QUANTIFYING SOURCES OF ERROR IN *SALMONELLA* THERMAL INACTIVATION MODELS FOR MEATS AND LOW-MOISTURE FOODS

By

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ABSTRACT

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Despite an incomplete understanding of the impact of experimental methodologies on resulting *Salmonella* thermal inactivation models, thermal resistance data and parameters continue to be generated, reported, and presumably applied to food safety analyses. Therefore, the objectives were to: (1) evaluate the impacts of varied experimental methods on the observed thermal resistance of *Salmonella*, (2) investigate the effects of substantively similar thermal inactivation methods on the quantification of *Salmonella* thermal resistance, and (3) investigate the effects of regression methods on the estimation of *Salmonella* thermal resistance parameters and their associated errors. These objectives were accomplished with two cross-laboratory comparison studies. The first study evaluated the effects of two *Salmonella* inactivation methods in ground beef on the resultant inactivation kinetics, based on data generated by two different laboratories. The two methods used yielded characteristically different *Salmonella* inactivation kinetics, regardless of the laboratory. The second study investigated the effects of five different inoculation methods on the subsequent stability and thermal resistance of *Salmonella* in wheat flour, and the repeatability of those results, based on data generated by two different laboratories. These methods yielded significantly different *Salmonella* thermal resistances, and only two yielded repeatable initial *Salmonella* populations and subsequent thermal resistances. Overall, thermal inactivation methods significantly impacted *Salmonella* thermal resistance in both meat and low-moisture food matrices.

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1. INTRODUCTION

1.1 Foodborne Disease and Regulations

Salmonella continues to be a public health concern for the U.S., with 52 food recalls from 2009-2014 (CDC 2015a). *Salmonella* is commonly implicated in foodborne disease outbreaks associated with meat and poultry products; however, recent outbreaks connected to peanut butter and nut products have increased awareness of potential *Salmonella* contamination of low-moisture foods. The potential impact of an outbreak from low-moisture foods is substantial, because many products are commonly used as ingredients in ready-to-eat (RTE) foods (such as nuts, flours, food powders, and pastes), which can increase the number of products implicated in outbreaks and/or recalls. Due to the frequency of outbreaks and severity of illness, nontyphoidal *Salmonella* has the largest economic burden (~$4.5 billion annually) compared to other foodborne diseases (Scharff 2012). Overall, the estimated annual health-related cost of foodborne illness in the U.S. is $51 billion; however, there are additional unquantified costs of foodborne illness for the food industry (e.g., recalls, development/application of safety protocols, public image) and public health agencies (e.g., inspections, enforcement) (Scharff 2012).

In 2011, the Food Safety Modernization Act (FMSA) was signed into law, with the intention to shift the focus of food safety regulation from reactive to preventative. FSMA has introduced mandatory science-based preventive controls for food facilities in order to minimize or prevent hazards (FDA 2014). While science-based food safety processing procedures were previously required for the reduction of *Salmonella* in RTE meats and poultry (USDA-FSIS 2001), similar standards were not required for low-moisture foods. The Almond Board of California has published guidelines for the development of science-based preventive controls

(Almond Board of California 2015); however, the behavior of *Salmonella* is insufficiently understood to establish preventive controls for most low-moisture foods.

1.2 Microbial Inactivation Kinetics – Development and Utilization

The scientific foundation for understanding *Salmonella* survival and inactivation kinetics often is developed in private or academic laboratories. Numerous independent laboratories have assessed the survival (e.g., Beuchat and Mann 2010; Burnett and others 2000; Danyluk and others 2005; Farakos and others 2014b; Jung and Beuchat 1999; Keller and others 2013; Kotzekidou 1998; Uesugi and others 2006) and inactivation (e.g., Archer and others 1998; Blackburn and others 1997; Du and others 2010; He and others 2013; Juneja and others 2001b; Li and others 2014; Murphy and others 1999; Murphy and others 2001; Stasiewicz and others 2008; Tuntivanich and others 2008; Veeramuthu and others 1998; Wesche and others 2005; Yohan and others 2009) of *Salmonella*, and a few have submitted their data to the Combined Database for Predictive Microbiology (ComBase 2014). *Salmonella* thermal inactivation studies are a significant portion of scientific literature because of the prevalence of thermal lethality processes in the food industry used for quality or safety controls.

Though not complete, previous research has investigated the effects of temperature, fat content, additives, water activity (a_w), serovar selection, stress adaptation, and product structure on *Salmonella* thermal resistance in meat and poultry products (e.g., Breslin and others 2014; Carlson and others 2005; Chambliss and others 2006; Juneja and Eblen 2000; Juneja and others 2001b; Juneja and others 2013; Mogollon and others 2009; Wesche and others 2005; Manios and Skandamis 2015; Murphy and others 2004a; Osaili and others 2013; Velasquez and others 2010; Schlisselberg and others 2013; Vasan and others 2014; Veeramuthu and others 1998; Yohan and

others 2009; Zhao and others 2014). The effects of the aforementioned factors on *Salmonella* thermal resistance are less understood in low-moisture foods (e.g., Archer and others 1998; Jeong and others 2009; Li and others 2014; Ma and others 2009; Villa-Rojas and others 2013). Despite the depth of research investigating thermal inactivation kinetics of *Salmonella*, discrepancies exist between the reported thermal resistances for *Salmonella* in similar food products, even with substantively identical experimental treatments, suggesting there are influences, outside of the explicit experimental treatments, that are not understood, expressed, or reported as contributing to the resultant thermal resistance.

The impact of methods used to generate *Salmonella* inactivation data, and subsequent lethality models, are incompletely understood. No prior study is known to have reported a direct comparison of *Salmonella* thermal inactivation across methods or across laboratories. Studies examining the effect of inactivation methods on other microorganisms often report substantial differences between methods (Basaran-Akgul 2013; Donnelly and others 1987; Fujikawa and others 2000; Zimmermann and others 2013). However, errors associated with laboratory methods and lethality models are under-reported.

