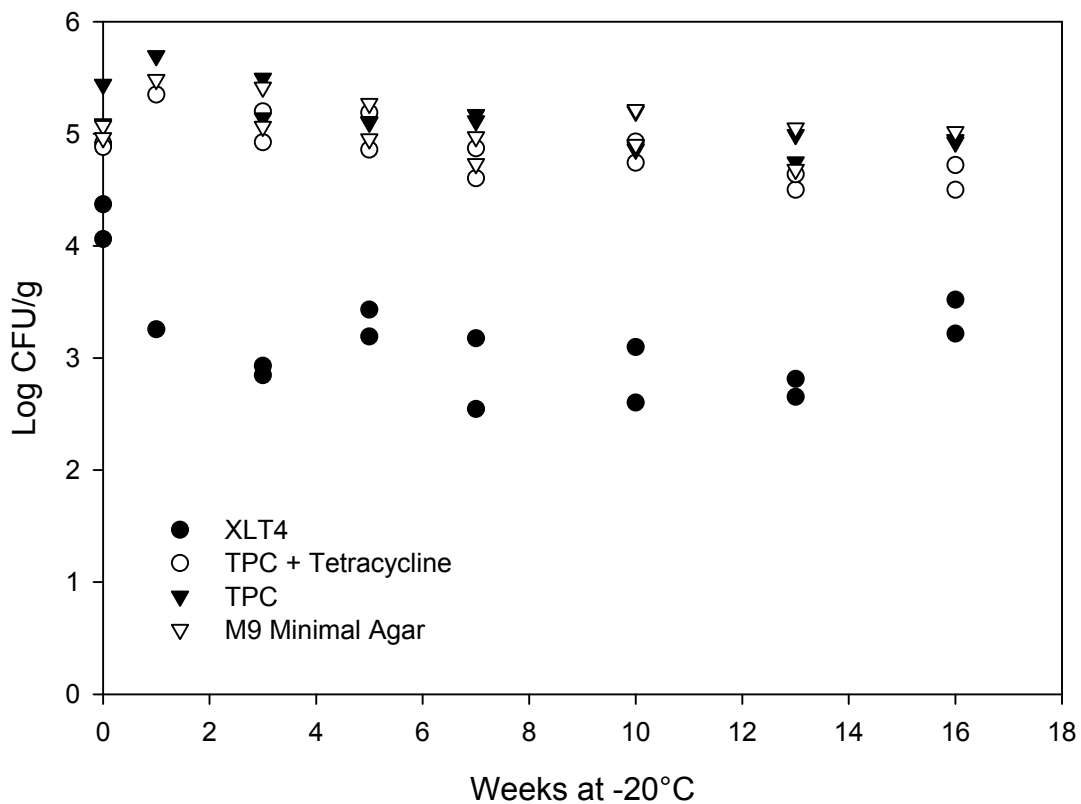
Figure 2.2: Concentration of cells in *Salmonella*-inoculated chicken strips during frozen storage as measured in different agars: ● (XLT4); ○ (TPC + tetracycline); ▼(TPC); and ▽ (M9 Minimal agar)
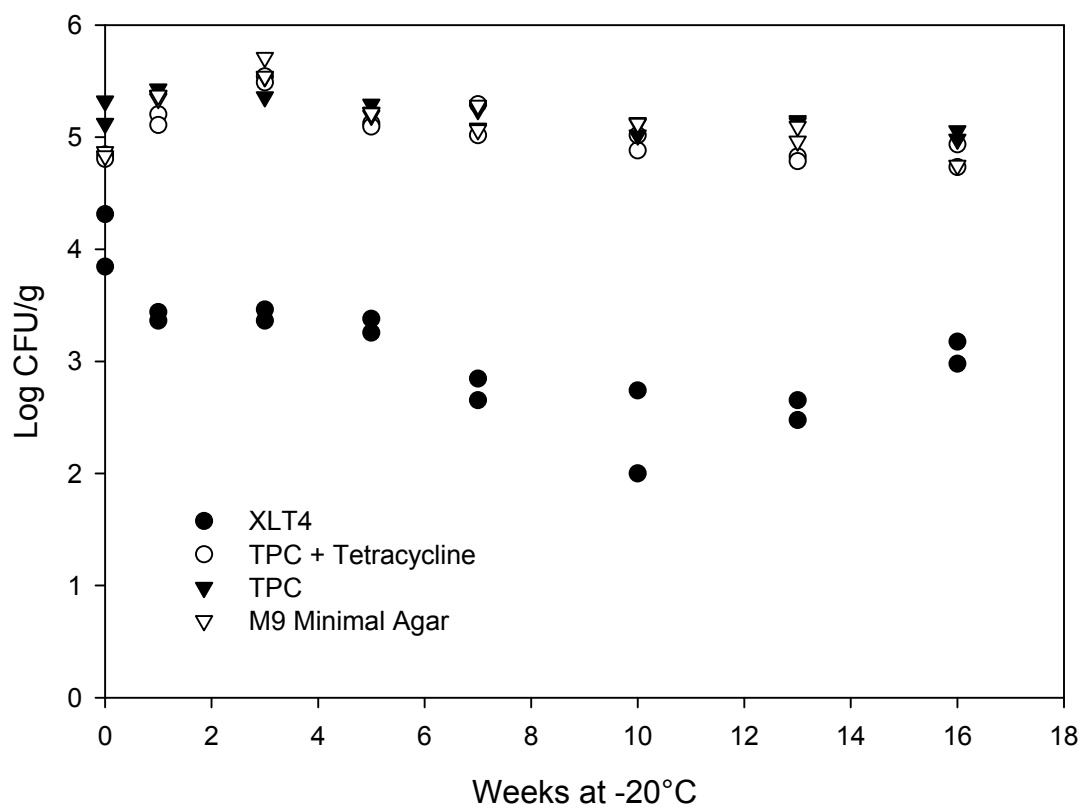
Chapter 3: The effect of lipoplysaccharide and porin mutations on the survival of

***Salmonella* during frozen storage in chicken products**

3.1. Abstract

The ability of *Salmonella* to survive frozen storage when inoculated in meat and chicken products has been previously demonstrated. In published studies, the outer membrane has been identified as the site of damage in Gram negative bacteria when subject to freezing stress. The objective of this study was to assess the effects of LPS and porins (OmpD and OmpC) in the survival of *Salmonella* during frozen storage, under common food processing conditions (-20°C), when inoculated in a chicken matrix.

Four isogenic *S.* Typhimurium LT2 *rfa* mutant strains with increasing LPS defects, and three non-isogenic *S.* Typhimurium LT2 *omp* mutant strains, lacking OmpD or OmpC, were characterized with respect to their tolerance to sodium lauryl sulfate (SLS). Each strain was inoculated onto a set of chicken strips ($\sim10^4$ CFU/g) and subject to storage at -20°C. During 10 weeks of frozen storage, cell populations were measured in Nutrient Agar, Nutrient Agar supplemented with varying concentrations of SLS, and XLT4 Agar.

Results indicate that LPS defects did not hinder the survival of *Salmonella* when inoculated on chicken strips and stored at -20°C. All four *rfa* mutant strains showed complete survival under the conditions tested, including a strain lacking the LPS saccharide chain almost entirely. The sensitivity to SLS of these LPS defective strains remained constant during frozen storage, suggesting no increasing outer membrane damage under the conditions tested. Two mutant strains lacking OmpD behaved

differently with respect to their tolerance to SLS and ability to form colonies on XLT4

Agar, possibly as a consequence of not being isogenic mutants. However, they both

showed high survival during storage at -20°C, and only a minor decrease in SLS

tolerance. The OmpC mutant strain studied showed the lowest threshold of SLS tolerance

before freezing. During freezing, its population decreased markedly after week 4 and its

tolerance to SLS decreased.

The results of this study indicate that the length of the LPS saccharide chain does

not appear to have any effect on the survival of *Salmonella* during frozen storage in

foods. Additionally, the lack of OmpC appeared to detrimentally affect the survival of

Salmonella during frozen storage in foods. OmpC is derepressed under high osmolarity

conditions, so its function may be important for cell tolerance to freezing stress,

preventing passage of ice crystals inside the cell. The overall high survival rates in this

frozen storage study may also be attributed to the protective nature of the chicken matrix.

A better understanding of the factors that contribute to the survival of *Salmonella*

and other Gram negative bacteria during freezing and frozen storage may provide useful

information for future work on the development of antibacterial treatments and

technologies for freezing processes.

3.2. **Introduction**

Previous research has shown that *Salmonella*, as well as other Gram-negative

species, are able to survive freezing and frozen storage, with varying degrees of injury

depending mainly on the freezing rate and temperature, and the freezing substrate , as

demonstrated in Chapter 2 of this dissertation and elsewhere (*15,43,47,51,57*). This injury

however is rapidly repaired and pathogens are able to regain their capacity to cause

disease once favorable environmental conditions return (*22,108*). The nature of the freeze-injury of Gram negative species has been shown to be structural rather than metabolic (*15,20*), but metabolic injury (*122*) or no injury of any kind (*43*) have also been reported. Metabolic and structural injury differ only in the degree of cell damage; all injured cells have their permeability barriers damaged, but in metabolically injured cells, functional components related to their metabolic activities are affected as well (*108*).

Extensive evidence points at damage to outer membranes as the major effect of freezing bacteria (*20,27,80,109,110*). The cell envelope of Gram-negative species is made up of 3 layers: the outer membrane, the peptidoglycan layer and the cytoplasmic membrane. The differential sensitivity of the cells to detergents and NaCl (*80*) has demonstrated that although the outer membrane is affected by freezing, the cytoplasmic membrane remains unharmed (*19*). The outer membrane of Gram-negative species is formed by lipopolysaccharides, phospholipids, porins, the enterobacterial common antigen, and the capsular polysaccharide M antigen (*96*). The lipopolysaccharide component (LPS) is composed of 3 sections: the proximal, hydrophobic Lipid A region; the distal, hydrophilic O antigen polysaccharide region; and the core polysaccharide region that connects the two (*96,118*).

Mutants with defined LPS core defects have been used in previous studies to determine the role of this molecule in the resistance of *Salmonella* to antibiotics (*110*) and to stresses such as freezing (*19*) and high pressure (*82*). *S*. Typhimurium LPS mutants range from "smooth", lacking only the O antigen and part of the distal oligosaccharide core (Ra, referring to the chemotype of the mutant LPS produced), to "rough" (Rb and Rc) and "deep rough" (Rd and Re) mutants, lacking larger portions of

the core closer to the proximal region (*96*). "Deep rough" mutants are hypersensitive to hydrophobic compounds, including a variety of antibiotics and detergents (*19,110*). These mutants exhibit less protein content (*3,19,119*). In "deep rough" mutants, the space that should be occupied by proteins is filled in with phospholipids (*96,119*). Bennett, *et al*. (*19*) demonstrated that freeze-thaw sensitivity in *S*. Typhimurium "deep rough" mutants (frozen to -196°C in solution) was significantly increased when compared with the wild type, however whether this was a result of the shorter LPS chains or of the reduced protein content was not investigated. Boziaris (*20*) observed that *S*. enteritidis PT7 was more sensitive to nisin during freezing (in solution) than PT4; it was hypothesized that the longer LPS chains of PT4 protected the cells from freeze-injury, however defined LPS mutants were not included in the study.

Outer membrane proteins ("Omp") are predominantly porins, transmembrane proteins that form non-specific channels that allow the passage of small hydrophilic molecules into the cell (*96*). Research has investigated the role of porins as adhesion molecules in host cell recognition during infection (*40,40,61*). Under normal growth conditions, *Salmonella* Typhimurium commonly expresses three porins: OmpC (36 kDa), OmpF (35 kDa) and OmpD (34 kDa) (*61,96*). The total amount of these porins is relatively constant and is very large; they can represent up to 2% of the total protein of the cell (*96*). OmpD is the most abundant outer membrane protein in *S*. typhimurium, and its abundance increases in response to anaerobiosis and decreases in response to low pH (*114*). OmpF is known to be repressed by high osmolarity and temperature, and OmpC derepressed by high osmolarity (*96*).

The objectives of this study were to assess the effects of LPS and porins (OmpD and OmpC) in the survival of *Salmonella* during frozen storage, under common food processing conditions (-20°C), when inoculated in a chicken matrix. A better understanding of the factors that contribute to the survival of *Salmonella* and other Gram negative bacteria during freezing and frozen storage may provide useful information for the development of bacterial reduction treatments under these conditions.

3.3. Materials and Methods

Processed chicken products. Frozen chicken strips containing raw poultry (~25g/strip) were used. The description in the product's label read: "Crunchy chicken strips. Contains uncooked poultry. Breaded strips-shaped chicken patties. Keep frozen." Chicken meat, water, sodium phosphates and salt were listed in the ingredients, as well as various breading components.

Bacterial strains. Four isogenic mutant strains of *Salmonella* Typhimurium LT2 with varying degrees of lipopolysaccharide (LPS) defects, i.e. with mutations affecting the length and branching of the saccharide chain, and three non-isogenic *Salmonella* Typhimurium LT2 mutant strains with no *rfa* mutations but lacking outer membrane proteins OmpD or OmpC, were obtained from *Salmonella* Genetic Stock Centre (SGSC) – University of Calgary, Canada (Table 1) and used in this study. Mutant strains SL3749, SL3748 and SL3789 have increasing LPS defects, and are part of an *rfa*-transductants set derived from the "smooth" strain SL3770 using phage ES18 (*73,110*). An isogenic set of *omp* mutant strains, with no *rfa* mutations, was not available at the time this research was conducted. Strains CH338 and SH7241 have mutations in *ompD* and o*mpC* respectively by transposon (Tn10) insertion into an unidentified *S.* Typhimurium LT2 parent strain

(*40*). Strain SH7782 carries a transposon-mediated (Tn5) mutation in o*mpD*, as indicated by SGSC and Hara-Kaonga and Pistole (*61*), however no reference for the origin of this strain was available. *S.* Typhimurium LT2 *ompF* mutants with no *rfa* defects were not available at the time this research was conducted.

Inoculation procedure. Each set of mutants was grown separately in Tryptic Soy Broth (Difco Laboratories, Detroit, MI) for 18-24 hours at 37°C. Stationary phase cultures were used in order to realistically represent the state of natural contaminants of meat, and are more resistant to freezing than exponential phase cultures (82). The day before inoculation, frozen chicken strips were thawed overnight at 4°C. For each set of mutants, a total of 0.3 ml of the overnight culture was inoculated in two spots (0.15ml each) of the thawed chicken strips using a syringe. An inoculation level of 10^5 CFU/g was targeted. Inoculation spots were located on the center and edge of the strips, at a depth equivalent to half their width (total width ~1cm). Seven sets of strips, each inoculated with only one type of mutant, were obtained.

Frozen storage. Immediately after inoculation, samples were placed in their original package and frozen for 10 weeks. A laboratory freezer set to -20°C (± 1°C) was used to store samples during frozen storage. Frozen poultry products are commonly stored at temperatures close to -20°C (1). Temperature inside the freezer was monitored at every sample collection point using a mercury thermometer in glycerol. The freezing rate was determined by placing a set of non-inoculated, thawed samples (initial temperature 3 ± 1°C) in the freezer and monitoring temperatures hourly using a calibrated infrared thermometer.

Microbial analysis. Each mutant strain was initially characterized with respect to their tolerance to increasing concentrations of sodium lauryl sulfate (*19,26*). The maximum concentration of sodium lauryl sulfate (SLS) that allowed unstressed populations to plate with >90% efficiency was determined (*19,26*): colony counts in Nutrient Agar with varying concentrations of SLS were compared to those in Nutrient Agar without supplements; the concentration of SLS that allowed equivalent (>90%) growth on Nutrient Agar was determined. During frozen storage, survival of each strain was monitored in Nutrient Agar supplemented with the previously determined SLS concentration. Changes in this tolerance were also evaluated by measuring growth in media with increasing concentrations of the detergent and comparing them to the initial SLS tolerance determined. Samples were analyzed immediately after inoculation and during frozen storage at frequencies ranging from weekly to monthly, depending on the stage of the study. The data was normalized dividing the mean observed growth at week X by the mean observed growth at week 0, giving a referential value of 1 at week 0. Seven chicken strips (each inoculated with one type of mutant strain only) were removed from the freezer for microbial analysis at each time point. Controls for not-inoculated strips were also determined. Frozen samples were stomached, serially diluted and plated on the media described below. The detection limit of the method is 50 CFU/g.

Microbiological media. Nutrient Agar (Difco Laboratories, Detroit, MI) was used as a complete, non-selective agar in this study. Nutrient Agar supplemented with filter-sterilized sodium lauryl sulfate (Fisher Scientific, Pittsburgh, PA) to reach final concentrations of 0.01%, 0.1% and 0.5% were used to characterize the detergent tolerance of outer membrane mutant strains (*19,26*). The tolerance of mutant strains to

higher concentrations (1-5%) of sodium lauryl sulfate (SLS) was also evaluated initially but not during the frozen storage period. Growth on selective XLT4 Agar (Difco Laboratories, Detroit, MI) was assessed when possible; not all mutant strains were able to produce colonies on this growth medium.

3.4. Results

Before freezing treatment, for strains SL3748 and SL3789 - with severe LPS defects, the maximum concentrations of SLS in the media that provided cell recovery equivalent to that on Nutrient Agar without SLS were 0.1 and 0.01% respectively (p<0.5; data not shown). This is in agreement with the results of Bennett, *et al.* (*19*). However, for strains SL3770 and SL3749 (with no or little LPS defect), an increased tolerance to SLS different than that reported by Bennett *et al.* (*19*) was observed. Bennett *et al.* (*19*) reported a maximum tolerance of 0.5% SLS for both strains, however our results indicate that these strains can tolerate 0.5% SLS as well as concentrations ≥ 1% SLS (data not shown). Media preparation and visibility of colonies on agar plates was compromised by inclusion of higher (≥ 1% SLS) concentrations of detergent. In addition, considering that this methodology (*19*) was only applied in our study in order to assess if SLS sensitivity, and therefore membrane damage, would increase after frozen storage we decided to use 0.5% SLS as a reference maximum tolerance for SL3770 and SL3749. For *omp* mutants, no reference was found regarding their tolerance to SLS. For strain CH338, results indicate that the maximum concentrations of SLS in the media that provided cell recovery equivalent to that on Nutrient Agar without SLS was 0.5% (p<0.5; data not shown). Similar to SL3770 and 3749, preliminary results also suggested that CH338 can tolerate ≥ 1% SLS (data not shown). For strains SH7782 and SH7241, equivalent growth

to that on Nutrient Agar without SLS was obtained with 0.01% SLS. SH7782 was able to grow in presence of SLS concentrations as high as 0.5% (albeit with decreased recovery), but SH7241 did not produce colonies in presence of ≥ 0.1% SLS.

Also before freezing, strains SL3770, SL3749, SL3748 and CH338 were able to grow profusely in XLT4 agar (Table 2). SH7782 and SH7241 were not able to produce the expected black colonies characteristic of *Salmonella* in XLT4 agar, but instead produced yellow colonies. SL3789 did not produce colonies of any type in XLT4 agar.