For example, in a group of 23 different studies investigating the thermal inactivation of *Salmonella* in low-moisture foods (collected by the author of this thesis), only 5 reported a model selection process, 2 reported model error (RMSE, etc.), 15 reported parameter error, and 11 reported replication error. None of the 23 studies reported all four measures of error. The differences between thermal inactivation methods, coupled with unknown replication error and artifacts, reduce the potential to compare results across studies and synthesize broader applications (e.g., meta-analyses, review articles, and industrial applications). For example, when van Asselt and Zwietering (2006) aggregated thermal resistance parameters from 20 studies

investigating *Salmonella* spp., the variations between studies were larger than many of the measured differences in experimental treatments (van Asselt and Zwietering 2006). The results of their analysis suggest that variations in laboratory methods have a noticeable but unquantified effect on thermal resistance determination. Overall, the effects of inactivation methods on *Salmonella* thermal resistance are generally unquantified or underreported, decreasing the utility of the results and lethality models.

Reliable and repeatable thermal lethality processes are essential for the RTE food industry to provide a safe product to the consumers. Because lethality models often focus on describing the effects of a single variable, a model-based process validation requires the utilization of findings from multiple lethality studies independently developed to separately describe the effects of product composition, product structure, and/or other treatment variations. As a result, the ad hoc prediction is accompanied by an unknown amount of model error and may not accurately predict the true inactivation of *Salmonella* in the modeled environment.

Unfortunately, very few studies have validated lethality models across similar products or in scaled-up processes (e.g., Breslin and others 2014; Farakos and others 2013; Jeong and others 2009); however, such studies generally report larger replication errors and model biases when applied to scaled-up processes. While larger replication error can result from larger variations in the scaled-up process, as opposed to a laboratory experiment, the model biases also could be the result of method artifacts that influenced thermal inactivation in laboratory studies differently than in the validation experiment. When the methods used to determine thermal inactivation kinetics of *Salmonella* are incompletely understood, the application of resulting models in the RTE food industry are adversely affected. Additional research is needed to characterize the

effects of experimental methods on inactivation kinetics, in order to develop a better foundation from which prior research can be compared and future research can be coordinated.

1.3 Goal and Objectives

The overall goal of this thesis project was to quantify the effects of different thermal inactivation methods on the resulting inactivation kinetics. The specific objectives were to:

(1) Evaluate the impacts of varied experimental methodologies on the observed thermal resistance of *Salmonella*.

(2) Investigate the effects of substantively similar thermal inactivation methodologies, applied in independent laboratories, on the quantification of *Salmonella* thermal resistance.

(3) Investigate the effects of regression methodology on the estimation of *Salmonella* thermal resistance parameters and their associated errors.

2. LITERATURE REVIEW

Salmonella inactivation is impacted by many factors including the organism, substrate, and treatment. However, given the pressing need to better understand thermal inactivation of *Salmonella* in meats and low-moisture foods, this literature review focuses primarily on prior studies and methodologies within those specific domains.

2.1 Introduction

Salmonella is among the leading causes of foodborne illness in the U.S. (Scharff 2012), and is estimated to impose the largest economic burden (Scharff 2012). *Salmonella* is a Gram-negative bacterium that, if a sufficient dose is consumed, can cause salmonellosis. Symptoms of salmonellosis, which can occur up to 72 h after ingestion, include nausea, vomiting, diarrhea, cramps, and fever (CDC 2015b). Symptoms can persist up to 4-7 days and can even result in hospitalization, chronic reactive arthritis, or death.

Salmonella outbreaks are traditionally associated with poultry and egg products; however, other foods have also been implicated, including meats, fruits, vegetables, and low-moisture foods (CDC 2015a). Salmonellosis from meat and egg products often is associated with the consumption of a raw or undercooked product. Despite limited ability to reproduce at low a_w (~0.97 a_w), *Salmonella* is capable of surviving for long periods in low-moisture foods having a $a_w < 0.60$ (Burnett and others 2000; Kimber and others 2012; Uesugi and others 2006). Since low-moisture foods are often used as ingredients (e.g., spices, flours, nuts) or retailed as ready-to-eat products (e.g., nuts, dates, seeds), the potential impact of an outbreak can include a wide range of related recalls.

The USDA and FDA have developed a series of guidelines and regulations for the food industry to enhance end-product safety. High risk RTE foods, including meats, poultry, and more recently low-moisture foods (FDA 2014; USDA-FSIS 2001), require a prevention or lethality process based on valid science-based controls. As a result, the demand for science-based preventative controls has driven research to investigate *Salmonella* survival and inactivation kinetics in these foods. Because thermal treatments (e.g., ovens, roasters, fryers, etc.) are commonly used in the food industry, a fairly large body of research has been devoted to quantifying the thermal resistance of *Salmonella* in various food matrices.

2.2 Quantification of Thermal Resistance

2.2.1 Laboratory-scale experiments

Standard methods for isolation and identification of *Salmonella* spp. are outlined in the Bacteriological Analytical Manual (BAM), which is made available online by the FDA (Maturin and Peeler 2001); however, there are no standard methods for quantification of *Salmonella* thermal resistance. As a result, thermal inactivation methods are non-uniform between laboratories and over time. Among these methods, different procedures are often used for cultivation, inoculation, thermal treatment, and enumeration of survivors (e.g., Abd and others 2012; Gurtler and Kornacki 2009; Harris and others 2012; Juneja and others 2001b; Kang and Fung 2000; Orta-Ramirez and others 2005; Porto-Fett and others 2009; Tenorio-Bernal and others 201X ; Wesche and others 2005).

As expected, experimental methods for the quantification of *Salmonella* thermal resistance in meats are commonly quite different from those in low-moisture foods, mainly because of product limitations and the *Salmonella* strains of interest (Chung and others 2007;

Chung and others 2008; Keller and others 2012; Uesugi and Harris 2006). For example, experiments involving ground meats often entail the addition of a liquid inoculum containing multiple *Salmonella* serovars, while experiments involving almond kernels often entail dip inoculation of the product with a single *Salmonella* serovar followed by drying (e.g., Du and others 2010; Juneja and Eblen 2000; Juneja and others 2001b; Lee and others 2006; Orta-Ramirez and others 2005; Wesche and others 2005).

Ideally, inactivation experiments are designed to mimic realistic contamination conditions; however, in practice, experiments typically are designed to evaluate the effects of treatment variables on products artificially contaminated with *Salmonella*. Invariably, the *Salmonella* inoculation level will exceed levels found with natural contamination events (Lambertini and others 2012). It is assumed that the initial *Salmonella* population concentration does not affect the thermal inactivation kinetics; however, data collection near the limit of detection may significantly affect the resulting inactivation parameters (Garces-Vega and Marks 2014).

In practice, the *Salmonella* culturing and inoculation methods used are variable. Drip inoculation of broth-cultivated *Salmonella* is predominantly used for ground meats (e.g., Juneja and Eblen 2000; Juneja and others 2013; Manios and Skandamis 2015; Mogollon and others 2009; Murphy and others 2001; Murphy and others 2004b; Orta-Ramirez and others 2005; Takhar and others 2009; Tuntivanich and others 2008). Inoculation methods for low-moisture foods are more varied, incorporating both broth- and agar-based cultures (e.g., Blessington and others 2013; Farakos and others 2014b; Ma and others 2009; Uesugi and others 2006; Villa-Rojas and others 2013). Growth conditions where the *Salmonella* culture is exposed to sublethal injury increases the subsequent thermal resistance of the culture (Alvarez-Ordonez and others

2010; Wesche and others 2005); however, the effect of non-selective growth media on the subsequent thermal resistance of *Salmonella* has not been widely reported. Keller et al. (2012) reported a difference in *Salmonella* stability and thermal inactivation kinetics in peanut butter between broth- and agar-cultivated cultures (Keller and others 2012). Blessington and others (2013) reported negligible difference in culture stability between dip and dry inoculation; however, thermal resistance was not quantified. Overall, the effect of different inoculation methods on *Salmonella* thermal resistance are incompletely understood or generally underreported.

Thermal treatments for laboratory-scale experiments often are classified as either isothermal or non-isothermal (dynamic)(e.g., Huang 2009; Ma and others 2009; Mattick and others 2001b; Mogollon and others 2009). Some thermal inactivation experiments quantify thermal resistance as the inactivation resulting from a single dynamic process (e.g., Dierschke and others 2010; Harper and others 2009). Isothermal experiments are more common and allow for easy sampling during the treatment duration; however, these experiments cannot be directly translated to an industrial process without the use of predictive lethality models. In addition, isothermal treatments have to overcome temperature-come-up-time uncertainty, which is dependent on the inactivation vessel material and geometry (Chung and others 2007). Dynamic temperature experiments are more data intensive, requiring temperature and survival profiles. (Dolan 2003; Peleg and Normand 2010). Experiments comparing the results for *Listeria* inactivation during isothermal and dynamic temperature profiles reported significant differences in thermal resistance and model selection (Huang 2009); however, no prior studies are known to have explicitly examined the impact of temperature profile on model selection for *Salmonella* inactivation.

Following thermal treatment, enumeration methods quantify the surviving *Salmonella* population. Direct plating is the most common *Salmonella* enumeration method (e.g., Gurtler and Kornacki 2009; Kang and Fung 2000; Leguerinel and others 2007; Wesche and others 2005), though most probable number (MPN) methods are a possible alternative. Trypticase soy agar (TSA) is frequently used in thermal inactivation experiments (e.g., Gurtler and Kornacki 2009; Jeong and others 2009; Juneja and others 2012); however, TSA often is supplemented with recovery promoters, which are non-uniform across laboratories and with varying effectiveness (Gurtler and Kornacki 2009). TSA can be supplemented with an anti-microbial compound to yield a selective media (Jordan and others 2011). Comparing enumerated *Salmonella* survivors on TSA to enumerated survivors on a selective medium, such as xylose lysine decarboxylase (XLD), yields an estimate for the portion of the population that is injured (e.g., Kang and Fung 2000; Lee and others 2006; Wesche and others 2005).

2.2.2 Pilot-scale experiments

Pilot-scale experiments incorporate the use of processing equipment that approximates industrial-scale equipment and focuses primarily on the impact of process conditions on *Salmonella* inactivation. Most pilot-scale inactivation studies either evaluate an existing lethality model (e.g., Breslin and others 2014; Jeong and others 2009; Tenorio-Bernal and others 201X) or an existing process where laboratory-scale experiments are impractical (e.g., Buege and others 2006; Harper and others 2009). Bacteriological methods used in pilot-scale studies are similar to methods utilized in laboratory-scale experiments, with the exception of sample size and thermal treatment. Because process and environmental conditions are difficult to control, pilot-scale

studies require continual and active monitoring of process conditions (e.g., temperature, humidity, air speed, a_w).

Reported results from pilot-scale studies often identify deficiencies in models generated in laboratory-scale experiments. For example, traditional lethality models for laboratory-scale experiments may not reflect the impact of process humidity on *Salmonella* inactivation on almonds (Jeong and others 2009) or the temperature path-dependency of *Salmonella* inactivation in meat products (Breslin and others 2014; Tenorio-Bernal and others 2013). In addition, the root mean square error (RMSE) for lethality models reported from a laboratory-scale experiments often are larger when validated with data from pilot-scale experiments (Breslin and others 2014; Juneja and others 2001b; Tenorio-Bernal and others 2013). Pilot-scale experiments are capable of connecting laboratory developed scientific principles to industry applications; however, products associated with *Salmonella* outbreaks are severely underrepresented in the literature from pilot-scale experiments.