After inoculation, samples were subject to frozen storage (-20 ± 1°C). The average freezing rate was 10.5°C/h until the freezing point was reached (about -12°C), and 7°C/h after it, until reaching the final storage temperature of -20°C. The total freezing process was completed in 4-5 hours for samples starting at 3°C.

During 10 weeks of storage at -20°C, SLS tolerance remained relatively unchanged for all strains studied; i.e. by week 10, strains were able to grow in presence of the same concentration of SLS used in week 0, suggesting no increased outer membrane damage caused by frozen storage. Thus, for each strain, during the frozen storage period studied, growth in the presence of only one concentration of SLS is presented (Figures 2 and 4), in addition to growth on Nutrient Agar (Figures 1 and 3) and XLT4 agar (Table 2). For observations in Nutrient Agar and Nutrient Agar supplemented with SLS, the raw data evidenced no dramatic change in cell populations over time during frozen storage, thus cell counts were normalized with reference to the original inoculum levels obtained ($\sim 10^{4.5}$ CFU/g) in order to visualize variations over time, if any (Figures 1-4).

Cell concentrations of all LPS defective strains (smooth parent SL3770 and increasingly defective LPS mutants SL3749, SL3748 and SL3789) remained constant during the 10 weeks of frozen storage studied (Figure 1), as measured in Nutrient Agar. These results suggest that defective LPS did not hinder the survival of *Salmonella* when inoculated in chicken nuggets and stored at -20°C. Similar results were obtained when cell concentrations for LPS defective strains were measured in the presence of SLS (Figure 2), indicating no increasing sensitivity to the detergent during frozen storage.

Cell concentrations of OmpD⁻ strains CH338 and SH7782 remained relatively constant over 10 weeks of frozen storage, as measured in Nutrient Agar (Figure 3). The concentration of OmpC⁻ strain SH7241 however showed a decreasing trend, and after 10 weeks of frozen storage a $\sim$0.3$\log_{10}$ reduction with respect to its concentration at week 0 was observed. SLS (0.5%) did not appear to affect survival of OmpD⁻ strain CH338 to frozen storage; the original population level was maintained during the 10 weeks studied as measured in Nutrient Agar with 0.5% SLS (Figure 4). For OmpD⁻ SH7782, a slight decrease ($\sim$0.2log) in population may be noted after 4 weeks at -20°C when measured in the presence of 0.1% SLS, however this was not observed in the presence of 0.01% (Figure 4), suggesting only a minor increase in the sensitivity of the strain to SLS. For OmpC⁻ strain SH7241, similar to results in Nutrient Agar, a decreasing trend was clear after 4 weeks of frozen storage when measuring population in presence of 0.1% SLS (Figure 4). This trend was less pronounced when cell concentration was measured in the presence of 0.01% SLS during the first 6 weeks of frozen storage however, by week 10 recovery with 0.1% and 0.01% SLS was almost equivalent. The differential recovery of

strain SH7241 in presence of 0.1% and 0.01% SLS suggests a certain degree of outer membrane injury during storage at -20°C.

Recovery in XLT4 agar was variable, and only the concentration of strains SL3770, SL3749, SL3748 and CH338 could be monitored in this medium during the 10 weeks of frozen storage studied. LPS defective strains SL3749, SL3748 and parent SL3770 showed similar recovery in XLT4 agar; a net decrease in population of $\sim$1log$_{10}$ was observed when monitoring survival in this medium (Table 2). Strain SH3789, as initially determined was not able to produce colonies in XLT4 agar. OmpD$^-$ CH338 showed complete survival when measured in XLT4 agar (Table 2). Strain SH7782 was occasionally in the limit of detection during the frozen storage period studied, whereas SH7241 remained undetected (Table 2).

3.5. Discussion

Salmonellla has been proven able to survive freezing, particularly when in non-fluid substrates with varying degrees of injury, in Chapter 2 of this dissertation and elsewhere (*15,43,47,51,57*). Under extreme freezing conditions however, and when inoculated into fluid substrates, severe damage to the outer membrane of *Salmonella* has been reported (*19,20,109,110*). Thus, it is believed that components of the outer membrane play a role in the survival/sensitivity of *Salmonella* to freezing, as well as other stresses.

The effects of freezing in LPS-defective *Salmonella* strains have been previously investigated (*19*), and we attempted to characterize the degree of outer membrane damage of *Salmonella* in chicken meat using this same methodology (*19*). Parent strain SL3770 and strain SL3749 were able to tolerate ≥1% SLS, conflicting with results

reported by Bennett *et al*. (*19*). It is possible that Bennett *et al.* (*19*) also observed this higher tolerance, but did not reach >90% plating efficiency, and therefore was not reported. The semi-quantitative nature of this method, which may introduce subjective judgment, could have also affected the results. The methodology did succeed however in giving an overall assessment of the degree of outer membrane damage in the mutant strains used, as well as monitoring this damage during frozen storage.

LPS defects did not appear to affect survival of *Salmonella* during storage at -20°C, as evidenced by recovery in Nutrient Agar, Nutrient Agar with SLS and XLT4 Agar. Strain SL3789, containing only one heptose in the LPS core (*73*), with an SLS tolerance of only 0.01% and unable to produce colonies on XLT4 Agar, showed complete survival after 10 weeks of storage at -20°C as measured in Nutrient Agar and Nutrient Agar with 0.01% SLS (Figures 1 and 2). Bennett *et al*. (*19*) also observed complete recovery for all LPS-defective strains studied as determined in Nutrient Agar, however recovery in the presence of SLS was variable. In agreement with our results, parent strain SL3770 and strain SL3749, with almost complete LPS, were completely recovered in presence of 0.5% SLS after slow freezing and thawing (*19*). Yet, marked decreases in population were reported by Bennett *et al*. (*19*) for SL3748 and SL3789 (>99%), as measured in the presence of 0.1% and 0.01% SLS respectively, conflicting with the complete survival for both strains observed in our study. Bennett, *et al*. (*19*) hypothesized, based on Nikaido (*96*), that freeze-thaw stress induced increased exposure of phospholipids in the outer membrane, allowing the passage of SLS into the cell. The conflict between our results and those reported by Bennett, *et al*. (*19*) for rough strains exhibiting shorter LPS chains (SL3748 and SL3789) may be attributed to different

factors. Both freezing temperatures (-20 and -196°C) may be considered of little influence, since it has been demonstrated that death rates are usually slow at or below -20°C, being more pronounced at temperatures just below the freezing point (*51,57*). However, regardless of the temperature, during frozen storage cells are subject to the growth of existing crystals by recrystallization and to the extended effect of the increased concentration of solutes (5,64). The freezing rate in our study may be considered slow (1-10 °C/h), and is characterized by extracellular ice formation and osmotic dehydration of the cell (14,64)(26). Rapid freezing (>50°C/h) is characterized by intracellular ice crystals formation (*22,109*). Yet, the results of Bennett *et al.* (*19*) show little influence of freezing rate on bacterial survival. Thus, the survival of rough strains SL3748 and SL3789 observed in our study may be attributed to the absence of thawing stress in our experimental design and/or to the nature of the freezing matrix, chicken strips, which may have played a protective role during this stress. Freeze/thaw cycles have been demonstrated to be detrimental for bacterial cells (*19,26,27,97*). Also, the lethal effects of freezing are significantly more evident in liquids than in solids (81, 83), and studies on the survival of *Salmonella* inoculated in meat products support this finding (Chapter 2 of this dissertation and references (*15,43,47,51,57*). Thus, our results suggest that, under the conditions tested, LPS did not influence the survival of *Salmonella* during freezing, as mutants lacking the saccharide chain of this molecule almost entirely showed equivalent survival to non-defective strains. Also, damage to the outer membrane allowing the entrance of detergent into the cell (potentially, increased exposure of phospholipids) was not observed under the conditions tested, as indicated by stable tolerance to SLS during frozen storage.

Salmonella strains lacking outer membrane proteins have been previously constructed in order to study their role during host infection (*40,40,61*).   The strains used in this study are the only ones we could obtain, that did not carry *rfa* mutations. We were unable to obtain information regarding the source of the parent strain(s) from the *Salmonella* Genetic Stock Centre. Before freezing, OmpD⁻ strain CH338 showed full recovery in the presence of 0.5% SLS, and was able to tolerate concentrations $\geq$ 1% SLS. SH7782, lacking OmpD as well, could also tolerate 0.5% SLS, however growth equivalent to that in Nutrient Agar was obtained in the presence of 0.01% SLS. In addition, CH338 was able to produce colonies on XLT4 Agar but SH7782 only occasionally. The difference in SLS tolerance and ability to grow on XLT4 Agar of these OmpD mutants is not clear, but could be attributed to the different parent *S.* Typhimurium LT2 strains they originated from. Both strains were constructed by transposon insertion in the targeted gene regions, using transposons of the Tn family. Although these elements often carry antibiotic-resistance genes, these are unlikely to have caused the differential SLS tolerance observed. Future research should utilize isogenic mutants to assess the impact of only the relevant mutations; but since such *omp* mutants are not available, they would need to be developed. OmpC⁻ strain SH7241 showed the highest sensitivity to SLS before freezing; it was fully recovered in presence of 0.01% SLS, however could not produce colonies at concentrations of SLS $\geq$ 0.1% nor in XLT4 Agar. Since this strain is not isogenic with the two other studied, other characteristics of the parent *S.* Typhimurium LT2 strain could have influenced this result apart from the OmpC deletion. While all parent strains were *S.* Typhimurium LT2, this reduces but does not eliminate any variability in inherent strain factors. OmpC has been

described as a narrower, more restrictive channel than OmpD and OmpF, derepressed by high osmolarity and which provides advantages when the cell encounters an environment rich in inhibitory substances (*96*). The increased sensitivity to SLS of strain SH7241 may be attributed to the absence of this narrower channel, which could have inhibited the passage of the detergent into the cell.

OmpD$^-$ strain CH338 showed no major change in population over the 10 weeks of storage at -20°C, when recovered in Nutrient Agar with/without SLS (0.5%) and XLT4 Agar. Similar results were obtained for OmpD$^-$ strain SH7782 when recovered in Nutrient Agar with/without SLS (0.01%). When using a concentration of SLS (0.1%) higher than that initially determined (0.01%) to recover SH7782, a slight decrease in population was measured after week 4 (Figure 4). OmpC$^-$ strain SH7241 decreased its population as measured in Nutrient Agar with/without SLS (0.01%), and this decrease was more pronounced when recovered in the presence of higher concentrations (0.1%) of SLS (Figure 4). Thus, SLS tolerance indeed declined during frozen storage, approximately after week 4, for strains SH7782 and SH7241. Changes in SLS sensitivity were not observed for LPS-defective strains, suggesting that the effect of frozen storage may be more significant in porins rather than LPS. Bennett *et al.* (*19*) proposed that the increased sensitivity to SLS may be caused by increased exposure of phospholipids or by disruption of phospholipid-protein-LPS interactions. The observed increased sensitivity to SLS was more pronounced for the OmpC$^-$ strain SH7241. In a frozen environment, conditions of high osmolarity are generated; the formation of ice crystals results in an increasing concentration of solutes (5,64). The lack of the more restrictive OmpC channel may result in a reduced barrier for the passage of ice crystals into the cell, as well as SLS

in this experiment, increasing the susceptibility of the cell to freezing stress. Indeed, OmpD⁻ strain CH338 maintained its population during the entire period studied, and SH7782 showed a minor decrease only when measured with higher SLS concentration than that originally set. Thus, under the conditions tested, the absence of the less restrictive OmpD channel had no apparent effect in the survival of these strains during frozen storage. Calcott and Calcott (*26*) reported an *E. coli ompB* mutant as more resistant to freezing (-70°C) and thawing than the isogenic wild type. In *E. coli*, OmpB is a peptidoglycan-linked transmembrane porin and, although other porins were not investigated, Calcott and Calcott (*26*) hypothesized that the presence of this porin channel is lethal to cells undergoing freezing and thawing since it allows a free passage for external ice.

3.6. Conclusions

The results of our study appear to indicate that, at relevant food frozen storage conditions (-20°C), porin proteins, rather than LPS, are the components of the outer membrane that influence the survival of *Salmonella* to freezing stress. In particular, the absence of OmpC resulted in decreased survival during frozen storage and increased sensitivity to SLS, perhaps due to the more restrictive nature of this channel. When present, OmpC may limit the passage of ice crystals and harmful molecules such as SLS therefore protecting the cell form these stresses. It must be noted however that the *omp* mutants' results of this study are only preliminary; the use of isogenic mutants is important as well as the inclusion of OmpF⁻ strains. In addition, although the entry of SLS into the cell is limited by permeability of the outer membrane (*80*) in Calcott and Calcott, the use of this detergent to assess porins function may not be entirely adequate,

considering that these Omp proteins typically allow the passage of small hydrophilic

molecules.

3.7. Tables

Table 3.1: *Salmonella* Typhimurium LT2 mutant strains used[a]

rfa mutants		
Strain	**LPS phenotype / chemotype**	**References**
SL3770	Rfa(+) / Smooth	(*73,110*)
SL3749	Rfa(-) / Ra	(*73,110*)
SL3748	Rfa(-) / Rb3	(*73,110*)
SL3789	Rfa(-) / Rd2	(*73,110*)

omp mutants		
Strain	**Omp phenotype**	**Reference**
CH338	OmpD(-)	(*40*)
SH7782	OmpD(-)	SGSC
SH7241	OmpC(-)	(*40*)

[a] Obtained from *Salmonella* Genetic Stock Centre (SGSC) – University of Calgary, Canada

Table 3.2: Recovery (LogCFU/g) of *Salmonella* Typhimurium LT2 mutant strains from

inoculated chicken strips in XLT4 agar during frozen storage

Weeks at -20°C	*Salmonella* Typhimurium LT2 strain						
	SL3770	SL3749	SL3748	SL3789	CH338	SH7782	SH7241
0	4.55	4.21	4.20	ND	4.49	NB	NB
1	3.90	3.74	4.02	ND	3.74	ND	ND
2	3.30	2.70	3.20	ND	4.42	1.70	ND
3	3.62	3.41	4.12	ND	4.52	ND	ND
4	3.48	2.30	3.04	ND	4.16	ND	ND
6	3.70	3.34	3.66	ND	4.09	ND	ND
10	3.78	3.66	3.08	ND	4.47	1.70	ND