2.2.3 *Validation of processes by surrogates*

The Grocery Manufacturers Alliance (GMA) identified two different validation approaches for preventative control validations: (1) a challenge study using appropriate *Salmonella* strains or surrogates with known thermal resistances or (2) measurement of physical characteristics of the process to be incorporated into an appropriate predictive model (GMA 2015). Regardless of how industry approaches process validation, sufficient laboratory- and pilot-scale experiments are required to develop a scientific foundation for reliable process validation. Potential surrogates for *Salmonella* include several nonpathogenic bacteria and chemical indicators (Bianchini and others 2014; Borowski and others 2009; Harris and others

2012; Jeong and others 2011; Maurer 2001). Careful consideration is required when selecting a surrogate to validate a process. Surrogates should be non-hazardous and exhibit inactivation characteristics that are statistically comparable to, or more resistant than, the pathogen of concern (e.g., *Salmonella*). *Enterococcus faecium* has been identified as a potential surrogate for *Salmonella* in meats and low-moisture foods (Bianchini and others 2014; Harris and others 2012; Jeong and others 2011; Ma and others 2007); however, the efficacy of *Enterococcus faecium* as a surrogate is influenced by the product and process (Jeong and others 2011; Ma and others 2007).

2.3 Model Development and Evaluation

Several monographs have extensively covered the field of predictive microbiology (Brul and others 2007; McKellar and Lu 2003a; McMeekin 1993; Peleg 2006). The following section will focus on topics and resources specific to model development and evaluation for *Salmonella* thermal inactivation.

2.3.1 Primary

Salmonella thermal resistance is typically reported as a model parameter, though some studies quantify thermal resistance as lethality due to a controlled process (Ha and others 2013; Harper and others 2009). Primary inactivation models provide an empirical approximation of *Salmonella* population as a function of time, given constant conditions (McKellar and Lu 2003b; Whiting 1995).

Log-linear inactivation kinetics of *Salmonella* during isothermal treatment has been previously reported (e.g., Blackburn and others 1997; Juneja and others 2001b; Ma and others 2007; Ma and others 2009). As a result, the log-linear model is commonly fit to data from

isothermal inactivation experiments and is commonly used in the following equation to predict microbial reduction during thermal processes.

$$\mathrm{Log}\left(N/N_o\right) = -t/D_T \quad (1)$$

where t is the time of the isothermal treatment at temperature T, D_T is the time required for a 1 log reduction of the population during isothermal treatment at T, and N and N_o are the populations (e.g., CFU/g) at times t and 0, respectively. The D_T-value often is reported as the measure of *Salmonella* thermal resistance.

Non-log-linear inactivation kinetics have also been described in numerous thermal inactivation studies (Peleg and Cole 1998). The most common inactivation model fitted to non-log-linear data is the Weibull model:

$$\mathrm{Log}\left(N/N_o\right) = -\left(t/\delta_T\right)^p \quad (2)$$

where t is time of the isothermal treatment at temperature T, δ_T represents the steepness of the inactivation curve during isothermal treatment at T, p (unitless) is the shape factor, and N and N_o are the populations (e.g., CFU/g) at times t and 0, respectively (Peleg and Cole 1998). Depending on the shape factor value, the Weibull model can be used to describe log-linear inactivation ($p=1$), tailing ($0<p<1$), or shouldering ($p>1$).

Other primary models are utilized to a lesser extent (Li and others 2007); however, models incorporating more than four parameters in the primary model prevent easy application to thermal treatment processes and have limited prediction power (Mafart and others 2002).

2.3.2 Secondary

Primary models are limited to describing inactivation kinetics as a function of time and require secondary models to describe the effects of environmental factors (e.g., temperature, a_w, pH) on the primary model parameters (Ross and Dalgaard 2003; Whiting 1995). The utility of the secondary model often depends on the modified parameter within the primary model and independent variables included within the secondary model; however, three common secondary model forms include response surface-type, Arrhenius-type, and Bigelow-type models.

Response surface secondary models are a purely empirical model where the modified primary model parameter is described by a polynomial with interaction terms. For example, a second-order response surface model with an interaction describing the log-linear model D-value as a function of temperature (T) and pH would be:

$$D = \beta_o + \beta_1 \cdot T + \beta_2 \cdot T^2 + \beta_3 \cdot pH + \beta_4 \cdot pH^2 + \beta_5 \cdot T \cdot pH \quad (3)$$

where coefficients β_i represent the impact of each variable or interaction. The response surface secondary model can be reduced with significance testing on the β parameters (Valdramidis and others 2006).

Arrhenius-type models describe the primary model parameter as an exponentiation of a polynomial relationship of the secondary model parameters. For example, Cerf et al. (1996) reported a Arrhenius-type secondary model represented as:

$$\ln(D) = C_o + {}^{C_1}/_{T} + C_3 \cdot pH + C_3 \cdot pH^2 + C_3 \cdot {a_w}^2 \quad (4)$$

where parameter coefficients C_i describe the effects of temperature (T), pH, and a_w on the decimal reduction time (D) (Cerf and others 1996). Farakos et al. (2013) used an Arrhenius-type

models to describe the effects of temperature and water activity on Weibull parameters δ and p (Farakos and others 2013).

The Bigelow-type model is most commonly used to describe the effect of temperature on the D-value within the log-linear model (Dolan and Mishra 2013). The Bigelow-type model accounting for only the temperature effect on the D-value is:

$$D_T = D_{ref} \times 10^{\left(\frac{T_{ref}-T}{Z_T}\right)} \quad (5)$$

where D_T (s) is the decimal reduction time at temperature T, Z_T is the difference in temperature required for a log change of D_T, and D_{ref} is the decimal reduction time at reference temperature T_{ref}. The Bigelow-type model can be expanded to include other factors, such as processing conditions, pH, and a_w (Jeong and others 2009; Valdramidis and others 2006).