ND = non detected; NB = not black colonies

3.8. Figures

Figure 3.1: Normalized concentration of cells in chicken nuggets inoculated with *S.* Typhimurium LT2 strains with defective lipopolysaccharide during frozen storage as measured in Nutrient Agar
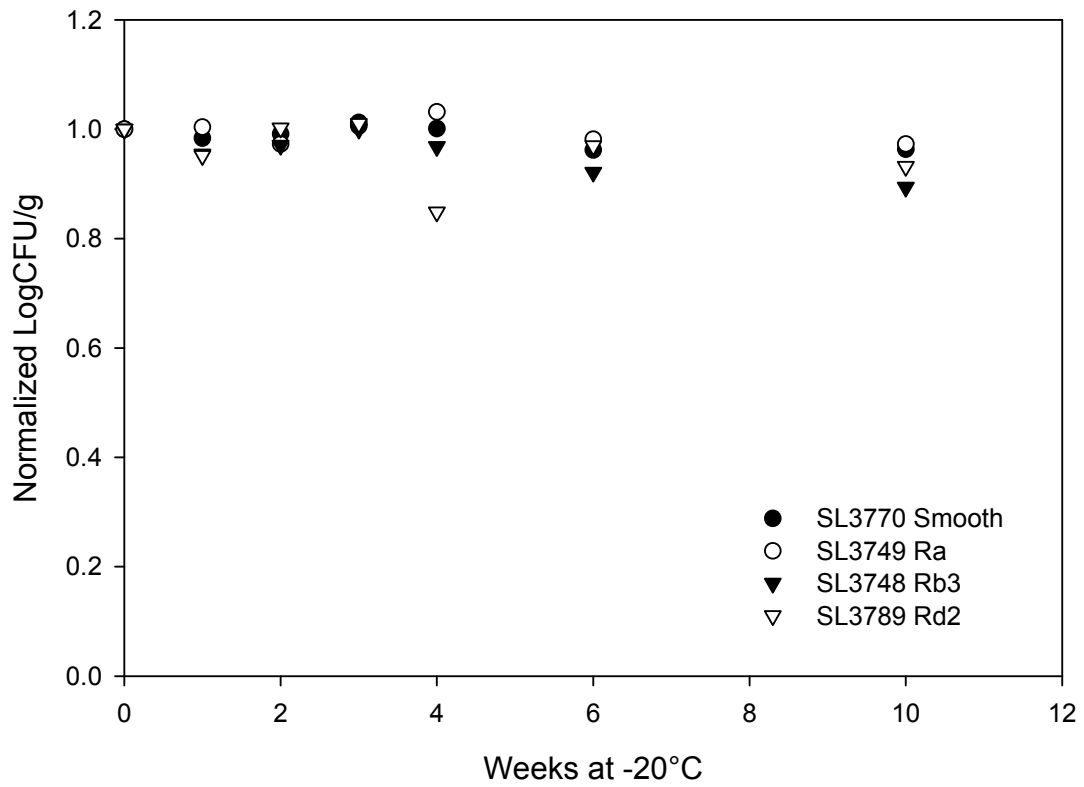
Figure 3.2: Normalized concentration of cells in chicken nuggets inoculated with *S.* Typhimurium LT2 strains with defective lipopolysaccharide during frozen storage as measured in Nutrient Agar supplemented with sodium lauryl sulfate
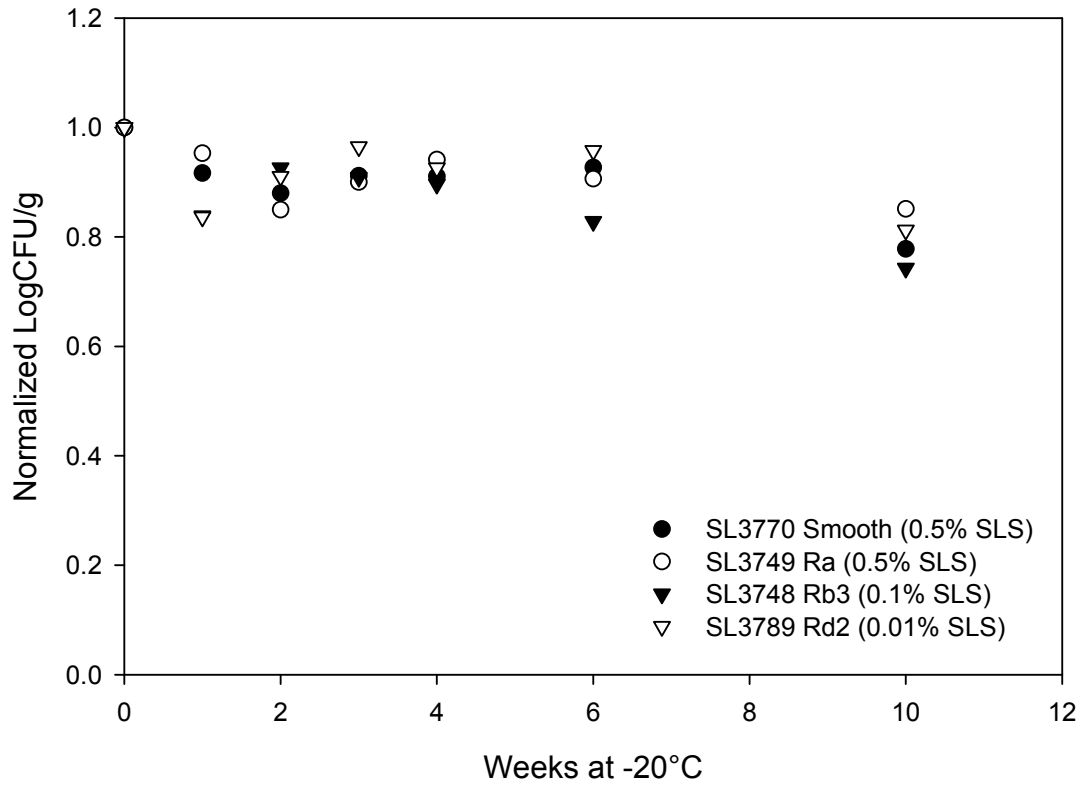
Figure 3.3: Normalized concentration of cells in chicken nuggets inoculated with *S.* Typhimurium LT2 strains lacking outer membrane proteins during frozen storage as measured in Nutrient Agar
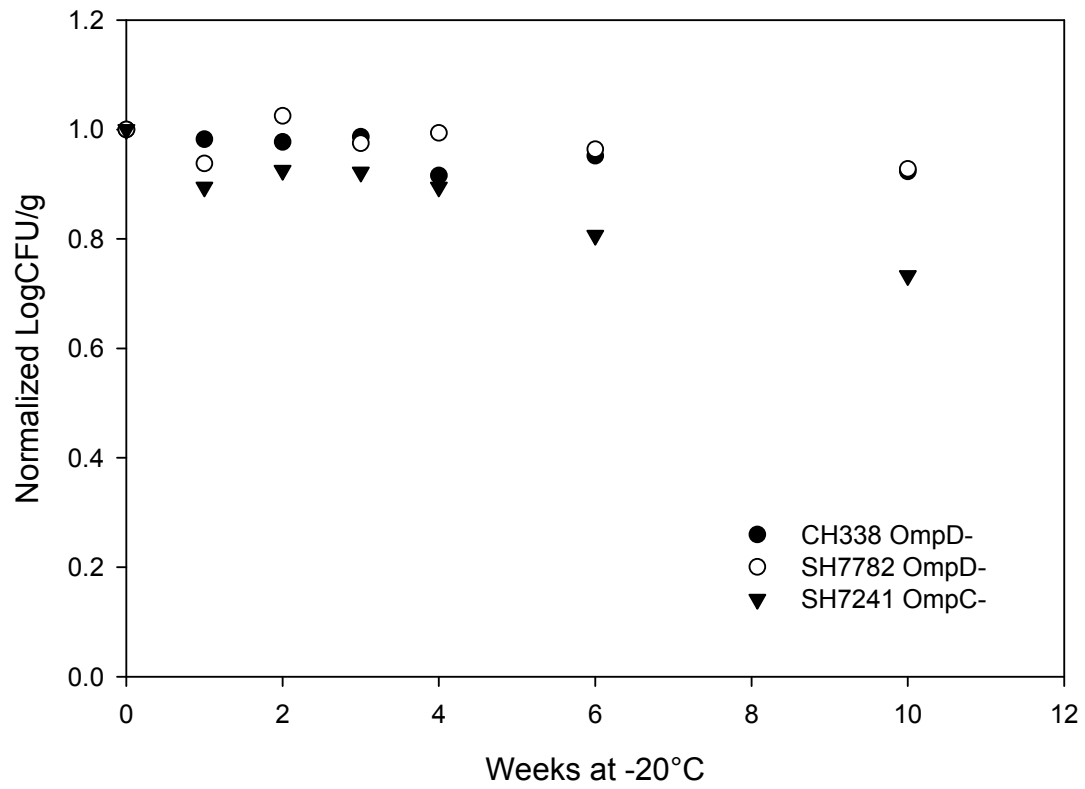
Figure 3.4: Normalized concentration of cells in chicken nuggets inoculated with *S.* Typhimurium LT2 strains lacking outer membrane proteins during frozen storage as measured in Nutrient Agar supplemented with sodium lauryl sulfate
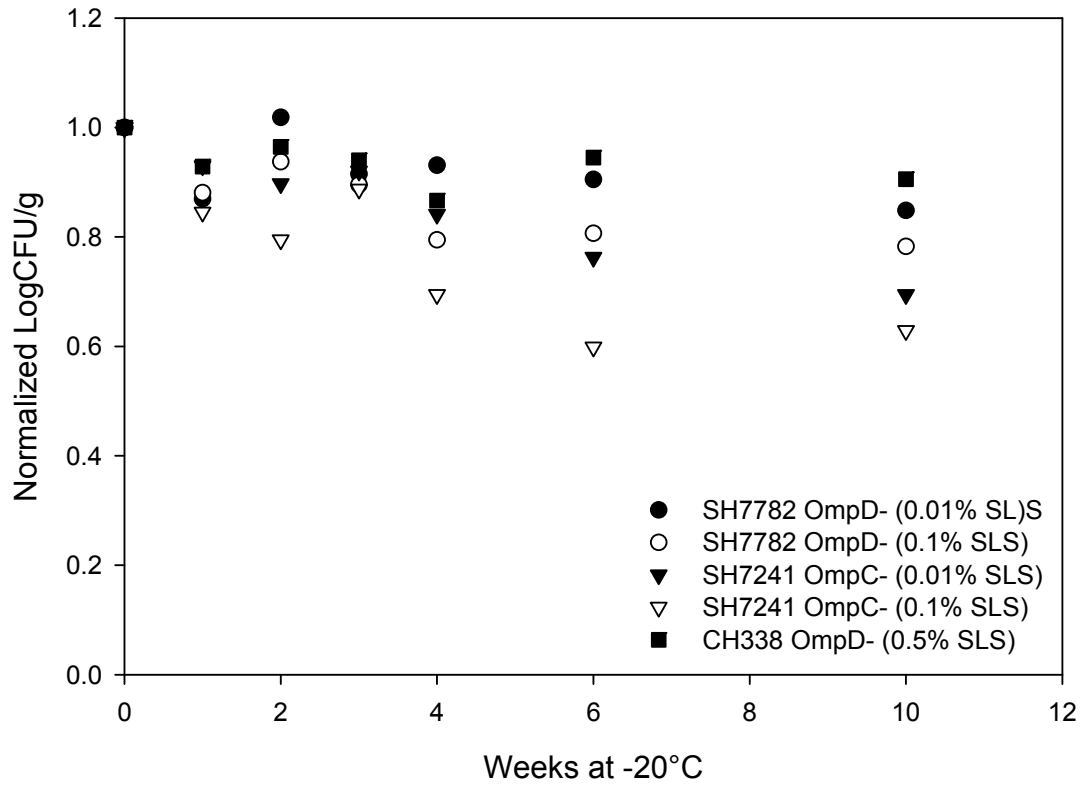
Chapter 4: The survival of *Salmonella* in frozen chicken products during microwave cooking

4.1. Abstract

Frozen chicken products have been recently identified as cause of salmonellosis. Epidemiological investigations have recognized improper cooking, often using a microwave oven, as a common consumer practice leading to infection.

The objective of this study was to assess the effect of microwave cooking at different wattages in the survival of *Salmonella*, when inoculated in a frozen entrée containing raw poultry and cooked following the label's instructions.

Salmonella strains isolated from poultry were inoculated into raw, frozen chicken entrées. Samples were cooked in a research microwave oven according to the instructions in the label, at varying oven wattages (500, 1000 and 1300 Watts). Temperatures in different locations of the entrée were monitored using fiberoptic thermometry.

Mean actual wattages for the microwave oven used (1300 Watts nominal, as stated by the manufacturer) ranged from 89 to 689 Watts (levels 1- 10). An oven efficiency of 53% was calculated. Frozen chicken entrees cooked at 1300 Watts reached mean final temperatures of 99.5-101°C, and 93-99°C when cooked in a 1000 Watt oven; *Salmonella* was not recovered from any of these trials. Frozen entrees cooked in a 500 Watt microwave oven reached mean final temperatures of 42-63.5°C; *Salmonella* was recovered in every case studied at this wattage.

The data presented in this study indicates that microwave oven wattage significantly affects the final cooking temperatures reached, and that the use of a low

wattage microwave oven for cooking frozen products containing raw poultry represents an unsafe practice.

4.2. Introduction

Frozen, breaded chicken products containing raw poultry have been recently identified as risk factors for *Salmonella* infection the (*32,76*). Within the last decade, salmonellosis outbreaks in Australia (*71*), British Columbia - Canada (*32,76*) and Minnesota – United States (*120*) have implicated raw, frozen chicken nuggets, strips and entrees as transmission vehicles of infection. The survival of *Salmonella* after freezing and frozen storage has been demonstrated in Chapter 2 and elsewhere(*15*) and may lead to foodborne infection if the product is insufficiently cooked. The cooked appearance of frozen breaded chicken products containing raw poultry makes them subject to undercooking; consumers are likely to identify them as fully cooked and only reheat them before consumption, as suggested by epidemiological investigations of recent outbreaks (*32,120*).

In response (at least in part) to the recently recognized risk of *Salmonella* infection associated with the improper cooking of processed chicken products that appear to be ready-to-eat but contain raw poultry, the National Advisory Committee on Microbiological Criteria for Foods has proposed guidelines for the safe cooking of poultry products (*92*). A single minimum internal temperature of 165°F (74°C) that would guarantee a 7-$\log_{10}$ reduction of *Salmonella* was recommended for cooking poultry products. This temperature must be achieved in all parts of the product (*54*). Microwave cooking was referred to as "not advisable" for cooking products containing not-ready-to-eat poultry (*92*). The uneven heating profile typical of microwave cooking and the

variability in equipment characteristics, particularly wattage, make it difficult to define standardized microwave oven cooking instructions (*116*). Currently, Not-Ready-To-Eat (NRTE) products containing raw poultry but indicating microwave oven cooking instructions on the label can still be found in the market. The FSIS (*56*) however has announced that if the label of such products does not discourage the use of a microwave oven for cooking purposes, an investigation will be conducted in order to assess the validation of the cooking instructions declared. It is evident that measures are being taken in order to reduce the risk of S*almonella* infection associated with the improper cooking of processed chicken products containing NRTE poultry. However, it is still likely that consumers will continue to use microwave ovens for cooking these products, in most cases without the use of a food thermometer, as suggested by epidemiological investigations of recent outbreaks (*120*).

The generation of temperature gradients within food products cooked in microwave ovens is well documented, particularly in multi component foods (*62,65,66,132*). Several factors related to the food product and equipment characteristics may influence the heating rates achieved in different parts of the product, such as dielectric and thermal properties as well as geometry of the food, its location in the oven, and oven wattage and usage. Evidently, for retail products the instructions declared on the label will also have an effect on the heating rates and final temperatures achieved. Thus, predicting the location of "cold" spots within a multi component product cooked in a microwave oven represents a difficult task. It is generally accepted that the death of microorganisms exposed to microwaves is due to thermal effects (*62*). Comparisons between *Salmonella* destruction obtained after heating in microwave ovens versus

conventional ovens or hot water has proven microwaves to be less effective (*12,29*). *Salmonella* were still detected in whole turkeys roasted in microwave ovens reaching internal temperatures of 76.6°C (*1*), suggesting uneven heating throughout the product. Heddleson (*63*) also detected surviving *Salmonella* after microwave heating of liquid food products reaching 74°C, when the samples were not stirred, proving the safety implications of the temperature gradients produced by microwave heating.

Microwave oven power has been identified as a major variable determining heating profiles and thus bacterial survival (*62*). Cooking instructions must be validated for different oven wattages, otherwise heating profiles may vary and the safety of the product can not be guaranteed (*92*). The only frequency approved for domestic microwave ovens in the United States is 2450 MHz (*65*) but power levels may vary. According to Edgar and Osepchuk (*44*), by 2001, consumer microwave ovens ranging from 500 to 1100 Watts were available in the marketplace. Presently, microwave ovens for consumer use are commercially available in up to 1500 Watts of nominal power.

The objective of this study was to assess the effect of microwave cooking in the survival of *Salmonella*, when inoculated in a frozen entrée containing raw poultry cooked following the label instructions. With this practical approach we intend to generate experimental data related to epidemiological investigations (*32,120*) which have recognized microwave cooking of chicken products containing raw poultry as an unsafe practice.

4.3. Materials and Methods

Processed chicken products. Frozen chicken entrees with broccoli cheese stuffing were used as samples in this study. Each entrée weighed approximately 170g and

had dimensions of 12.5 cm long, 5 cm wide and 3 cm tall (as measured frozen), with a

certain degree of piece to piece variation. The entrees were described in the label (as of

December 2007) as: "Breaded boneless breast of chicken with rib meat, stuffed with

broccoli, Swiss and American cheeses. Microwavable. Not pre-cooked". These entrees

were chosen because they contained raw poultry and microwave oven cooking

instructions were declared on the label. These entrees were used for all the experiments

presented in this study; however, microwave oven cooking instructions were removed

from the label in 2008 in response to the association of this product with a salmonellosis

outbreak.

Microwave oven. The microwave oven used was a Panasonic Model NN-

S760WA with 1300W of nominal power, with inverter technology (Panasonic North

America, Seacaucus, New Jersey).

Determination of temperatures. The FISOCommander OSR System ® (FISO

Technologies, Quebec, Canada) including fiber optic sensors (Figure 4.1), and the

FISOCommander Microwave Workstation Edition ® (Fiso Technologies, Quebec,

Canada) software for data acquisition were used to monitor temperatures during

microwave cooking.

Microwave oven actual wattage determination. The standardized wattage

determination method for household microwave ovens developed by the International

Electrotechnical Commission (*68*) was followed (Appendix 1). Briefly, wattage is

calculated based on the time required to increase the temperature of a 1000ml load of

water by 10°C. Considering the 1300-Watt nominal power of the microwave oven used

in this study, and the actual power measured at the highest power level, the oven's

efficiency was calculated. The actual wattage for each of the 10 power levels was measured, and those equivalent to 500, 1000 and 1300 Watts were identified and selected for the microwave cooking experiments. All experiments were conducted considering a maximum continued usage of the microwave oven of 30 minutes, with a minimum 6-hour interval between experiments.

Microwave heating profiles. Cooking instructions were specified on the entrees' label (as of December 2007) as follows: "For one portion: cook upside down on High power for 2 minutes. Turn over, rotate and cook on High power for an additional 1 ¾ minutes. Cook to an internal temperature of 165°F. Microwave cooking times will vary". Two cooking methods were simulated: (1) Samples were cooked following the label instructions in detail, and (2) samples were cooked following the label instructions but ignoring the turning and rotation steps (i.e. during 3 ¾ continued minutes), simulating a worst-case scenario. To obtain the corresponding heating profiles, temperatures in "hot" and "cold" spots of the chicken entrée (Figure 4.2) were monitored during the cooking process using fiber optic thermometry. Frozen samples were drilled in order to insert the fiber optic probes. The terms "hot" and "cold" spot are used in this study to describe areas on the entrée that achieve faster and slower heating rates, respectively. Preliminary trials were conducted in order to identify these "hot" and "cold" spots, monitoring temperatures in multiple zones throughout the entrée. The "cold" zone referred to in this study, located on the interface between the stuffing and meat component, was selected based on its slower heating rate and likelihood of contamination with *Salmonella* under real processing conditions. The "hot" zone referred to in this study, located on the bottom section of the entrée, in the interface between the meat component and external breading,

achieved the highest heating rates among the zones of the entrée evaluated and is also a potential area of pathogen contamination. All experiments were conducted considering a maximum continued usage of the microwave oven of 30 minutes, with a minimum 6-hour interval between experiments.

Bacterial strains. Two strains of *Salmonella* Kentucky and two strains of *Salmonella* Typhimurium isolated from chicken (*31*) with and without antibiotic resistance were used (Table 1.1).

Inoculation procedures. Cells were grown to stationary phase on Tryptic Soy Broth (Difco Laboratories, Detroit, MI) or Tryptic Soy Broth (Difco Laboratories, Detroit, MI) with 0.01g/L of tetracycline for 18 hours at 37°C. After incubation, all four strains were combined in one inoculation cocktail. The day before inoculation, the frozen chicken products were thawed overnight at 4°C. A total of 0.3 ml of the *Salmonella* cocktail was inoculated using a syringe (~3cm long) in one spot (0.15 ml each) of a thawed entrée corresponding to the identified "cold" or "hot" spot (Figure 4.2). A set of samples was tested immediately for initial population determination. All other samples were placed on a -20°C freezer and stored frozen for a minimum of 1 week before proceeding with the microwave cooking experiments. An inoculation level of 10^5 CFU/g was targeted.

Survival of *Salmonella* after microwave cooking. Entrees were inoculated as described above, frozen and heated at three power levels equivalent to microwave ovens of 500, 1000 and 1300 Watts of nominal power. When cooking inoculated samples, probes were placed immediately adjacent to the inoculation spots.

Microbiological analysis. For initial population determination and immediately after microwave heating, samples were stomached, serially diluted and plated on XLT4 agar, Plate Count Agar and Plate Count Agar with 0.01g/L tetracycline. A slice of an entrée (~20 g) containing the inoculated region was cut with a sterile knife and used for microbial analysis. Controls for not-inoculated entrees were also determined. The detection limit of the method is 50 CFU/g (1.69 $\log_{10}$ CFU/g).

Microbiological media. The agars used in this study were: Plate Count Agar (Difco Laboratories, Detroit, MI) – referred to as PCA in this document, Plate Count Agar (Difco Laboratories, Detroit, MI) with 0.01g/L of tetracycline (Acros Organics, Pittsburgh, PA) - referred to as PCA-Tet in this document, and XLT4 Agar (Difco Laboratories, Detroit, MI).

Statistical analysis. The software SPSS for Windows version 16.0 (SPSS Inc., Chicago IL) was used in the statistical analysis of the data. Means were compared using ANOVA ($p<0.05$) with Duncan multiple range as post-hoc test, if applicable.

4.4. Results

Microwave oven actual wattage determination. Following the International Electrotechnical Commission (*68*) methodology, the average actual wattages of the microwave oven used in this study were determined based on three experimental measurements for each of its 10 power levels (Figure 4.3). Average actual wattages measured ranged from 89 (±6) Watts for power level 1, to 689 (±102) Watts for power level 10. Comparing the average actual wattage measured at the highest power level (i.e. level 10; 989 Watts) to the oven's nominal power of 1300 Watts, an average oven efficiency of 53% was estimated. Considering this efficiency, power levels equivalent to

nominal powers of 500 and 1000 Watts were identified. For example, the average actual power for level 3 was measured as 267 Watts; taking into account the 53% oven efficiency previously determined, level 3 was assumed to represent a microwave oven of ~500 Watts nominal power. Similarly, level 5 was assumed to represent a microwave oven of ~1000 Watts nominal power.

Microwave heating profiles. For each combination of wattage (1300, 1000 and 500 Watts), location in the product ("cold"/"hot" spot) and adherence to cooking instructions (turning/not turning the entrée) a minimum of four heating profiles were obtained. These profiles showed a certain degree of variability; however, the curves were consistent within treatments (data not shown). Initial temperature of entrees at the time of microwave cooking was -7.8 ± 1.5°C. The heating profiles shown in Figures 4.3,4. 4 and 4.5 represent those that provided intermediate results for each wattage and treatment studied.

At 1300 Watts, average final temperatures reached with the four treatments studied ranged from 99.5 ± 0.1°C to 101 ± 0.6°C (Table 4.1) and proved to be not significantly different at a 95% confidence level. The fastest heating rates were achieved in the "hot" spot regardless of compliance with the turning step (Figure 4.3). The turning step can be visualized as a plateau or depression in the curves around 120 seconds. Temperatures monitored in the "cold" spot when the turning step was not followed show a similar heating profile to those in the "hot" spot, with only a slight decrease in the slope. A delay in the heating rate however is clear after the entrée is turned. The turning step implicates the relocation of the "cold" spot into a position approaching the "hot"

zone, and although it occurred only at ~ 55°C, the final temperature was reached ~1 minute before the end of the cooking time.

At 1000 Watts, average final temperatures ranged from 93 ± 9°C to 99 ± 1°C (Table 4.1), and were also considered not significantly different (p<0.05). An overall decrease in slopes and time at the final temperature (99°C) can be noted (Figure 4.4), as compared to heating profiles at 1300 Watts. The effect of the turning step is evident at 1000 Watts, delaying the achievement of the final temperature. In specific, for the "hot" spot temperature curve, after the turning step (~120 seconds, ~40°C) the slope is markedly decreased resulting in lower final temperatures reached. In this case, it is clear that the relocation of the "hot" spot into a location closer to the "cold" area had a detrimental effect in the heating profile and final temperature achieved.

At 500 Watts (Table 4.1), the highest temperatures (63.5 ± 15°C) were reached in the "hot" spot when the turning step was not followed. Temperatures achieved with this treatment were significantly different (p<0.05) than those with the other three studied, i.e. in the "cold" spot regardless of compliance with the turning step, and in the "hot" spot following the turning step, which only reached temperatures of 47 ± 15°C, 47 ± 9°C and 42 ± 9°C respectively. Results shown in Table 4.1 suggest that variability in average final temperatures achieved, measured as standard deviation, is the highest when cooking at lower wattages.

Survival of *Salmonella* after microwave cooking. Controls of not-inoculated entrees detected no growth on XLT4 and PCA-Tet, and 10^2 - 10^3 CFU/g on PCA. Entrees were inoculated with *Salmonella* in the identified "hot" and "cold" spots (Figure 4.2). An inoculum level of 5.5 ± 0.3 log CFU/g was obtained as measured in XLT4 agar, 5.7 ± 0.2

log CFU/g in PCA-Tet, and 6.1 ± 0.04 log CFU/g in PCA. After inoculation, samples were stored at -20°C for periods ranging from 1 – 12 weeks before microwave cooking. Cell concentrations measured after frozen storage averaged 3.7 ± 0.3 log CFU/g as measured in XLT4 agar, 4.6 ± 0.1 log CFU/g in PCA-Tet, and 4.8 ± 0.1 log CFU/g in PCA (Table 4.2, labeled as "Before microwaving") and were not significantly different (p<0.05) from each other over time. However, mean cell concentrations were significantly (p<0.05) reduced after storage at -20°C as compared to the initial inoculum. Also, cell concentrations after frozen storage measured in XLT4 agar were significantly (p<0.05) lower than those on PCA and PCA-Tet.

Inoculated, frozen entrees were cooked following the label instructions (as of December 2007). Temperatures in areas immediately adjacent to the inoculation spots were monitored during cooking; this data was incorporated on the heating profiles analysis described previously. *Salmonella* was not detected after microwave cooking at 1300 and 1000 Watts, for any condition studied (data not shown). *Salmonella* was recovered after all treatments at 500 Watts (Table 4.2). No significant differences (p<0.05) existed between cell concentrations measured before and after microwave treatment, regardless of the agar used to assess recovery, for all conditions tested except when *Salmonella* was inoculated in the "hot" spot and the entrée was not turned during cooking (Table 4.2). In this case, overall recovery was significantly lower than cell concentrations before microwave cooking (p<0.05), and recovery in XLT4 and PCA-Tet was significantly lower (p<0.05) than recovery on PCA.

4.5. Discussion

In this study, we attempted to experimentally evaluate a high-risk factor identified in epidemiological investigations of recent salmonellosis outbreaks associated with consumption of undercooked frozen poultry products: cooking these products in a microwave oven. The effect of location within a frozen chicken entrée and adherence to cooking instructions specified on the label on the heating profiles achieved and the survival of *Salmonella* were investigated.

The microwave oven used in this study (1300 Watts nominal power) was characterized following the IEC (*68*) standard method, by determining experimentally the actual wattage of each of its 10 power levels. An oven efficiency of 53% was determined, which can be defined as the percentage of the total power available for generation of microwaves by the oven that was actually transmitted to the water load and used to produce heat. Although crucial, microwave oven characterization is not frequently reported in the published literature. An efficiency of about 50% has been previously estimated for consumer microwave ovens (*44*). Heddleson *et al.* (*64*) reported 80-90% microwave oven efficiencies however, a different methodology was followed. The wide range of actual wattages that can be obtained with one single microwave oven must be noted (Figure 4.2) and heating profiles and final temperatures achieved when cooking a food product may be significantly affected by the power level selected. Heddleson *et al.* (*64*) observed that different oven power levels resulted in different final temperatures when heating milk for a constant time, however wattages were not calculated for the different power levels.

Power levels 3, 5 and 10 were selected as representative of microwave ovens of 500, 1000 and 1300 Watts nominal power respectively, in an attempt to simulate

compact, common and high-wattage consumer microwave ovens. We emphasize that variability exists in the heating profiles achieved at each wattage, and can be largely attributed to difficulties in precisely replicating the locations ("hot"/"cold" spots) in every trial, as well as reliability of oven performance. Large variability in final temperatures achieved when cooking food products in microwave ovens has been previously reported and most studies approach this issue by adjusting cooking times in order to achieve standardized final cooking temperatures in every trial (*1,12,29,52,63,65*).

The "cold" and "hot" spots referred to in this study were selected as indicative of the existence of differential heating rates within the product however do not intend to represent individual areas of absolute slowest or fastest heating rates. The "cold" and "hot" spots were also selected considering the likelihood of contamination with *Salmonella* under normal processing conditions, i.e. assuming that the potential source of pathogen would be the poultry component and that the interior of the chicken matrix is free of bacterial contaminants. Previous studies in non-fluid foods have often measured only post-cooking temperatures in the geometric center or most internal point of the product (*1,12*), however bacteria was still recovered after cooking to internal temperatures of 64-76.6°C suggesting that these were not the areas reaching the lowest temperatures in the product.

For experiments at 1300 Watts, the similarity between heating profiles for the "hot" and "cold" spots suggests a non-relevant difference between temperatures in these two locations (Figure 4.3). In addition, turning the entrée appeared to have a minor effect probably because by the time the entrée was due to be turned, it had already reached its final temperature (~100°C) in most locations. At 1000 Watts however the effect of

compliance with the turning step on the heating profiles and final temperatures achieved is evident (Figure 4.4). Despite differences in the heating profiles obtained for each condition studied at 1300 and 1000 Watts, mean final temperatures ranged from 93-101°C (Table 4.1) and are therefore safe for cooking products containing raw poultry, according to the NACMCF (*126*) recommendations. Thus, at 1300 and 1000 Watts, cooking instructions declared on the product's label may be considered valid based on the final temperatures achieved. It should be noted however that if consumers do not follow the cooking instructions provided in detail, for example reducing cooking time, risk of not achieving safe cooking temperatures increases (Figures 4.3 and 4.4).

At 500 Watts, cooking instructions declared on the label of the entrée used in this study could not be validated. Heating profiles for both the "hot" and "cold" spots, regardless of compliance with the turning step, revealed final temperatures (63.5 ± 15°C) well below the NACMCF (*126*) recommended safe cooking temperature of 74°C for products containing raw poultry (Table 4.1). Heddleson *et al.* (*64*) report final temperatures of 63°C for the highest power setting of a 600 Watt microwave oven. The dramatic difference between results obtained at 1300 and 1000 Watts versus those at 500 Watts highlights the major influence of microwave oven wattage on the heating profiles and final cooking temperatures achieved. The use of microwave ovens covering a range of wattages for the validation of cooking instructions for NRTE products has been recommended by GMA (*116*). Also, our results support those of epidemiological investigations (*120*) suggesting the high risk of cooking products containing raw poultry in low wattage microwave ovens.

Experiments were also conducted with *Salmonella*-inoculated samples, which were subject to a period of frozen storage before microwave cooking. A reduction in total bacterial population after storage at -20°C was observed, which may be attributed to the initial freezing stress and potentially slow freezing rates achieved, considering the dimensions of the entrée and freezer temperature (-20°C). It has been demonstrated that slow freezing (1-10 °C/h) - characterized by extracellular ice formation and osmotic dehydration of the cell - has a detrimental effect on cell viability (*11,22,46,109*). Also, recovery in XLT4 agar was lower than recovery in PCA and PCA-Tet, which has been previously reported as a result of structural injury of the cells after freezing in Chapter 2 and elsewhere (*15*).

Not surprisingly, experiments conducted with *Salmonella*-inoculated samples were in agreement with the temperature profiles previously discussed. No cells were recovered from inoculated entrees cooked at 1300 or 1000 Watts (mean final temperatures > 93°C; ~5-log$_{10}$ reduction). Baker *et al.* (*12*) reported microwave cooking as an efficient method for destroying *Salmonella* in chicken loaves and roasted chicken. In this study however the final temperature of 64°C was measured only in the geometric center of the samples and the oven's wattage was not determined (*12*); higher temperatures may have been reached in other areas of the products being cooked. Contrary to results at 1300 and 1000 Watts, at 500 Watts no significant reduction ($p<0.05$) or only partial reduction (~2 -log$_{10}$) in initial population was obtained. In the latter case, when entrees inoculated in the "hot" spot were cooked at 500 Watts not following the turning step (final temperature 63.5±15°C), approximately 3-log$_{10}$ in PCA, and 2-log$_{10}$ in XLT4 and PCA-Tet were still recovered, rendering the entrees unsuitable

for consumption. Baker *et al.* (*12*) concluded that microwave cooking was not effective

for destroying *Salmonella* in chicken burgers reaching 64°C in the center of the product

however, large reductions were obtained. Similarly, Aleixo *et al.* (*1*) report significant

reductions of *Salmonella* from turkeys cooked in a 600 Watts microwave oven however,

the pathogen was not completely eliminated; final temperatures reached 76.6°C as

measured in only two internal sections of the turkey breast. It is logical to suggest that

although lethal temperatures were reached in the locations chosen for measurement in the

studies described before, these temperatures were not homogeneous throughout the

products and areas of slower heating rates must have allowed for the survival of

Salmonella. Heddleson *et al.* (*64*) found that significantly less destruction of *Salmonella*

in milk was achieved when using a 450 Watts microwave oven (final temperature 36°C),

as compared to a 700 Watts oven. We also observed that, for entrees inoculated in the

"hot" spot and cooked at 500 Watts not following the turning step, recovery in XLT4 and

PCA-Tet was significantly lower than recovery in PCA, suggesting a certain degree of

cell injury. Heddleson and Doores (*63*) found that injured *Salmonella* cells, as indicated

by reduced recovery in selective agar as compared to complete agar, could be detected

after heating beef broth to temperatures as high as 72°C when the samples were not

stirred and a standing time was allowed. This was attributed to the non-uniform heating

achieved in beef broth due to its high dissolved salt concentration.

4.6. Conclusions

The results presented in this study make evident the variable nature of microwave

cooking, and the challenges it poses for developing safe cooking instructions for NRTE

foods. As indicated by our results at 500 Watts, the use of low wattage microwave ovens

for cooking food products containing raw poultry represents a risk to the consumer, and may result in foodborne illness, due to the inadequate final temperatures reached. Our results support those of epidemiological investigations of recent outbreaks linked to raw, frozen chicken products, which identified undercooking in microwave ovens as a risk factor for salmonellosis.

4.7 Tables

Table 4.1: Final temperatures reached in selected locations of a frozen chicken entrée after microwave treatments at different oven wattages [a]

Location and Treatment	Final Temperature (°C) ± Standard Deviation		
	1300 W	**1000 W**	**500 W**
Cold spot, without turning	99.5 ± 0.3 [A]	99 ± 1 [a]	47 ± 9 [α]
Cold spot, turning	99.5 ± 0.1 [A]	94 ± 9 [a]	47 ± 15 [α]
Hot spot, without turning	101.0 ± 0.6 [A]	99 ± 1 [a]	64 ± 15 [β]
Hot spot, turning	100.0 ± 0.1 [A]	93 ± 9 [a]	42 ± 9 [α]

a. Within columns, means with different letters are considered significantly different ($p<0.05$)

Table 4.2: Recovered cells (average log CFU/g ± standard deviation) from selected locations of *Salmonella*-inoculated chicken entrees after microwave treatments at 500 Watts as measured in different agars [a,b]

	Before microwaving[A]	**Cold spot, without turn**[A]	**Cold spot, turning**[A]	**Hot spot, without turn**[B]	**Hot spot, turning**[A]
XLT4	3.7 ± 0.3	3.9 ± 0.3	3.5 ± 0.8	1.9 ± 0.4	3.6 ± 0.8
PCA-Tet	4.6 ± 0.1	4.4 ± 0.6	4.0 ± 0.5	2.1 ± 0.8	4.2 ± 0.3
PCA	4.8 ± 0.1	4.8 ± 0.5	4.4 ± 0.4	3.1 ± 0.5	4.7 ± 0.2

a. Treatments with different letters are considered significantly different ($p<0.05$), regardless of the agar used to assess recovery.
b. No detection was reported as 1.68 log CFU/g, immediately below the detection limit of the method (1.69 log CFU/g).

4.8. Figures

Figure 4.1: FISOCommander OSR System ® (FISO Technologies, Quebec, Canada) and microwave oven workstation[a]

a. With only one fiberoptic probe connected. Not showing connection to computer.

Figure 4.2: Diagram of "hot" and "cold" zones in a transversal section of the frozen chicken entrée used in this study
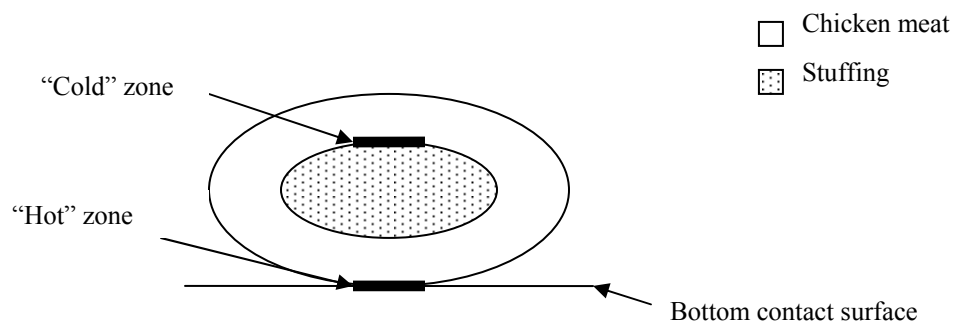
Figure 4.3: Actual wattage of power levels 1-10 of the microwave oven used in this study (1300 Watts of nominal power)
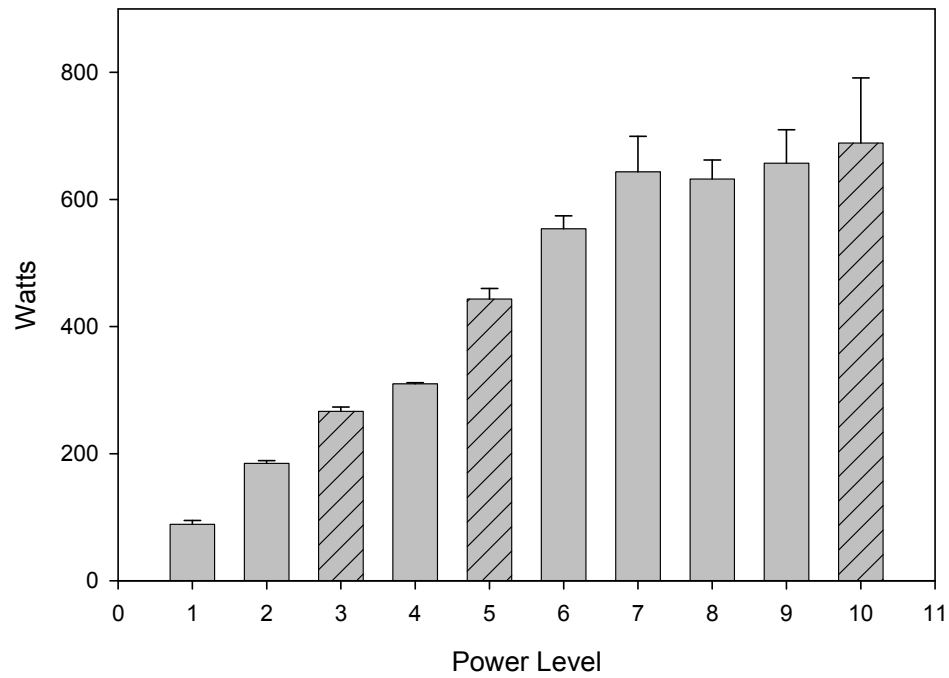
Figure 4.3: Representative heating profile for a frozen chicken entrée during microwave cooking at 1300 Watts
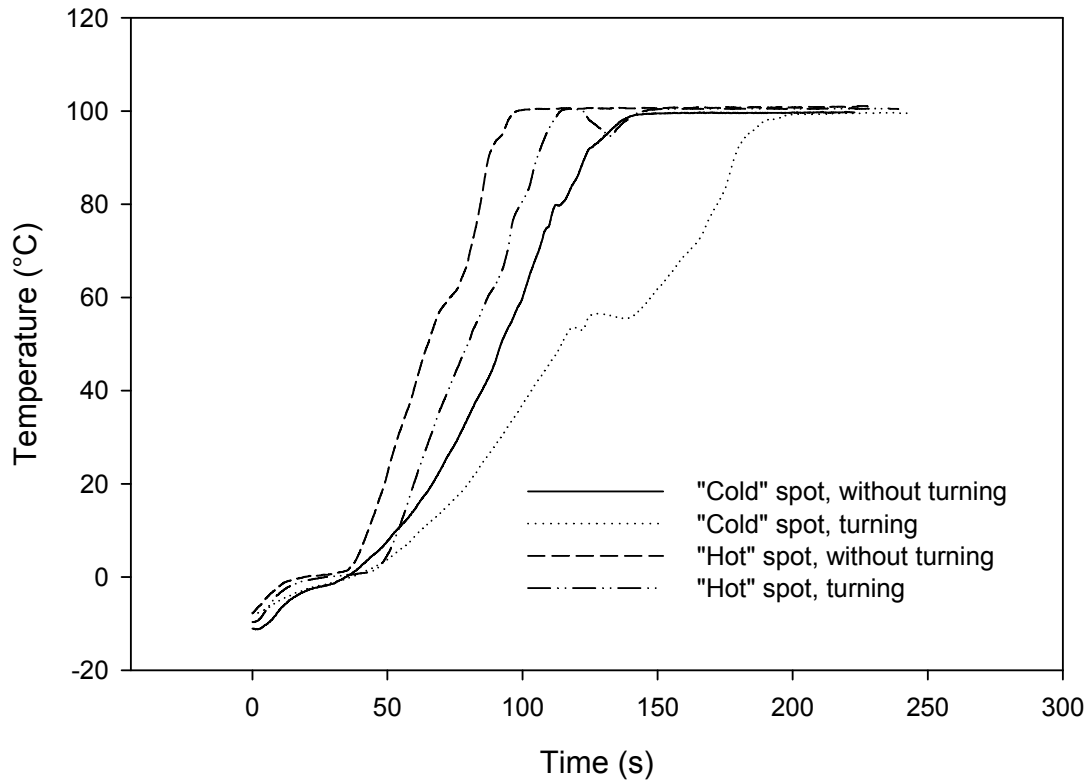
Figure 4.4: Representative heating profile for a frozen chicken entrée during microwave cooking at 1000 Watts
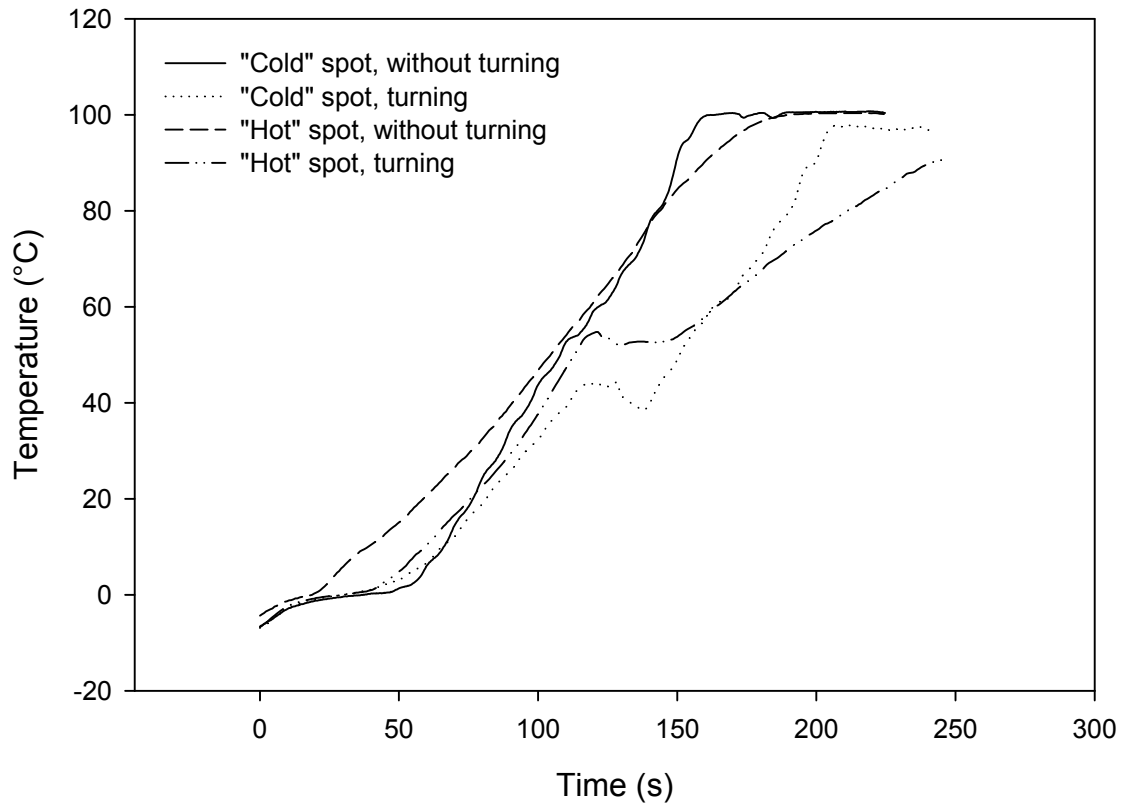
Figure 4.5: Representative heating profile for a frozen chicken entrée during microwave