2.3.3 Model regression and selection

Model regression methods can be categorized as multiple-step or global. Global regressions estimate all parameters in the complete inactivation model (including primary and secondary models) with one regression (Jewell 2012; Martino and Marks 2007; Motulsky and Christopoulos 2004). Multi-step regressions require estimation of primary model parameters in order to subsequently estimate secondary model parameters (Jewell 2012; Martino and Marks 2007). Inactivation studies utilizing dynamic temperature profiles require a global regression to estimate model parameters (Mattick and others 2001b; Peleg and Normand 2010). Despite several studies reporting that multi-step regressions produce inferior prediction models (Jewell 2012; Martino and Marks 2007), most studies estimating primary and secondary model

parameters utilize multi-step regressions (e.g., Blackburn and others 1997; Farakos and others 2013; Juneja and others 2003; Smith and others 2001).

Model selection statistics often are not utilized or reported in thermal inactivation studies. In a group of 23 studies (analyzed by the author of this thesis) investigating thermal inactivation of *Salmonella* in low-moisture foods, only 5 studies reported a model selection process. Model selection criteria include comparing adjusted coefficients of determination (R^2_{adj}) (eqn. 6), bias and accuracy factors (Baranyi and others 1999), the Akaike Information Criterion (AIC) (eqn. 7) (Motulsky and Christopoulos 2004), or using preliminary data testing (van Boekel 2002).

$$R^2{}_{adj} = 1 - \frac{\sum\left(\log(N)_{predicted} - \log(N)_{observed}\right)^2}{\sum\left(\log(N)_{predicted} - \log(N)_{average}\right)^2} \cdot \frac{n - p - 1}{n - 1} \tag{6}$$

$$AIC = n\,log\left(\frac{\sum\left(log\left(N/N_o\right)_{predicted} - log\left(N/N_o\right)_{observed}\right)^2}{n}\right) + 2K \tag{7}$$

In eqns. 6 and 7, p represents the number of model parameters, K is the number of parameters plus 1, n is the number of observations, and N and N_o are the populations at times t and 0, respectively. Despite being commonly reported, R^2_{adj} is considered inappropriate as a model selection criteria for non-linear models (Ratkowsky 2003).

2.3.4 Validation of models

Model validation studies should be designed to quantify the performance of models, in terms of key statistical performance criteria. In most prior research, validation work has evaluated model performance in the same study in which the model was developed or reported (e.g., Farakos and others 2013; Jeong and others 2009; Tenorio-Bernal and others 2013); few

studies have examined the accuracy of models reported in prior literature (e.g., Tenorio-Bernal and others 201X). Currently, no reports are known where an entire inactivation study was duplicated in a separate independent laboratory for the purpose of quantifying cross-laboratory errors and/or model validity.

Additionally, because laboratory methods and experimental treatments change over time, results from a particular study rarely can be directly validated against another study conducted in a different time or place. For example, USDA-ARS ERRC (United States Department of Agriculture-Agricultural Research Service Eastern Regional Research Center) studies published between 2000-2013 report *Salmonella* thermal resistance results using multiple experimental methods which involved different culture, inoculation, thermal treatment, or enumeration techniques, or differences in product, environment, or strain selection (Chambliss and others 2006; Juneja and Eblen 2000; Juneja and others 2001a; Juneja and others 2001b; Juneja 2003; Juneja and others 2003; Juneja 2007; Juneja and others 2010; Juneja and others 2012; Juneja and others 2013). While each study reported novel results, limited potential exists to validate a model from one study against data from another, due to the unknown effects of methods variability.

There are relatively few validation studies, when compared to a much larger number of studies reporting models, with only a few products represented in the literature (e.g., Breslin and others 2014; Farakos and others 2013; Grijspeerdt and Herman 2003; Jeong and others 2009; Mak and others 2001; Murphy and others 2004b; Tenorio-Bernal and others 201X). Although prior modeling studies include the effects of factors such as fat content, a_w, temperature, temperature history, and additives in inactivation models (e.g., Cerf and others 1996; Farakos and others 2013; Juneja and Eblen 2000; Juneja and others 2013; Tenorio-Bernal and others 2013), there is limited validation of such models against independent data, which limits the

application of such models to substantively similar products and conditions. For example, studies reporting that meat structure has an effect on *Salmonella* thermal resistance do not report *Salmonella* thermal resistance as a function of product structure, but rather report two separate lethality models (Orta-Ramirez and others 2005; Mogollon and others 2009), so that a given model (and associated parameters) can be applied only to equivalent products and conditions..

Overall, *Salmonella* inactivation model validation studies are critically underrepresented in the literature, resulting from the specificity of lethality models. In one example, models developed for isothermal inactivation of *Listeria* were validated with data generated from a dynamic temperature profile, but the model over-estimated inactivation and was considered not suitable (Huang 2009). Similarly, models reported from *Salmonella* isothermal inactivation studies have been shown to overestimate inactivation during pilot-scale slow-cooking of meats (Breslin and others 2014). Ultimately, independent validation of microbial inactivation models remains a significant gap in the literature.

2.4 Estimating Error in Inactivation Data and Models

2.4.1 Lab and model error

Sources of error in inactivation models can come from the laboratory methods used and *Salmonella* strain variability. Random, sampling, and systematic errors associated with laboratory methods can impact experimental uncertainty. Experimental uncertainty is most commonly reported as replication error of data; however, error is underreported in the literature. For example, in a sample of 50 *Salmonella* survival/inactivation studies (analyzed by the author of this thesis), only 28 reported any measure of experimental error.