cooking at 500 Watts
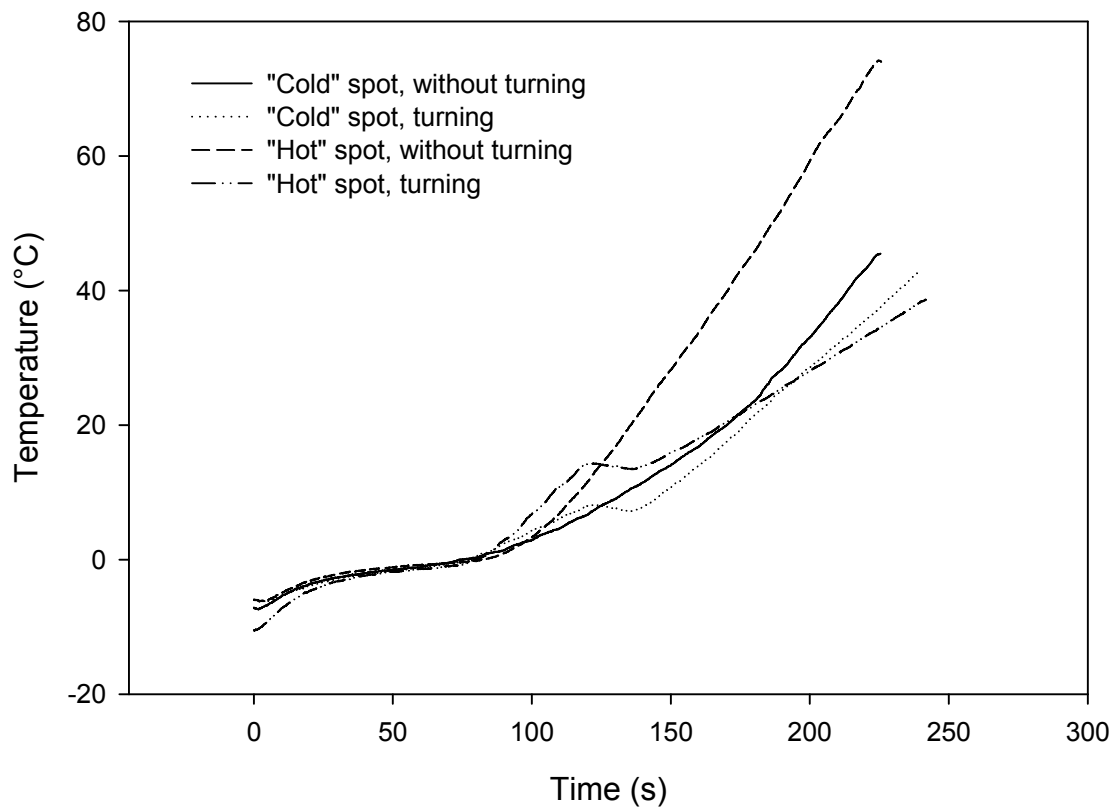
Chapter 5: Quantitative risk assessment for *Salmonella* in raw, frozen chicken products

5.1. Abstract

Salmonella is frequently associated with poultry, and its presence in raw frozen chicken products may pose an infection risk if improperly cooked. Recent salmonellosis outbreaks have been associated with consumption of frozen products containing raw poultry. Epidemiological investigations report that consumers perceive such products as being precooked and improper cooking (often using a microwave oven) has been identified as a common practice leading to infection. Microwave heating produces an uneven temperature distribution, and its use for cooking frozen chicken products containing raw poultry is currently a matter of concern.

The objective of this study was to provide a quantitative estimation of the risk of salmonellosis associated with consumption of raw, frozen chicken products cooked in a low wattage microwave oven, utilizing published and newly collected data.

A quantitative risk assessment using @Risk was developed. The following events were considered in the model: prevalence and concentration of *Salmonella* on chicken entrees at retail, probability of using low wattage microwave ovens for cooking raw, frozen chicken entrees, and *Salmonella* inactivation during low wattage microwave cooking. Data on *Salmonella* prevalence and concentration, consumer cooking practices and microwave use was taken from the published literature. When necessary, surrogate data was used or assumptions were made. Temperature profiles for low wattage microwave oven cooking were collected in our laboratory and incorporated in this risk

assessment. The model outputs were integrated in a Beta-Poisson *Salmonella* dose-response model and a probability of illness was calculated.

A 2005 outbreak in Minnesota linked to frozen chicken entrees (4 reported cases; 202,252 servings) was simulated using our risk model. The number of reported cases predicted by the model ranged from 5-7 for 100 simulations of this outbreak. Despite certain limitations in availability of data, the proposed model's predictions were in agreement with reported outbreak data, suggesting a realistic modeling approach. The risk assessment model developed may provide useful quantitative data relevant for risk management initiatives, ultimately aiming at controlling the risk of salmonellosis from raw, frozen chicken products.

5.2. Introduction

Salmonella is one of the leading causes of foodborne illness in the United States and the most frequently reported. As reported through the Foodborne Disease Outbreak Surveillance System, *Salmonella* was responsible for 585 outbreaks, 16,821 cases and 20 deaths in the United States between 1998 and 2002 (*75*). For this 5-year period, 50 salmonellosis outbreaks were associated with poultry. The real number of total cases however may be more than 30 times larger than that reported, considering the underreporting of milder illnesses (*86,86,94*). It is estimated that approximately 1.4 million nontyphoidal *Salmonella* infections occur in the United States every year (*86*).

Frozen, breaded chicken products containing raw poultry have been recently identified as risk factors for *Salmonella* infection (*32,76,77,77*). Within the last decade, salmonellosis outbreaks in Australia (*72*),(*71*) British Columbia - Canada (*32,76,77,77*) and Minnesota – United States (*120*) have implicated raw, frozen chicken nuggets, strips

and entrees as transmission vehicles of infection. The cooked appearance of these products makes them potentially dangerous; consumers are likely to identify them as fully cooked and only reheat them before consumption, as suggested by epidemiological investigations of recent outbreaks (*32,120,120*). Previous studies (*15,42,42,43,51*) and Chapter 2 of this dissertation have demonstrated the survival of *Salmonella* during frozen storage, as well as during microwave cooking at low wattages when inoculated into processed chicken products (Chapter 4).

  Salmonella are facultative anaerobic, gram-negative rods, which belong to the *Enterobacteriaceae* family. *Salmonella* have an optimum growth temperature of 37°C, however they readily adapt to extreme conditions. Growth at temperatures as low as 5.9°C (*83*) and as high as 54°C (*41*) have been reported for specific experimental conditions. Extended survival under freezing temperatures when inoculated in non-fluid matrixes has been demonstrated in previous studies (*15,42,43,51,57*) and Chapter 2 of this dissertation. Survival to heat treatment, especially in low water activity foods, is currently a matter of concern. *Salmonella* has an optimum growth pH of 6.5-7.5, however it can grow at pH values of 4.5-9.5. Poultry meat and eggs are the most common reservoir of *Salmonella*, with the characteristic hen-to-egg transovarian transmission of serovar Enteritidis posing a major difficulty in the control of this pathogen in eggs. However, in recent years the occurrence of salmonellosis outbreaks linked to fresh produce has increased. Non-typhoidal human salmonellosis is often characterized by the onset of non-bloddy diarrhea and abdominal pain 8-72 hours from ingestion of the pathogen. The mechanism by which *Salmonella* infection generates these symptoms in the human host has not yet been fully elucidated. The illness is usually self-limiting,

however in extreme cases it can lead to systemic infections and chronic conditions. A *Salmonella* dose-response curve based on outbreaks data has been constructed by FAO/WHO (*49*) and indicates that even very low doses can cause illness. Extensive information on the identification, growth and survival of *Salmonella,* as well as on human salmonellosis can be found on specific review chapters (*34,90*).

Quantitative microbial risk assessment (QMRA) is a methodology used to organize and analyze relevant data in order to estimate the public health consequences associated with microbiological risk (*38*). QMRA considers some or all of the various stages in the food production process, and the main outcome of QMRA is traditionally defined as the estimated probability of illness from the consumption of the food product under study (*38*). Monte Carlo simulation is currently the most widely used technique for conducting microbial risk assessments. This methodology uses a stochastic approach, where key factors in the model are represented by distributions and a set of output values in the form of a distribution is generated as a result of multiple iterations (*38*). Because high-risk scenarios often arise from outlying data points rather than average results (*130*), Monte Carlo simulation has the potential to provide a more realistic estimation of risk compared to a strictly deterministic approach. In addition, taking into account the variability described by a frequency distribution produces a more realistic assessment of risk than one based on a sole discrete value, such as the mean or worst case, at each step modeled (*23*).

QMRA typically consists of four stages: (1) hazard identification, in which the pathogenic microorganisms potentially present in the food product are identified; (2) hazard characterization, which describes the adverse health effects associated with the

microorganism if consumed; (3) exposure assessment, which provides an estimated

frequency of consumption of the food in study, and the probable number of

microorganisms per serving; and (4) risk characterization, where hazard characterization

and exposure assessment are integrated to provide an estimated risk of infection

associated with the consumption of the food product.

The objective of this study was to provide a quantitative estimation of the risk of

salmonellosis associated with consumption of raw, frozen chicken products cooked in a

microwave oven, based on published and newly collected data and utilizing Monte Carlo

simulation techniques. Stages 1 and 2 of the QMRA have been described above; stages 3

and 4 will be discussed in the following sections.

5.3. Materials and methods

A retail-to-consumption risk assessment model for salmonellosis linked to

consumption of raw, frozen chicken products cooked in low wattage microwave ovens

was constructed in Excel (Microsoft, Redmond, WA) and simulated using the @Risk

software package (version 4.5, Palisade Corporation, Ithaca, NY). The scope of the

model is depicted in Figure 5.1. Inputs for each of the model stages (Table 5.1) are

described in the following subsections (1-5). The exposure assessment step is outlined in

subsections 1, 2 and 3. The risk characterization step is outlined in subsection 4.

1. Prevalence and concentration of *Salmonella* on raw, frozen chicken entrees at

    retail. Data for the prevalence of *Salmonella* in frozen chicken products was

    extracted from Bucher *et al.* (*25*), who reported 25 *Salmonella*-positive out of 92

    retail and wholesale raw, frozen chicken nuggets and strips prepared at nine

    different establishments in Canada. Although these results are not specifically for

frozen chicken entrees in the United States, in the absence of any other available data, we believe these data are an appropriate surrogate for the data required. Eglezos *et al.* (*45*) surveyed the bacteriological profile of raw, frozen chicken nuggets in Australia, finding 26 of 300 samples positive for *Salmonella.* This study however sampled products from only one Australian facility (*45*). The probability of finding a *Salmonella*-positive entrée was modeled as a Beta distribution with parameters α (25+1) and β (92-25+1) based on the results of Bucher *et al.* (*25*). This distribution is commonly used to represent the uncertainty about the prevalence of a positive sample, based on the total number of samples collected and the number of positive samples observed and is expressed as Beta (positive+1, negative+1) (*115*). A Binomial distribution was incorporated to simulate whether an entrée would or would not be contaminated. To estimate the concentration of *Salmonella* in a contaminated entrée, the USDA-FSIS baseline data for *Salmonella* in ground chicken (*5*) was used as a surrogate. Studies on the occurrence of *Salmonella* in raw, frozen chicken products exist however enrichment steps are required and the actual concentration of the pathogen has not been quantified (*25,45*). Considering that the main potential source of *Salmonella* in raw, frozen chicken entrees is the chicken meat component, and that it is often formed by reconstitution of chicken pieces rather than a single cut (*45,77*), we assumed ground chicken to be an appropriate surrogate. The USDA-FSIS (*5*) reports a 1.27 $\log_{10}$ geometric mean for the baseline concentration of *Salmonella* per gram of ground chicken, with a standard deviation of 0.05 $\log_{10}$. These values were used as parameters for a Normal distribution to model the concentration of

Salmonella in chicken entrees at retail ($\log_{10}$CFU/serving). The weight of one entrée was considered 170 grams, as reported in the label for several brands at retail. In order to segregate false-negative (0 $\log_{10}$CFU/serving) or undefined values ($\log_{10}$ of 0), an error-trapping "If" function was used.

2. Use of low wattage microwave ovens for cooking raw, frozen chicken entrees. In response to a 2003 salmonellosis outbreak in Canada associated with frozen chicken products, MacDougall *et al.* conducted an epidemiological investigation (*77*). Of 36 subjects interviewed (cases and controls), 10 report use of a microwave oven to cook these products. Smith *et al.* (*120*) surveyed microwave oven use for cooking raw, frozen chicken entrees in the United States in Minnesota's 1998 and 2005 outbreaks, however only cases' responses were reported. Similarly, Kenny *et al.* (*72*) conducted an epidemiological investigation for a chicken nuggets salmonellosis outbreak in Australia, but only cases were questioned regarding microwave oven use. Thus, we described the probability of microwave oven use among frozen chicken entrees consumers with a Beta distribution with parameters α (10+1) and β (36-10+1), according to the results of MacDougall *et al.* (*77*). As observed in a previous study, the use of a low wattage microwave oven (i.e. 500 Watts) to cook raw, frozen chicken entrees was concluded to be an unsafe practice, whereas in higher wattage ovens (> 1,000 Watts) safe final cooking temperatures were achieved (Chapter 4). Thus, in this risk assessment we decided to model the risk associated with cooking raw, frozen chicken products in low wattage microwave ovens only. Microwave ovens for consumer use currently in the market range from compact 500 Watts ovens to

2,000 Watts large capacity ovens, with ~ 1,000 Watts being the most commonly

found (*9*). Estimations of the proportion of different wattage microwave ovens in

the United States' households were not available; however, the American Frozen

Food Institute has developed a list of current models in the market (*9*). Based on

this list, we constructed a histogram (Figure 5.2) and calculated the prevalence of

600-850 Watts ovens in the market (10.4%). The previously described Beta

distribution for the overall prevalence of microwave oven use was then multiplied

by this percentage in order to estimate the probability of using a low wattage

microwave only. Although this implies that all microwave oven models are

equally distributed among consumers (i.e. equivalent sales per model), we

decided to use this assumption. A Binomial distribution was included in order to

simulate whether an entrée would or would not be cooked in a low wattage

microwave oven.

3. *Salmonella* inactivation during low wattage microwave cooking. In a previous

   study, we generated temperature profiles during microwave oven cooking of raw,

   frozen chicken entrees at different wattages, following the product's label

   instructions (Chapter 4). Final temperatures achieved when cooking the products

   at 500 Watts ranged from 29 to 82°C, with a mean of 50°C and a standard

   deviation of 14°C (Figure 5.3). These values were used as parameters for a

   Normal distribution to describe final cooking temperatures achieved in a low

   wattage microwave oven. *D*-values for *Salmonella* serovars isolated from raw,

   frozen chicken products in Canada have been experimentally determined by

   Bucher *et al.* (*24*). The thermal resistance of the strains studied by Bucher *et al*.

(*24*) was lower than those reported in other studies, however the strains were

isolated from raw, frozen chicken products and *D*-values were determined using

chicken meat as heating menstrum, making their results relevant to this risk

assessment. A linear relationship between the logarithm of *D*-value (minutes) and

temperature (°C) was constructed using the *D*-values for *S.* Heidelberg reported

by Bucher *et al.* (*24*), and the anti-log of this function was included in our model:

D-value $= 10^{(-0.2103*T) + 12.205}$ **Equation 5.1**

For a given microwave oven cooking temperature from the Normal distribution

previously described, a corresponding *D*-value was selected in the simulation.

Bucher *et al.* (*24*) report very similar D-values for other *Salmonella* strains as well

however, we selected the data presented for *S.* Heidelberg as this strain was the

most common isolate in their previous study (*25*). *S.* Heidelberg has also been

identified, among other strains, in recent salmonellosis outbreaks associated with

frozen chicken products in the United States (*120*). Relevant epidemiological

investigations (*32,71,72,72,76,77,77,120,120*) indicate that consumers tend to

disregard the cooking instructions declared in raw, frozen chicken products'

labels and rather, arbitrarily select a cooking method and time. The cooking time

declared in the raw, frozen chicken entrees used in a previous study (Chapter 4)

was 3 minutes and 45 seconds, and it was observed that before this time was

completed the entrees were hot to the touch and cheese from the stuffing had

melted. Kenny *et al.* (*72,72*) surveyed cases involved in a salmonellosis outbreak

in Australia and one victim stated that they usually heat six nuggets in the microwave oven for 2 minutes. Due to the very limited published information regarding consumer's arbitrarily selected microwave oven cooking times, we modeled cooking time using a Normal distribution with a mean of 2 minutes and a standard deviation of 1 minute, partially based on the observations of Kenny *et al.* (*72*). The number of log reductions achieved during cooking in a low wattage microwave oven was determined dividing cooking time by *D*-value. The final *Salmonella* concentration ($\log_{10}$CFU/serving) in the entrée after cooking was calculated by subtracting this value from the initial concentration at retail. This result was then converted to CFU/serving. However, if the bacterial destruction achieved during microwave cooking was larger than the initial concentration in the entrée, a negative value of no real significance was calculated ("-X$\log_{10}$CFU/serving") for the *Salmonella* concentration after cooking. An error-trapping "If" function including a Binomial distribution was used in order to correct for these values.

4. Dose-response relationship and probability of illness. The FAO/WHO (*49*), as part of their risk assessments of *Salmonella* in eggs and broiler chickens, proposed a dose-response relationship derived from reported outbreaks data and fitted to a Beta-Poisson model. This Beta-Poisson model was able to fit the outbreaks data more accurately than three previously published models. In the Beta-Poisson model, it is assumed that the pathogen cells in the food consumed are Poisson distributed and that each cell has a probability of causing infection defined by a Beta distribution (α, β), considering the variability between hosts

(*16,16,17,111*). Thus, we described the probability of illness according to this model as follows:

Probability of illness $= 1 - (1 + c/\beta)^{-\alpha}$ **Equation 5.2**

Where c is the number of *Salmonella* cells in one serving of chicken entrée at the time of consumption, and α and β are the parameters of the FAO/WHO (*49*) Beta-Poisson model 0.1324 and 51.45, respectively. The calculated probability of illness was then multiplied by the probability of using a low wattage microwave oven in order to account only for this cooking method.

5. Simulation settings. Data from a 2005 salmonellosis outbreak in Minnesota associated with consumption of raw, frozen entrees (*120*) was used to verify the performance of our model. In this outbreak, 75,800 pounds of product were recalled which, assuming an entrée serving of 170g, is equivalent to 202,252 servings. Four confirmed cases were reported. We simulated this outbreak 100 times, running 202,252 iterations per simulation. The number of illnesses predicted per simulation was determined and compared to the actual number reported in the 2005 Minnesota outbreak.

5.4. Results

The proposed risk assessment's predictions, and therefore the overall model performance, were evaluated by comparison against actual outbreak outcomes. A 2005 salmonellosis outbreak in Minnesota associated with consumption of raw, frozen entrees (*120*) was selected due to the availability of data, particularly number of servings

involved. For 100 simulations of this outbreak, the proposed risk assessment model predicted between 190-270 illnesses (Figure 5.4). In order to estimate the number of reported illnesses, the total number of illnesses predicted was divided by 38: the salmonellosis under-reporting factor proposed by Mead *et al*. (*86,87*). This factor takes into account the likelihood of not reporting mild illnesses caused by *Salmonella* infection. Therefore, in our 100 simulations of the Minnesota outbreak, the 190-270 total number of illnesses predicted would have resulted in 5-7 illnesses reported. This is in very close agreement with the actual outcome of the outbreak, suggesting a relatively reliable model.

5.5. Discussion

The risk assessment model proposed successfully predicted the outcome of an actual salmonellosis outbreak. This is indicative of a sound model, however certain limitations exist. As discussed previously, the availability of specific data for the food product in study as well as consumer practices was overcome by the use of surrogate data and/or assumptions. The lack of specific data is often encountered when developing quantitative microbial risk assessment models however, the cautious use of surrogate data, assumptions and expert opinions has been shown to represent valid model inputs in several cases (*38*). When possible, the generation of original experimental data is the desired approach in order to create appropriate model inputs when facing limited availability of published data. This approach was taken in our study for the final cooking temperatures achieved when cooking frozen chicken entrees in a low wattage microwave oven (Chapter 4). For other cases, such as the concentration of *Salmonella* in frozen chicken entrees at retail, this poses significant challenges (e.g. extensive testing of

numerous samples and improved pathogen detection methods) however it may be feasible (*16,16*). Model inputs regarding variable consumer behaviors, such as cooking time for frozen chicken entrees heated in a microwave oven, may require targeted behavioral studies. These studies often carry high variability and for risk assessment purposes, expert opinions and/or assumptions may represent a valid alternative.

In the proposed risk assessment model, the prevalence of low wattage microwave oven use was derived from the proportion of 600-850 Watts ovens (10.4%) currently in the market (*9*). This implies that every model has equivalent sales share, which is not necessarily true. In addition, it does not take into account older microwave oven models that may not be presently in the market but are certainly used in a number of households. Older microwave oven models have characteristically lower wattages than those currently in the market (*44*), and extended usage could have further decreased their performance. In a previous study, we assessed the performance of different oven wattages (Chapter 4). We observed that 500-Watt ovens led to unsafe final cooking temperatures (<74°C), whereas 1,000 and 1,500-Watt ovens reached safe temperatures ($\geq$74°C). A single minimum internal temperature of 165°F (74°C) that would guarantee a 7-$\log_{10}$ reduction of *Salmonella* has been recommended for cooking poultry products (*93*). Ovens between 500 and 1,000 Watts were not tested. We assume in this risk assessment that ovens between 600 and 850 Watts may result in unsafe final cooking temperatures, however it is possible that this range is an overestimation, if wattages for example higher than 600 actually result in safe final temperatures. When evaluating this, in an alternative approach, we simulated the same 2005 Minnesota outbreak but considering the prevalence of low wattage microwave ovens derived only from the proportion of 600

Watts ovens in the market (1.2%), according to the data collected by the American

Frozen Foods Institute (*9*). The results of these simulations predicted between 16 and 40

cases (data not shown) which, considering a 38 under-reporting factor (*86*), results in a

non-detected outbreak. This suggests that the actual prevalence of low wattage ovens in

households may be higher than 1.2%, probably due to the use of older, low wattage oven

models not included in the AFFI survey. Alternatively, this may imply that undercooking

in a low wattage microwave oven was not the major risk source in this outbreak.

Epidemiological investigations of recent outbreaks have found that consumers of

raw, frozen chicken products tend to identify them as fully cooked and only reheat them

before consumption, often using a microwave oven (*32,120,120*). In our risk assessment

model, we considered only undercooking in a low wattage microwave oven as critical

step in the occurrence of salmonellosis associated with consumption of raw, frozen

chicken products. Other practices however may also lead to infection with *Salmonella*

after consumption of these products, such as increased concentration of the pathogen in

the raw material and/or product at retail or undercooking when using a conventional

oven, which were not considered in the scope of this risk assessment. Thus, predictions of

our risk assessment may be considered an underestimation of the total salmonellosis risk

associated with these products. However, it was our goal to particularly investigate the

effect of undercooking in a low wattage microwave oven, being this a characteristic risk

factor identified in association with consumption raw, frozen chicken products

(*32,55,92,93,93,120,120,127*). Comparison with the 2005 Minnesota outbreak seems to

indicate that microwave oven undercooking may indeed be a predominant risk source.

The proposed risk assessment model was compared to the reported outcome of a 2005 Minnesota outbreak (*120*). Validation of risk assessment models evidently provides strength and reliability, however this is often not possible, since it depends on the availability of outbreaks or other relevant observed data. Application of the model for the analysis of different scenarios will be evaluated, for example exploring the consequences of different initial *Salmonella* concentration levels, evaluation of microwave oven cooking instructions for new products/packaging material, changes in *Salmonella* lethality requirements, among others.

5.6. Conclusions

The developed risk assessment may constitute a powerful tool for the evaluation of different strategies aiming at controlling the risk posed by raw, frozen chicken products as well as creating awareness of this risk among the consumers. The latter is of particular importance since although regulatory agencies have taken measures to discourage the inclusion of microwave oven cooking instructions in the labels of products containing raw poultry (*55,93,93*), two similar outbreaks occurred in 2008 (*7,8*).

5.7. Tables

Table 5.1: Risk assessment model inputs

		Variable	Value	Units	Source
B	1	Total number sampled at retail	92	Products	Bucher et al 2007
B	2	Positive at retail	25	Products	
B	3	Probability of pathogen	RiskBeta(B2+1,B1-B2+1)		
B	4	Does the sample contain pathogens?	RiskBinomial(1,B3)		
B	5	One serving = one entrée	170	Grams	Label information
B	6	Mean concentration/gram	1.27	CFU/g	FSIS 1996
B	7	Mean concentration/serving	B6*B5	CFU/serving	
B	8	Standard deviation concentration/gram	0.05	CFU/serving	FSIS 1996
B	9	Standard deviation concentration/serving	B8*B5	CFU/serving	
B	10	Concentration if present/serving	RiskNormal(B7,B9)	CFU/serving	
B	11	Log concentration/serving	LOG(B10)	LogCFU/serving	
B	12	Actual log concentration	B11*B5	LogCFU/serving	
B	13	Actual log concentration with error trapping	IF(B12=0,-5,B12)	LogCFU/serving	
B	15	How many were interviewed?	36	Persons	McDougall et al 2004
B	16	How many used a microwave?	10	Persons	
B	17	Probability of microwave oven use	RiskBeta(B16+1,B15-B16+1)		
B	18	Probability of low wattage microwave oven use	B17*0.104		AFFI 2008
B	19	Does the consumer use a low wattage oven?	RiskBinomial(1,B18)		
B	21	Mean exposure time to low wattage cooking/serving	2	Minutes	User input/Kenny et al 1999
B	22	Standard deviation exposure time	1	Minutes	User input
B	23	Exposure time	RiskNormal(B21,B22)	Minutes	
B	24	Mean final low wattage cooking temperature	50	°C	Experimental data
B	25	Standard deviation temperature	14	°C	
B	26	Temperature low wattage cooking	RiskNormal(B24,B25)	°C	
B	27	D-value	10^((-0.2103*B26)+12.205)	Minutes	Bucher et al 2008
B	28	Number of log-reductions given exposure time	B23/B27	Log-reductions	
B	29	Concentration/serving after cooking	B13-B28	LogCFU/serving	
B	30	Concentration/serving after cooking with error trapping	IF(B29<0,RiskBinomial(1,10^B29),10^B29)	CFU/serving	
B	32	α	0.1324		
B	33	β	51.45		FAO/WHO 2002
B	34	Probability of infection/serving, all low wattage oven	1-(1+B30/B33)^-B32		
B	35	Probability of infection/serving, only low wattage oven	B34*B19		
B	36	Is the person ill?	RiskBinomial(1,B35)		

5.8. Figures
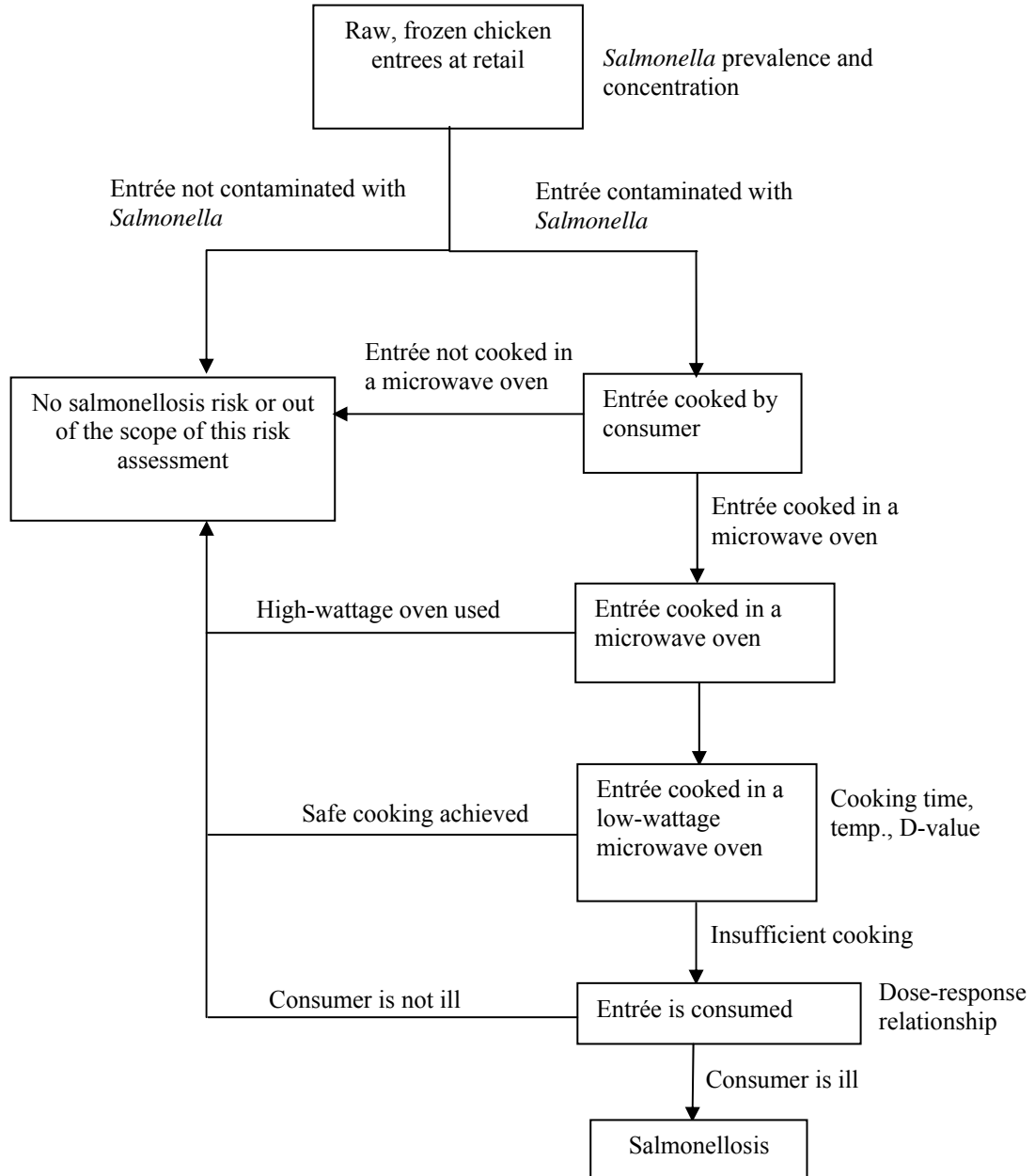
Figure 5.1: Risk assessment model scope

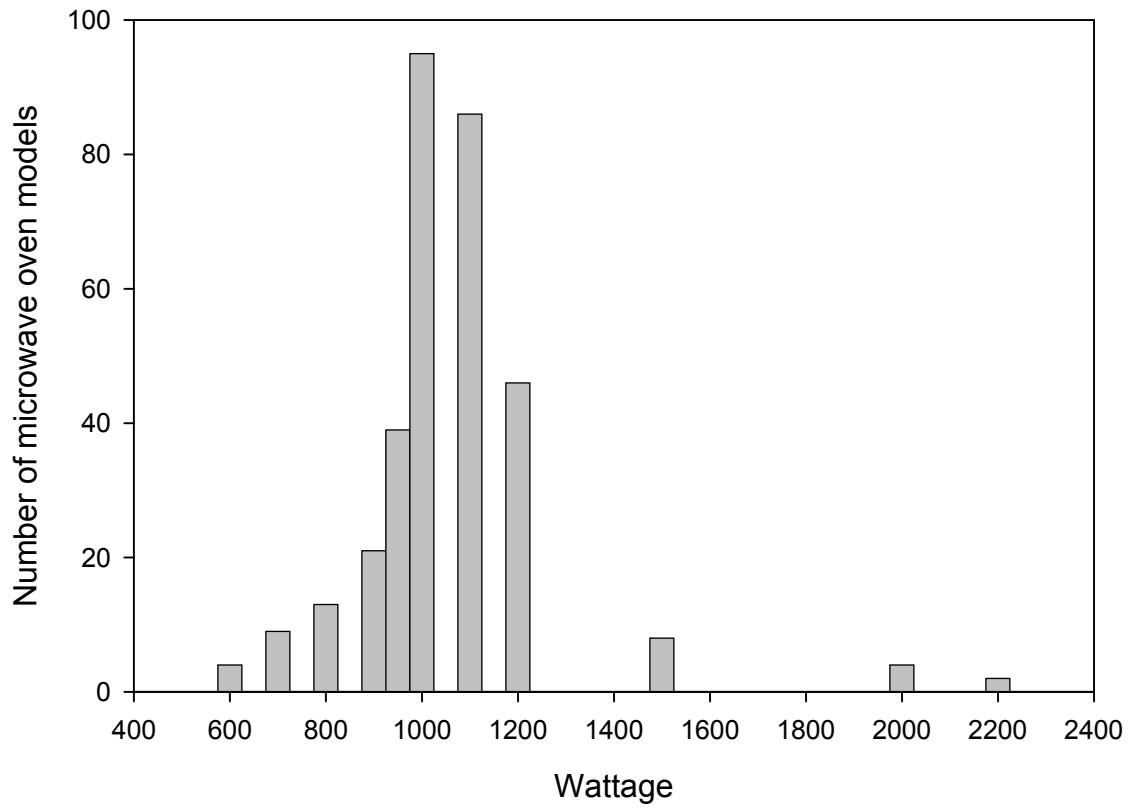Figure 5.2: Distribution of microwave oven models in the market according to their wattage