Few studies have investigated the impact of experimental methods and *Salmonella* variability on the quantification of thermal resistance (e.g., Aspridou and Koutsoumanis 2015; Keller and others 2012; Li and others 2014); fewer studies have investigated the effects of experimental methods and *Salmonella* variability on the inherent error associated with the quantification of *Salmonella* thermal resistance (e.g., Aspridou and Koutsoumanis 2015). No prior studies are known to have investigated, quantified, and reported the sources of error resulting from *Salmonella* inactivation methods. Prior work has demonstrated that data-handling practices, particularly at low populations, can increase variability and significantly bias models fit to the data (Aspridou and Koutsoumanis 2015; Garces-Vega and Marks 2014). Variability in experimental methods and across laboratories limits adequate estimation of replication error. For example, studies using the Danyluk et al (2005) almond inoculation methods as a template for almond kernel inoculation reported initial mean *Salmonella* populations ranging between 7.8 and 8.9 log CFU/g, but replication error was not reported for each study (Abd and others 2012; Danyluk and others 2005; Du and others 2010; Harris and others 2012; Jeong and others 2012; Uesugi and others 2006).

Model error is the product of experimental error, model selection, and the regression method. Model error reported in the literature is commonly described with the coefficient of determination (R^2) (eqn. 8) or RMSE (eqn. 9); however, model validation experiments also describe model error as fail-safe or fail-dangerous (Breslin and others 2014; Farakos and others 2013; Grijspeerdt and Herman 2003; Ross 1996).

$$R^2 = 1 - \frac{\sum\left(\log(N)_{predicted} - \log(N)_{observed}\right)^2}{\sum\left(\log(N)_{predicted} - \log(N)_{average}\right)^2} \quad (8)$$

$$RMSE = \sqrt{\frac{\sum\left(\log(\mathrm{N})_{\mathrm{predicted}} - \log(\mathrm{N})_{\mathrm{observed}}\right)^2}{\mathrm{n} - 1}} \quad (9)$$

In eqns. 8 and 9, n is the number of observations and N is the population. Despite being used as a measure of model error, the coefficient of determination does not quantify model error, but instead indicates the portion of the total variance that is explained by the model.

The goodness-of-fit of a model can be examined by model error; however, parameter error is important when applying the model to an independent process. Similar to model error, parameter error is influenced by model selection and regression methods, but can also be impacted by the experimental design (Dolan and Mishra 2013). An estimation of parameter error is required in order to generate confidence intervals for the prediction.

2.4.2 Unknown error (neither measured nor reported)

Food product, temperature, water activity, food additives, and serovar are some of the variables known to affect the thermal resistance of *Salmonella* (e.g., Archer and others 1998; Baird-Parker and others 1970; Corry 1974; Juneja and others 2001b; Manas and others 2003; Mattick and others 2001a; Mogollon and others 2009; Smith and others 2001; Wesche and others 2005). However, when van Asselt and Zwietering (2006) aggregated thermal resistance parameters from 20 studies investigating *Salmonella* spp., the variations between studies were larger than many of the measured differences in experimental treatments. For example, differences in strain and food product often did not significantly affect the thermal resistance of *Salmonella*, relative to the large variability across the studies. The results of van Asselt and

Zwietering's analysis suggest that variations in laboratory methods and techniques have a noticeable but unquantified effect on thermal resistance determination.

The impact of experimental methods on reported thermal resistance is incompletely understood. Keller et al. (2012) reported that the growth medium impacts *Salmonella* stability and thermal resistance in peanut butter. Li et al. (2014) examined the thermal inactivation kinetics of *Salmonella* in peanut butter inoculated with a peptone water-based or a peanut oil-based inoculum and reported faster *Salmonella* inactivation using the peptone water-based inoculum. Gurtler and Kornacki (2009) reported different populations when different recovery media were used to enumerate *Salmonella* survivors at a single time point during heating. This limited knowledge does not extend to all products and methods, and no studies are known that systematically evaluate a single thermal inactivation methodology.

As a specific example of the impact of using different experimental methodologies, numerous prior studies investigating thermal inactivation of *Salmonella* in meat products have been published by researchers at Michigan State University and the USDA-ARS-ERRC (Carlson and others 2005; Chambliss and others 2006; Juneja and Eblen 2000; Juneja and others 2001a; Juneja and others 2001b; Juneja 2003; Juneja and others 2003; Juneja 2007; Juneja and others 2010; Juneja and others 2012; Juneja and others 2013; Mogollon and others 2009; Orta-Ramirez and others 2005; Smith and others 2001; Tenorio-Bernal and others 2013; Velasquez and others 2010; Wesche and others 2005). This prior research reported by these two laboratories has focused on investigating factors contributing to the thermal resistance of *Salmonella* in meat products, and reporting the resulting thermal inactivation models and parameters. Although the laboratory methodologies are substantively the same (i.e., both isothermal, small sample size, identical *Salmonella* strains, same lethality models), a 300% difference was observed in one case

for the thermal resistance of *Salmonella* spp. in a comparable ground beef product at 60°C (Juneja and others 2001b; Mogollon and others 2009). This discrepancy between the reported thermal resistances for *Salmonella* in similar food products with identical experimental treatments suggests that other influences, outside of the explicit experimental treatments, are not understood, expressed, or reported as contributing to the resultant thermal resistance. However, no prior study has reported a direct comparison for *Salmonella* thermal inactivation results across methods and laboratories.

There are studies directly comparing the effects of thermal inactivation methods on other microorganisms (Donnelly and others 1987; Fujikawa and others 2000; Sorqvist 1989; Zimmermann and others 2013). Donnelly et al. (1987), using two previously established *Listeria* thermal inactivation methods, reported that one method yielded a sharp population decline and the other maintained persistent survivors (Donnelly and others 1987). Zimmermann et al. (2013) reported that "test methods are at least as important in determining thermal processes as the micro-organisms and media used" (Zimmermann and others 2013).

2.4.3 Quantification of error and artifacts

AOAC International develops standard methods for laboratory testing (Advanced Solutions International 2015). The methods undergo rigorous proficiency testing in order to determine scientific consensus and establish known performance requirements. Equivalent standards for microbial thermal inactivation studies do not exist; however, similar protocols might be used to help understand the effects of experimental methods on the quantification of *Salmonella* thermal resistance. Similar to studies investigating the effects of experimental methods on other microorganisms (Donnelly and others 1987; Fujikawa and others 2000;

Sorqvist 1989; Zimmermann and others 2013), a comparison between multiple experimental methods could yield insight on the variability of thermal resistance resulting from the methods. Additionally, multiple laboratories would be required to replicate experiments in order to quantify method repeatability.

2.5 Summary

The current state of *Salmonella* thermal inactivation research has been developed largely without the basic understanding of how the methods to quantify thermal resistance may be influencing the results. Despite indications that there are systematic inconsistencies among data from different experimental methods, no prior study has investigated the impacts of different experimental methods previously reported in literature on the resultant data and inactivation models. Considering the negative impact of salmonellosis on public health, there is a need to improve the fundamental understanding of the research that contributes to enhancing food safety. An evaluation of thermal inactivation methods and potential effects on *Salmonella* thermal resistance, stability, and experimental error is critical in order to fully utilize prior and future research. The completion of the objectives addressed in this thesis will contribute to a foundation for understanding the effects of thermal inactivation methods on observed *Salmonella* thermal resistance, and the resulting utility of thermal inactivation models.

3. CROSS-LABORATORY COMPARATIVE STUDY OF THE IMPACT OF EXPERIMENTAL AND REGRESSION METHODOLOGIES ON *SALMONELLA* THERMAL INACTIVATION PARAMETERS IN GROUND BEEF

3.1 Objective

The objective of this study was to evaluate the effects of laboratory and regression methodologies on *Salmonella* thermal inactivation data generation, interpretation, modeling, and inherent error, based on data generated in two independent laboratories. The study was specifically designed to compare the overall effects of two isothermal inactivation methods reported in several prior studies.

3.2 Materials and Methods

The overall experimental design consisted of a cross-laboratory comparison using two independent laboratories (MSU and USDA-ERRC laboratories under the direction of Dr. Bradley Marks and Dr. Vijay Juneja, respectively), both conducting isothermal inactivation studies at 55, 60, and 62°C. Both laboratories used previously published methods (ERRC: (Juneja 2007; Juneja and others 2010), MSU: (Mogollon and others 2009; Orta-Ramirez and others 2005; Velasquez and others 2010)). The two laboratory methods were substantively similar (i.e., identical *Salmonella* cocktail, identical food matrix, similar culture methods, small sample sizes for isothermal inactivation, recovery of survivors by plating on non-selective media); however, there were some differences (Table 1), which are described below. Two primary models (the log-linear and Weibull models) with one secondary model (the modified Bigelow model) were fit to the data generated by both laboratories using three regression methodologies described below.

Table 1. Key comparisons between ERRC and MSU laboratory methods

Methodology Procedure*	MSU	ERRC
Salmonella Selection*	Eight strain cocktail (from ERRC)	Eight strain cocktail
Food Matrix*	Ground beef	Ground beef (from MSU)
Culture Media	TSB with yeast extract	BHI broth and agar slant
Culture Development	Prepared from stock weekly	Prepared from stock monthly
Inoculum Preparation	Suspended in salt/phosphate sol.	Suspended in peptone water
Treatment Vessel	Sterile brass tubes (12 mm inner dia)	Sterile filter bags (~1 mm thick)
Sample Size	6.0-6.5 g	3 g
Thermal Treatment*	Isothermal in water bath	Isothermal in water bath
Enumeration Media	TSA with yeast extract, ammonium ferric citrate, and sodium thiosulfate	TSA with yeast extract and sodium pyruvate

* Indicates the specific procedures that were identical across the two methodologies.

3.2.1 Common materials and properties

All inactivation tests were conducted with the same eight-strain *Salmonella enterica* cocktail, used in previous studies by both laboratories, which originated from the ERRC, and consisted of *Salmonella* Thompson FSIS 120 (chicken isolate), *Salmonella* Enteritidis H3527

and H3502 (clinical isolates, phage types 13A and 4, respectively), *Salmonella* Typhimurium DT 104 H3380 (human isolate), *Salmonella* Hadar MF60404 (turkey isolate), *Salmonella* Copenhagen 8457 (pork isolate), *Salmonella* Montevideo FSIS 051 (beef isolate), and *Salmonella* Heidelberg F5038BGI (human isolate). All strains were individually maintained at -80°C in vials containing tryptic soy broth (TSB; Difco, BD, Sparks, MD) or brain heart infusion broth (BHI; Difco, BD, Sparks, MD) supplemented with 20 or 15% vol/vol glycerol for the MSU and ERRC methodologies, respectively.

The ground beef used in both laboratories originated from the MSU Meat Laboratory. On the day of slaughter, top round was ground twice through a 4.8-mm-hole plate and then once through a 3.2-mm-hole plate (model 4146 meat grinder, Hobart Corp., Troy, OH), vacuum packaged in sterile plastic bags, frozen at -20°C, and irradiated using an X-ray food irradiator (Rayfresh Foods Inc., Ann Arbor, MI) to approximately 40 kGy to reduce background microflora. Samples taken prior to vacuum packing were used to determine fat percentages using AOAC method 960.39 (AOAC International, Gaithersburg, MD) and were plated in duplicate on modified trypticase soy agar (mTSA; Difco, BD, Sparks, MD) supplemented with yeast extract (0.6%), ammonium ferric citrate (0.05%), and sodium thiosulfate (0.03%), followed by incubation at 37°C for 48 h to quantify the presence of background microflora. A portion of the irradiated meat batch was sent frozen overnight from MSU to the ERRC. Samples were stored at -20°C at both locations. The specific materials, utensils, and treatment vessels required for thermal inactivation that were not commercially available were transferred between laboratories to reduce variability.