Figure 5.3: Experimental final microwave oven cooking temperatures at 500 Watts
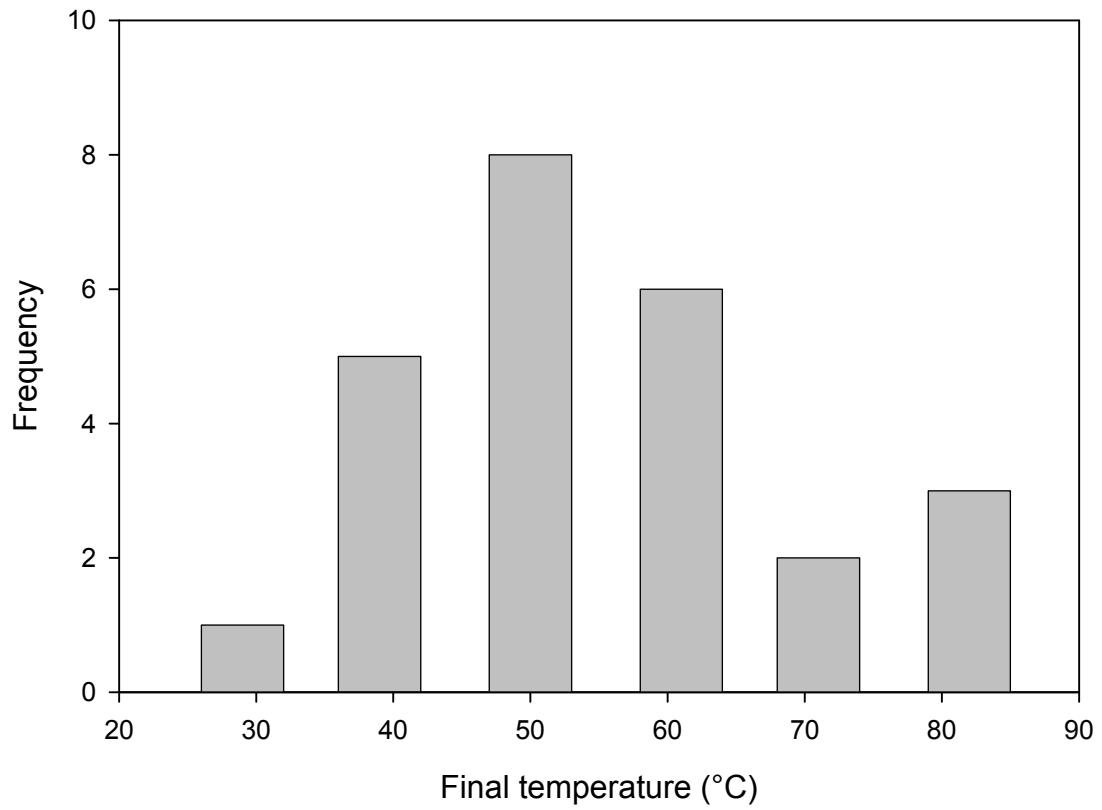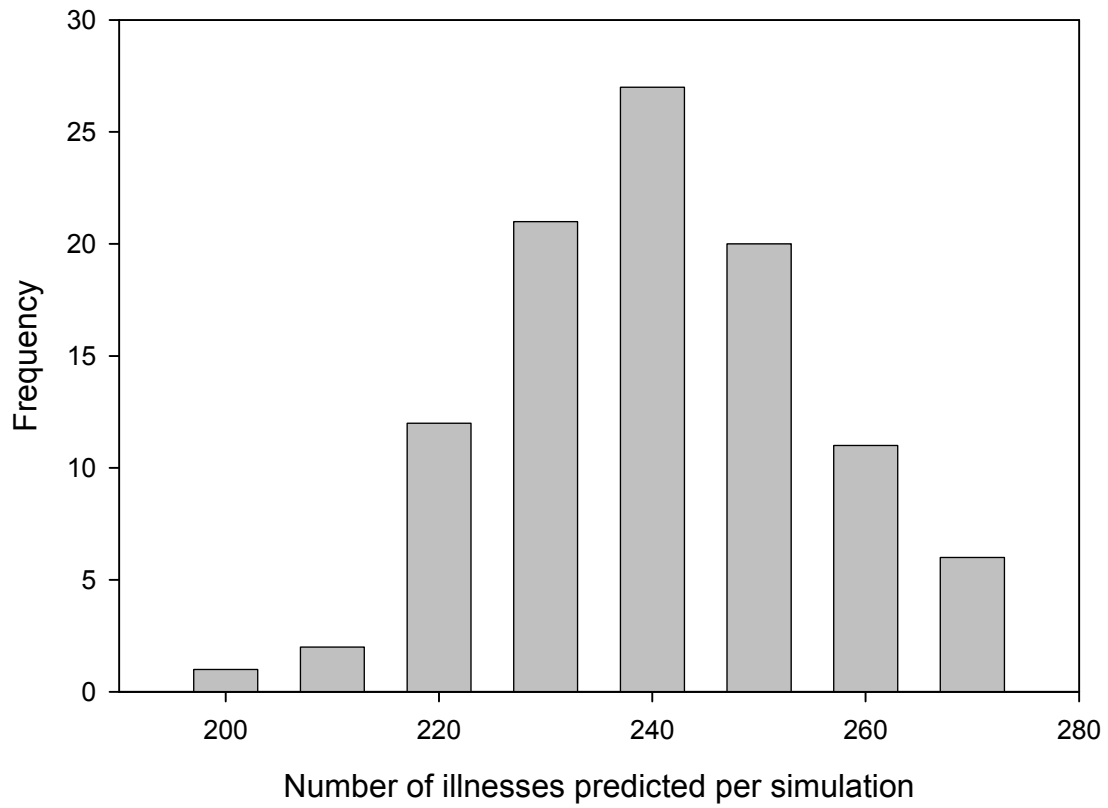
Figure 5.4: Number of illnesses predicted for 100 simulations of 2005 Minnesota outbreak

Overall conclusions

Different factors influencing the growth and survival of *Salmonella* on poultry products were studied in this dissertation. Through an analysis of relevant literature, we selected data on the growth of *Salmonella* related to meat and poultry products and used it to develop a predictive growth model. We demonstrated that when published data exist, their use for modeling purposes is a valid alternative to the generation of new data. The developed model was validated against experimental observations and can be safely used to predict the growth of *Salmonella* on raw poultry at temperatures 10-28°C.

We also investigated the effect of frozen storage (-20°C) on the survival of *Salmonella* inoculated into chicken products. The pathogen was able to survive these conditions during the 16 weeks of storage studied, with only structural injury as a consequence. The involvement of different outer membrane components in the survival of *Salmonella* to frozen storage was further investigated. Mutations in the lipopolysaccharide molecule or outer membrane proteins (ompC and ompD) deletions produced no significant changes in the survival of *Salmonella* to frozen storage. Previous studies had implicated lipopolysachharide in the pathogen's response to frozen storage under extreme conditions *(18)*, however this was not observed in our experiments. Thus, storage of chicken products at -20°C (average consumer freezer temperature) does not drastically impair the viability of *Salmonella*, if present. If selective media are used to recover *Salmonella* however, such methods may fail to detect low concentrations of the organism.

Microwave oven cooking of frozen chicken products at different wattages was another risk factor investigated in this dissertation. Results of experiments conducted at different wattages indicate that low-wattage ovens (i.e. 500 Watts) did not reach safe final cooking temperatures for adequate destruction of *Salmonella.* These results support the findings of epidemiological investigations of salmonellosis outbreaks linked to frozen chicken products, which identified microwave oven cooking as a potential consumer practice leading to infection *(76, 120).* We also constructed a quantitative microbial risk assessment for *Salmonella* in raw, frozen chicken products focusing on the use of low-wattage microwave ovens for cooking these products. A 2005 salmonellosis outbreak linked to frozen entrees was simulated using the proposed model, and the predicted number of reported cases (5-7) was in close agreement to the actual number (4). The proposed model may constitute a useful tool for evaluating a variety of scenarios, such as changes in pathogen concentration in the product or lethality of different microwave oven cooking instructions, among others.

Appendices

Appendix 1: International Electrotechnical Comission. Household microwave ovens -

Methods for measuring performance *(68)*

1. Microwave oven power output (wattage) measurement is made with the oven operated at rated voltage (120 vac) on high power with a load of $1000 \pm 5g$ water.

2. The water is contained in a cylindrical borosilicate glass container having a maximum material thickness of 3mm and an outside diameter of approximately 190mm.

3. The oven and empty container are at ambient temperature prior to starting the test. Refrigerate water over night to bring the temperature down before use. A quicker way is to cool water with ice, but make sure all the ice is dissolved because any ice chunks, no matter how small, will skew the results. The initial temperature of the water must be $10°C$ ($\pm 2°C$). The water temperature is measured immediately before it is added to the container. After the water is added to the container it is placed in the center of the oven.

4. The time (t) for the temperature of water to rise by a value of $10°C$ ($\pm 2°C$) is measured, where t is the time in seconds and ΔT is the actual temperature rise:

   $\Delta T = T_f - T_i$

   Where T_i = initial temperature $10°C$ ($\pm 2°C$), and T_f = actual temperature rise after microwave heating (between $16°C$ and $24°C$).

5. The microwave power output P is calculated from the following formula:

   $P = [4187 * (T_f - T_i)] / t$

References

1. Aleixo, J.A.G.**,** B. Swaminathan, and D.E. Pratt**.** 1985. Destruction of pathogenic bacteria in turkeys roasted in microwave ovens. *J. Food Sci.* 50**:**873

2. Alford, J.A. and J.D. Palumbo**.** 1969. Interaction of salt, pH, and temperature on the growth and survival of salmonellae in ground pork. *Appl. Microbiol.* 17**:**528-532.

3. Ames, G.F.L.**,** E.N. Spudich, and H. Nikaido**.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: Effect of lipopolysaccharide mutations. *J. Bacteriol.* 117**:**406-416.

4. Angelotti, R.**,** M.J. Foter, and K.H. Lewis**.** 1961. Time-temperature effects on salmonellae and staphylococci in foods. *Am J Public Health.* 51**:**76-88.

5. Anonymous. 1996. Nationwide raw ground chicken microbiological survey.USDA FSIS,

6. Anonymous.2007. ComBase - A combined database for predictive microbiology. Available at: http://www.combase.cc/.

7. Anonymous.2008. Minnesota Department of Health (MDH) Bug Bytes. Available at: http://www.health.state.mn.us/divs/idepc/newsletters/bugbytes/0807bb.html.

8. Anonymous.2008. *Salmonella* cases linked to raw, frozen chicken entrees; Consumers urged to cook raw chicken products thoroughly and to avoid using microwave. Available at: http://www.health.state.mn.us/news/pressrel/salmonella100308.html.

9. Anonymous.2009. Microwave Oven Facts. Available at: http://www.microwaveovenfacts.com/.

10. Archer, D.L. 2004. Freezing: an underutilized food safety technology? *Int. J. Food Micro.* 90**:**127-138.

11. Archer, G.P.**,** C.J. Kennedy, and A.J. Wilson**.** 1995. Position paper: towards predictive microbiology in frozen food systems - a framework for understanding microbial population dynamics in frozen structures and in freeze-thaw cycles. *Int. J. Food Sci. Technol.* 30**:**711-723.

12. Baker, R.C.**,** W. Poon, and V. Vadehra**.** 1983. Destruction of *Salmonella typhimurium* and *Staphylococcus aureus* in poultry products cooked in a conventional and microwave oven. *Poult. Sci.* 62**:**805-810.

13. Baranyi, J.**,** P.J. McClure**,** J.P. Sutherland, and T.A. Roberts**.** 1993. Modeling bacterial growth responses. *J. Indust. Microbiol.* 12**:**190-194.

14. Baranyi, J., C. Pin, and T. Ross. 1999. Validating and comparing predictive models. *Int. J. Food Micro.* 48:159-166.

15. Barrell, R.A.E. 1988. The survival and recovery of *Salmonella typhimurium* phage type U285 in frozen meats and tryptone soya yeast extract broth. *Int. J. Food Micro.* 6:309-316.

16. Bemrah, N., H. Bergis, C. Colmin, A. Beaufort, Y. Millemann, B. Dufour, J.J. Benet, O. Cerf, and M. Sanaa. 2002. Quantitative risk assessment of human salmonellosis from the consumption of a turkey product in collective catering establishments. *Int. J. Food Microbiol.* 80:17-30.

17. Bemrah, N., H. Bergis, C. Colmin, A. Beaufort, Y. Millemann, B. Dufour, J.J. Benet, O. Cerf, and M. Sanaa. 2002. Quantitative risk assessment of human salmonellosis from the consumption of a turkey product in collective catering establishments. *Int. J. Food Micro.* 80:17-30.

18. Bennett, G.M., A. Seaver, and P.H. Calcott. 1981. Effect of defined lipopolysaccharide core defects on resistance of *Salmonella typhimurium* to freezing and thawing and other stresses. *Applied and Environmental Microbiology.* 42:843-849.

19. Bennett, G.M., A. Seaver, and P.H. Calcott. 1981. Effect of defined lipopolysaccharide core defects on resistance of *Salmonella typhimurium* to freezing and thawing and other stresses. *Appl. Environ. Microbiol.* 42:843-849.

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

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

22. Brown,M.H. 1991. Microbiological aspects of frozen foods, p. 15-25. *In* W.B.Bald (ed.), Food freezing: Today and Tomorrow. Springer-Verlag, London.

23. Brown, M.H., K.W. Davies, C.M.P. Billon, C. Adair, and P.J. McClure. 1998. Quantitative microbiological risk assessment: principles applied to determining the comparative risk of salmonellosis from chicken products. *J. Food Protect.* 61:1446-1453.

24. Bucher, O., J.Y. D'Aoust, and R.A. Holley. 2008. Thermal resistance of *Salmonella* serovars isolated from raw, frozen chicken nuggets/strips, nugget meat and pelleted broiler feed. *Int. J. Food Micro.* 124:195-198.

25. Bucher, O., R.A. Holley, R. Ahmed, H. Tabor, C. Nadon, L.K. Ng, and J.Y. D'Aoust. 2007. Occurrence and characterization of *Salmonella* from Chicken nuggets, strips, and pelleted broiler feed. *J. Food Protect.* 70:2251-2258.

26. Calcott, P.H. and K.N. Calcott**.** 1984. Involvement of outer membrane proteins in freeze-thaw resistance of *Escherichia coli. Can. J. Microbiol.* 30**:**339-344.

27. Calcott, P.H. and R.A. MacLeod**.** 1975. The survival of *Escherichia coli* from freeze-thaw damage: permeability barrier damage and viability. *Can. J. Microbiol.* 21**:**1724-1732.

28. Castillejo-Rodriguez, A.M.**,** R.M.G. Gimeno**,** G.Z. Cosano**,** E.B. Alcala, and M.R.R. Perez**.** 2002. Assessment of mathematical models for predicting *Staphylococcus aureus* growth in cooked meat products. *J. Food Protect.* 65**:**659-665.

29. Chen, T.C.**,** J.T. Culotta, and W.S. Wang**.** 1973. Effects of water and microwave energy precooking on microbiological quality of chicken parts. *J. Food Sci.* 38**:**155-157.

30. Cody, S.H.**,** S.L. Abbott**,** A.A. Marfin**,** B. Schulz**,** P. Wagner**,** K. Robbins**,** J.C. Mohle-Boetani, and D.J. Vugia**.** 1999. Two outbreaks of multidrug-resistant Salmonella serotype typhimurium DT104 infections linked to raw-milk cheese in Northern California. *Jounnal of the American Medical Association.* 281**:**1805-1810.

31. Cui, S.**,** B. Ge**,** J. Zheng, and J. Meng**.** 2005. Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens from Maryland retail stores. *Appl. Environ. Microbiol.* 71**:**4108-4111.

32. Currie, A.**,** L. MacDougall**,** J. Aramini**,** C. Gaulin**,** R. Ahmed, and S. Isaacs**.** 2008. Frozen chicken nuggets and strips and eggs are leading risk factors for *Salmonella* Heidelberg infections in Canada. *Epidemiology and Infection.* 133**:**809-816.

33. D'Aoust,J.Y. and J.Maurer**.** 2007. Salmonella Species, p. 187-219. *In* M.P.Doyle and L.R.Beuchat (ed.), Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, DC.

34. D'Aoust,J.Y. and J.Maurer**.** 2007. Salmonella Species, p. 187-219. *In* M.P.Doyle and L.R.Beuchat (ed.), Food Microbiology: Fundamentals and Frontiers. ASM Press, Washington, DC.

35. Devlieghere, F.**,** A.H. Geeraerd**,** K.J. Versyck**,** H. Bernaert**,** J.F. Vanimpe, and J. Debevere**.** 2000. Shelf life of modified atmosphere packed cooked meat products: addition of Na-lactate as a fourth shelf life determinative factor in a model and product validation. *Int. J. Food Micro.* 58**:**93-106.

36. Dickson, J.S.**,** G.R. Siragusa, and J.E. Wray**.** 1992. Predicting the growth of *Salmonella typhimurium* on beef by using the temperature function integration technique. *Appl. Environ. Microbiol.* 58**:**3482-3487.

37. Dominguez,S. and D.W.Schaffner**.** 2009. Microbiological Quantitative Risk Assessment, p. 591-614. *In* F.Toldrá (ed.), Safety of Meat and Processed Meat. Springer, New York.

38. Dominguez,S. and D.W.Schaffner**.** 2009. Microbiological Quantitative Risk Assessment, p. 591-614. *In* F.Toldrá (ed.), Safety of Meat and Processed Meat. Springer, New York.

39. Dominguez, S.A. and D.W. Schaffner**.** 2007. Development and validation of a mathematical model to describe the growth of Pseudomonas spp. in raw poultry stored under aerobic conditions. *Int. J. Food Micro.* 120**:**287-295.

40. Dorman, C.J.**,** S. Chatfield**,** C.F. Higgins**,** C. Hayward, and G. Dougan**.** 1989. Characterization of porin adn ompR mutants of a virulent strain of Salmonella typhimurium: ompR mutants are attenuated in vivo. *Infection and Immunity.* 57**:**2136-2140.

41. Droffner, M.L. and N. Yamamoto**.** 1991. Procedure for isolation of *Escherichia, Salmonella*, and *Pseudomonas* mutants capable of growth at the refractory temperature of 54°C. *J. Microbiol. Method.* 14**:**201-206.

42. Dykes, G.A. and S.M. Moorhead**.** 2001. Survival of three *Salmonella* serotypes on beef trimmings during simulated commercial freezing and frozen storage. *J. Food Safety.* 21**:**87-96.

43. Dykes, G.A. and S.M. Moorhead**.** 2001. Survival of three *Salmonella* serotypes on beef trimmings during simulated commercial freezing and frozen storage. *J. Food Safety.* 21**:**87-96.

44. Edgar,R.H. and J.M.Osepchuk**.** 2001. Consumer, commercial and industrial microwave ovens and heating systems, p. 215-277. *In* A.K.Datta and R.C.Anantheswaran (ed.), Handbook of microwave technology for food applications. Marcel Dekker, Inc., New York.

45. Eglezos, S.**,** B. Huang, and E. Stuttard**.** 2007. A survey of the microbiological quality of kangaroo carcasses processed for human consumption in two processing plants in Queensland, Australia. *J. Food Protect.* 70**:**1249-1251.

46. El-Kest, S.E. and E.H. Marth**.** 1992. Freezing of *Listeria monocytogenes* and other microorganisms: a review. *J. Food Protect.* 55**:**639-648.

47. Escartin, E.F.**,** J.S. Lozano, and O.R. Garcia**.** 2000. Quantitative survival of native *Salmonella* serovars during storage of frozen raw pork. *Int. J. Food Micro.* 54**:**19-25.

48. FAO/WHO. 2002. Risk assessments of *Salmonella* in eggs and broiler chickens.

49. FAO/WHO. 2002. Risk assessments of *Salmonella* in eggs and broiler chickens.

50. Farrell, G.M. and M.E. Upton**.** 1978. The effect of low temperature on the growth and survival of *Staphylococcus aureus* and *Salmonella typhimurium* when inoculated on to bacon. *J. Food Technol.* 13**:**15-23.

51. Foster, R.D. and G.C. Mead**.** 1976. Effect of temperature and added polyphosphate on the survival of Salmonellae in poultry meat during cold storage. *J. Appl. Bacteriol.* 41**:**505-510.

52. Fruin, J.T. and L.S. Guthertz**.** 1982. Survival of bacteria in food cooked by microwave oven, conventional oven and slow cookers. *J. Food Protect.* 45**:**695-698.

53. FSIS**.** 1996. Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems; Proposed Rule. *Fed. Reg.* 60**:**6773-6889.

54. FSIS. 2006. Information on Validation of Labeled Cooking Instructions for Products Containing Raw or Partially Cooked Poultry.

55. FSIS. 2006. Information on Validation of Labeled Cooking Instructions for Products Containing Raw or Partially Cooked Poultry.

56. FSIS. 2006. Supplemental Q's and A's to address products affected by FSIS Notice 75-06 verification instructions for changes in label requirements for uncooked and raw, frozen, breaded boneless poultry products.FSIS,

57. Georgala, D.L. and A. Hurst**.** 1963. The survival of food poisoning bacteria in frozen foods. *J. Appl. Bacteriol.* 3**:**346-358.

58. Georgsson, F.**,** A.E. Thornorkelsson**,** M. Geirsdottir**,** J. Reiersen, and N.J. Stern**.** 2006. The influence of freezing and duration of storage on Campylobacter and indicator bacteria in broiler carcasses. *Food Microbiol.* 23**:**677-683.

59. Gibson, A.M.**,** N. Bratchell, and T.A. Roberts**.** 1988. Predicting microbial growth: growth responses of *Salmonellae* in a laboratory medium as affected by pH, sodium chloride and storage temperature. *Int. J. Food Micro.* 6**:**155-178.

60. Goepfert, J.M. and H.U. Kim**.** 1975. Behavior of selected food-borne pathogens in raw ground beef. *J. Milk Food Tech.* 38**:**449-452.

61. Hara-Kaonga, B. and T.G. Pistole**.** 2004. OmpD but not OmpC is involved in adherence of *Salmonella enterica* serovar Typhimurium to human cells. *Can. J. Microbiol.* 50**:**719-727.

62. Heddleson, R.A. and S. Doores**.** 1994. Factors affecting microwave heating of foods and microwave induced destruction of foodborne pathogens - a review. *J. Food Protect.* 57**:**1025-1037.

63. Heddleson, R.A. and S. Doores. 1994. Injury of *Salmonella* species heated by microwave energy. *J. Food Protect.* 57:1068-1073.

64. Heddleson, R.A., S. Doores, and R.C. Anantheswaran. 1994. Parameters affecting destruction of *Salmonella* spp. by microwave heating. *J. Food Sci.* 59:447-451.

65. Heddleson, R.A., S. Doores, R.C. Anantheswaran, and G.D. Kuhn. 1996. Viability loss of *Salmonella* species, *Staphylococcus aureus*, and *Listeria monocytogenes* in complex foods heated by microwave energy. *J. Food Protect.* 59:813-818.

66. Heddleson, R.A., S. Doores, R.C. Anantheswaran, G.D. Kuhn, and M.G. Mast. 1991. Survival of *Salmonella* species heated by microwave energy in a liquid menstruum containing food components. *J. Food Protect.* 54:637

67. Ikeme, A.I., B. Swaminathan, M.A. Cousin, and W.J. Stadelman. 1982. Extending the Shelf-Life of Chicken Broiler Meat. *Poult. Sci.* 61:2200-2207.

68. International Electrotechnical Commission. 2006. Household microwave ovens - Methods for measuring performance.

69. Juneja, V.K., M.V. Melendres, L. Huang, V. Gumudavelli, J. Subbiah, and H. Thippareddi. 2007. Modeling the effect of temperature on growth of *Salmonella* in chicken. *Food Microbiol.* 24:328-335.

70. Juneja, V.K., J.S. Novak, H.M. Marks, and D.E. Gombas. 2001. Growth of *Clostridium perfringens* from spore inocula in cooked cured beef; development of a predictive model. *Innov. Food Sci. Emerg. Technol.* 2:289-301.

71. Kenny, B., R. Hall, and S. Cameron. 1999. Consumer attitudes and behaviours - key risk factors in an outbreak of *Salmonella* Typhimurium phage type 12 infection sourced to chicken nuggets. *Australian and New Zealand Journal of Public Health.* 23:164-167.

72. Kenny, B., R. Hall, and S. Cameron. 1999. Consumer attitudes and behaviours - key risk factors in an outbreak of *Salmonella* Typhimurium phage type 12 infection sourced to chicken nuggets. *Australian and New Zealand Journal of Public Health.* 23:164-167.

73. Kuo, T.T. and B.A.D. Stocker. 1972. Mapping of rfa genes in *Salmonella typhimurium* by ES18 and P22 transduction and by conjugation. *J. Bacteriol.* 112:48-63.

74. Li, K.-Y. and J.A. Torres. 1993. A research note: water activity relationships for selected mesophiles and psychrotrophs at refrigeration temperature. *J. Food Protect.* 56:612-615.

75. Lynch, M., J. Painter, R. Woodruff, and C. Braden. 2006. Surveillance for Foodborne-Disease Outbreaks - United States, 1998-2002. *Morbid. Mortal. Week. Rep.* 55:1-48.

76. MacDougall, L., M. Fyfe, L. McIntyre, A. Paccagnella, K. Cordner, A. Kerr, and J. Aramini. 2004. Frozen chicken nuggets and strips - A newly identified risk factor for *Salmonella* Heidelberg infection in British Columbia, Canada. *J. Food Protect.* 67:1111-1115.

77. MacDougall, L., M. Fyfe, L. McIntyre, A. Paccagnella, K. Cordner, A. Kerr, and J. Aramini. 2004. Frozen chicken nuggets and strips - A newly identified risk factor for *Salmonella* Heidelberg infection in British Columbia, Canada. *Journal of Food Protection.* 67:1111-1115.

78. Mackey BM, Roberts TA, Mansfield J, and Farkas G. 1980. Growth of *Salmonella* on chilled meat. *J. Hyg.* 85:115-124.

79. Mackey, B.M. and A.L. Kerridge. 1988. The effect of incubation temperature and inoculum size on growth of salmonellae in minced beef. *Int. J. Food Micro.* 6:57-65.

80. MacLeod,R.A. and P.H.Calcott. 1976. Cold shock and freezing damage to microbes, p. 81-109. *In* T.R.G.Gray and J.R.Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge, UK.

81. MacLeod,R.A. and P.H.Calcott. 2007. Cold shock and freezing damage to microbes, p. 81-109. *In* T.R.G.Gray and J.R.Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge, UK.

82. Masschalck, B., D. Deckers, and C.W. Michiels. 2003. Sensitization of outer-membrane mutants of Salmonella typhimurium and Pseudomonas aeruginosa to antimicrobial peptides under high pressure. *J. Food Protect.* 66:1360-1367.

83. Matches, J.R. and J. Liston. 1968. Low temperature growth of *Salmonella. J. Food Sci.* 33:641-645.

84. McMeekin, T.A. 2007. Predictive microbiology: Quantitative science delivering quantifiable benefits to the meat industry and other food industries. *Meat Sci.* 77:17-27.

85. McMeekin, T.A., J. Brown, K. Krist, D. Miles, K. Neumeyer, D.S. Nichols, J. Olley, K. Presser, D.A. Ratkowsky, T. Ross, M. Salter, and S. Soontranon. 1997. Quantitative microbiology: a basis for food safety. *Emerg. Infect. Diseases.* 3:541-549.

86. Mead, P.S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, P.M. Griffin, and R.V. Tauxe. 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases.* 5:607-625.

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

88. Mellefont, L.A., T.A. McMeekin, and T. Ross. 2003. Performance evaluation of a model describing the effects of temperature, water activity, pH and lactic acid concentration on the growth of *Escherichia coli*. *Int. J. Food Micro.* 82:45-58.

89. Montville,T.J. and K.R.Matthews. 2004. Salmonella Species, p. 97-113. Food Microbiology: An Intoduction. ASM Press, Washington, DC.

90. Montville,T.J. and K.R.Matthews. 2004. Salmonella Species, p. 97-113. Food Microbiology: An Intoduction. ASM Press, Washington, DC.

91. Muermans, M.L.T., F.K. Stekelenburg, M.H. Zwietering, and J.H.J. Huis in't Veld. 1993. Modeling the microbiological quality of meat. *Food Control.* 4:216-221.

92. NACMCF. 2006. Response to the questions posed by FSIS regarding consumer guidelines for the safe cooking of poultry products.

93. NACMCF. 2006. Response to the questions posed by FSIS regarding consumer guidelines for the safe cooking of poultry products.

94. National Center for Zoonotic, V.-B.a.E.D.Z.2008. Salmonellosis. Available at: http://www.cdc.gov/nczved/dfbmd/disease_listing/salmonellosis_gi.html#8.

95. Neumeyer, K., T. Ross, and T.A. McMeekin. 1997. Development of a predictive model to describe the effects of temperature and water activity on the growth of spoilage pseudomonads. *Int. J. Food Micro.* 38:45-54.

96. Nikaido,H. and M.Vaara. 1987. Outer membrane, p. 7-19. *In* F.C.Neidhardt, J.L.Ingraham, K.B.Low, B.Magasanik, M.Schaechter, and H.E.Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology. American Society for Microbiology, Washington, D.C.

97. Obafemi, A. and R. Davies. 1986. The destruction of *Salmonella typhimurium* in chicken exudate by different freeze-thaw treatments. *J. Appl. Bacteriol.* 60:381-387.

98. Oscar, T.P. 1999. Response surface models for effects of temperature and previous growth sodium chloride on growth kinetics of *Salmonella typhimurium* on cooked chicken breast. *J. Food Protect.* 62:1470-1474.

99. Oscar, T.P. 1999. Response surface models for effects of temperature and previous temperature on lag time and specific growth rate of *Salmonella* Typhimurium on cooked ground chicken breast. *J. Food Protect.* 62:1111-1114.

100. Oscar, T.P. 1999. Response surface models for effects of temperature, pH, and previous growth pH on growth kinetics of *Salmonella typhimurium* in brain heart infusion broth. *J. Food Protect.* 62**:**106-111.

101. Oscar, T.P. 2002. Development and validation of a tertiary simulation model for predicting the potential growth of *Salmonella typhimurium* on cooked chicken. *Int. J. Food Micro.* 76**:**177-190.

102. Oscar, T.P. 2005. Development and validation of primary, secondary, and tertiary models for growth of *Salmonella* Typhimurium on sterile chicken. *J. Food Protect.* 68**:**2606-2613.

103. Oscar, T.P. 2006. Validation of a tertiary model for predicting variation of *Salmonella* Typhimurium DT104 (ATCC 700408) growth from a low initial density on ground chicken breast meat with a competitive microflora. *J. Food Protect.* 69**:**2048-2057.

104. Oscar, T.P. 2007. Predictive models for growth of *Salmonella typhimurium* DT104 from low and high initial density on ground chicken with a natural microflora. *Food Microbiol.* 24**:**640-651.

105. Pooni, G.S. and G.C. Mead**.** 1984. Prospective use of temperature function integration for predicting the shelf-life of non-frozen poultry meat products. *Food Microbiol.* 1**:**67-78.

106. Raj, H. and J. Liston**.** 1961. Survival of bacteria of public health significance in frozen sea foods. *Food Technol.*429-433.

107. Ratkowsky, D.A.**,** J. Olley**,** T.A. McMeekin, and A. Ball**.** 1982. Relationship between temperature and growth rate of bacterial cultures. *J. Appl. Bacteriol.* 149**:**1-5.

108. Ray, B. 1979. Methods to detect stressed microorganisms. *J. Food Protect.* 42**:**346-355.

109. Ray, B. and M.L. Speck**.** 1973. Freeze-injury in bacteria. *CRC Critical Reviews in Clinical Laboratory Sciences.*161-213.

110. Roantree, R.J.**,** T.T. Kuo, and D.G. MacPhee**.** 1977. The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium.* *J. Gen. Microbiol.* 103**:**223-234.

111. Rosenquist, H.**,** N.L. Nielsen**,** H.M. Sommer**,** B. Norrung, and B.B. Christensen**.** 2003. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. *Int. J. Food Micro.* 83**:**87-103.

112. Ross, T. 1996. Indices for performance evaluation of predictive models in food microbiology. *J. Appl. Bacteriol.* 81**:**501-508.

113. Ross,T. 1999. Predictive Microbiology for the Meat Industry.Meat and Livestock Australia, Sydney, Australia.

114. Santiviage, C.A., C.S. Toro, A.A. Hidalgo, P. Youderian, and G.C. Mora. 2003. Global regulation of the *Salmonella enterica* serovar Typhimurium major porin, OmpD. *J. Bacteriol.* 185:5901-5905.

115. Schaffner, D.W. 2004. Mathematical frameworks for modelling *Listeria* cross-contamination in food-processing plants. *J. Food Sci.* 69:R155-R159

116. Scott,J., L.Hontz, and Y.Chen. 2008. GUIDELINES FOR VALIDATION OF CONSUMER COOKING INSTRUCTIONS FOR NOT-READY-TO-EAT (NRTE) PRODUCTS.Grocery Manufacturers of America, Washington DC.

117. Silliker,J.H. and D.A.Gabis. 1986. *Salmonella*, p. 209-227. Advances in Meat Research, Vol. 2.

118. Sirisena, C.M., P.R. MacLachlan, S.L. Liu, A. Hessel, and K.E. Sanderson. 1994. Molecular analysis of the rfaD gene, for heptose synthesis, and the rfaF gene, for heptose transfer, in lipopolysaccharide synthesis in Salmonella typhimurium. *J. Bacteriol.* 176:2379-2385.

119. Smit, J., Y. Kamio, and H. Nikaido. 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. *J. Bacteriol.* 124:942-958.

120. Smith, K.E., C. Medus, S.D. Meyer, D.J. Boxrud, F. Leano, C. Hedberg, K. Elfering, C. Braymen, J.B. Bender, and R.N. Danila. 2008. Outbreaks of Salmonellosis in Minnesota (1998 through 2006) Associated with Frozen, Microwaveable, Breaded, Stuffed Chicken Products. *J. Food Protect.* 71:2153-2160.

121. Smith, M.G. 1985. The generation time, lag time, and minimum temperature of growth of coliform organisms on meat, and the implication for codes of practice in abattoirs. *J. Hyg. Camb.* 94:289-300.

122. Sorrells, K.M., M.L. Speck, and J.A. Warren. 1970. Pathogenicity of *Salmonella gallinarum* after metabolic injury by freezing. *Appl. Microbiol.* 19:39-43.

123. Speck, M.L. and B. Ray. 1977. Effects of freezing and storage on microorganisms in frozen foods: A review. *J. Food Protect.* 40:333-336.

124. Thomas, L.V. and J.W.T. Wimpenny. 1996. Competition between *Salmonella* and *Pseudomonas* species growing in and on agar, as affected by pH, sodium chloride concentration and temperature. *Int. J. Food Micro.* 29:361-370.

125. Tiwari, N.P. and R.B. Maxcy**.** 1972. Comparative growth of salmonellae, coliforms, and other members of the microflora of raw and radurized ground beef. *J. Milk Food Tech.* 35**:**455-460.

126. Unknown. 2007. NACMCF Struggles with Safe Seafood Cooking Advice . Food Prot. Rep. 23(7/8)**:**3.

127. USDA-FSIS**.** 2007. Title 9 - Animals and Animal Products Chapter III - Food Safety and Inspection Service, Department of Agriculture Part 38 - Poultry Products Inspection Regulations - Subpart I - Operating Procedures. *Code of Federal Regulations.* 9**:**451-452.

128. Vankerschaver, K.**,** F. Willocx**,** C. Smout**,** M. Hendrickx, and P. Tobback**.** 1996. The influence of temperature and gas mixtures on the growth of the intrinsic micro-organisms on cut endive: Predictive versus actual growth. *Food Microbiol.* 13**:**427-440.

129. Wei, Q.K.**,** T.J. Fang, and W.C. Chen**.** 2001. Development and validation of growth model for *Yersinia enterocolitica* in cooked chicken meats packaged under various atmosphere packaging and stored at different temperatures. *J. Food Protect.* 64**:**987-993.

130. Whiting, R.C. 1997. Microbial database building: What have we learned? *Food Technol.* 51**:**82

131. Wijtzes, T.**,** P.J. McClure**,** M.H. Zwietering, and T.A. Roberts**.** 1993. Modeling bacterial growth of *Listeria monocytogenes* as a function of water activity, pH and temperature. *Int. J. Food Micro.* 18**:**139-149.

132. Zhang,H. and A.K.Datta**.** 2001. Electromagnetics of microwave heating: Magnitude and uniformity of energy absorption in an oven, p. 33-67. *In* A.K.Datta and R.C.Anantheswaran (ed.), Handbook of microwave technology for food applications. Marcel Dekker, New York.

Curriculum vita

Silvia A. Dominguez Risco

1997 - 2001	Universidad Nacional Agraria La Molina, Lima, Peru
	B.S., Food Industries Engineering
2002	Universidad Nacional Agraria La Molina, Lima, Peru
	Agribusiness Diploma
2004 - 2007	Rutgers, The State University of New Jersey, New Brunswick, NJ
	M.S., Food Science
2007 - 2009	Rutgers, The State University of New Jersey, New Brunswick, NJ
	Ph.D., Food Science
2004 -2009	Rutgers, The State University of New Jersey, New Brunswick, NJ
	Department of Food Science
	Graduate Assistant

- Dominguez S.A. and Schaffner D.W. 2009. Chapter 26 – "Microbiological quantitative risk assessment", in book: *Safety of Meat and Processed Meat*, F. Toldra, editor. Food Microbiology and Food Safety Series, Springer, New York, USA
- Dominguez S.A. and Schaffner D.W. 2008. "Modeling the growth of *Salmonella* in raw poultry stored under aerobic conditions" *Journal of Food Protection* 71 (12): 2429-2435
- Dominguez S.A. and Schaffner D.W. 2007. "Development and validation of a mathematical model to describe the growth of *Pseudomonas* spp. in raw poultry stored under aerobic conditions" *International Journal of Food Microbiology* 12 (3): 287-295