3.2.2 MSU laboratory methodologies

The eight strains were transferred from frozen culture using a sterile transfer loop to individual 9 ml tubes of TSB supplemented with yeast extract (0.6%) (TSBYE) followed (after 24 h at 37°C) by a consecutive transfer to separate 9 ml of TSBYE and a second 24 h, 37°C incubation period. The eight cultures (9 ml) were then combined and centrifuged at 3800 × *g* for 15 min at 4°C. The supernatant was discarded, and the pelletized culture was re-suspended in 500 ml of sterile salt/phosphate solution (3.2% NaCl, 0.8% potassium phosphate). Samples of frozen irradiated ground beef (100 g) were thawed at room temperature (~2 h) after which 12 ml of inoculum was added to the meat in a sterile beaker. The sample was then mixed by hand using sterile gloves for 3 min. The salt/phosphate suspension medium was included in the inoculum to be consistent with prior studies (Maurer 2001; Mogollon and others 2009; Orta-Ramirez and others 2005; Velasquez and others 2010) that were comparing commercial-like products (e.g., whole-muscle vs. ground beef).

Samples of the inoculated meat (6.0-6.5 g) were aseptically distributed into sterile brass tubes (12 mm id, 10 cm long). Sterile rubber stoppers were inserted at both ends and sealed with teflon tape. All tubes within a series (for an individual replication) were simultaneously submerged into a circulating water bath for isothermal treatment at 55, 60, or 62°C, with the temperature of the water bath set 0.5°C higher than the treatment temperature. Prior to submersion, a thermocouple (1.0 mm; Type T, Omega Engineering, Stamford, CT) was inserted into the center of one tube within each replication series and monitored for thermal lag time (defined as the time when the sample core temperature was within 0.5°C of the target temperature). After the thermal lag time (~180 s), 8-10 sample tubes were removed from the

water bath at uniform intervals and immediately placed in an ice-water bath, reducing the sample temperature below 38°C in ~30 s.

Using a sterile spatula, the treated samples were extracted from the brass tubes and placed into individual sterile plastic bags. Samples were diluted 1:5 in 0.1% sterile peptone water and homogenized by stomaching for 3 min (Stomacher Model 0410, IUL Instruments USA, Inc., Cincinnati, OH). The samples then were serially diluted in 0.1% peptone water and plated in duplicate on mTSA. After 48 h of incubation at 37ºC for 48 h, all colonies with characteristic black centers were counted as *Salmonella*. The resulting limit of detection (LOD) was ~1.4 log CFU/g.

3.2.3 ERRC laboratory methodologies

Vials of frozen stock cultures were thawed, and 100 µl of the culture was transferred to individual 10 ml tubes of BHI (Difco, BD, Sparks, MD) broth, which were incubated at 37ºC for 24 h. Using a sterile transfer loop, cultures were streaked onto individual BHI (Difco, BD, Sparks, MD) slants, where cultures were grown at 37ºC for 24 h and then, maintained at 4ºC for up to one month. At the beginning of each week of experimentation, a working broth culture for each serovar was prepared by transferring a loop of culture from the BHI slant into 10 ml tubes of BHI broth followed by incubation at 37ºC for 24 h and then, maintained at 4ºC for up to 5 days. A day prior to experimentation, 50 ml of BHI broth was inoculated from the working culture for each serovar and incubated at 37ºC for 18 h. Each culture was centrifuged at 3000 × *g* for 15 min at 4ºC. The supernatant was discarded, and the pellet was suspended in 10 ml of 0.1% peptone water. The cultures were again centrifuged at 3000 × *g* for 15 min at 4ºC. Each culture was then suspended in 2 ml of sterile 0.1% peptone water and combined. Frozen irradiated ground beef (50 g) was thawed at room temperature prior to inoculation with 100 µl of the

cocktail, then mixed by hand massaging the sample bag for 2 min, then stomaching in the bag for 2 min.

Inoculated meat samples (3 g) were aseptically placed into sterile filter bags (BagPage 100, Interscience, St. Nom la Bretêche, France). Samples were flattened into a thin layer (~1 mm thick) and vacuum packaged. All samples within a series and individual replication were placed in a wire mesh basket, so that water could freely move between the samples, and submerged into a circulating water bath at 55, 60, or 62°C, with the water bath set at the experimental temperature. Thermal lag time was measured and was considered negligible (~25 s), relative to the durations of the full treatment; 8-10 samples were pulled at uniform intervals after initial submersion into the water bath and placed immediately into an ice-water bath.

Thermally treated samples were opened with sterile scissors and diluted with 3 g of sterile 0.1% peptone water. The samples then were hand-mixed and stomached for 2 min. The samples were serially diluted in 0.1% peptone water and plated in duplicate on modified trypticase soy agar supplemented with yeast extract (0.6%) and sodium pyruvate (1.0%). The plates were incubated at 37°C for 48 h and then colonies were enumerated. The resulting LOD was ~1.2 log CFU/g.

3.2.4 Pre-regression data processing

All data were processed by a single individual to reduce bias. From all the data sets, one outlier, as determined by the Grubb's outlier test (Grubbs 1950), was removed ($n_{removed} = 1$; $P < 0.05$). In addition, any data points observed or occurring after the *Salmonella* population was below the LOD were removed (Garces-Vega and Marks 2014). Data series were transformed into log survivor ratios by subtracting the log of the initial population for a given replication from the log of the population at each time.

3.2.5 Model regression

Two common inactivation models were employed: the log-linear (eqn. 10) model and a Weibull-type model (eqn. 11), respectively:

$$\log\left(\mathrm{N}/\mathrm{N}_0\right) = -\frac{\mathrm{t}}{\mathrm{D}_\mathrm{T}} \tag{10}$$

$$\log\left(\mathrm{N}/\mathrm{N}_0\right) = -\left(\frac{\mathrm{t}}{\delta_\mathrm{T}}\right)^{\mathrm{p}} \tag{11}$$

where N and N_0 (CFU/g) are the *Salmonella* populations at times t and 0, respectively, t (s) is the isothermal heating time, D_T (s) is the decimal reduction time at temperature T (°C), δ_T (s) is the Weibull scale parameter at temperature T (°C), and p (unitless) is the shape factor for the Weibull model.

A modified Bigelow secondary model was applied to account for the effect of temperature on D_T (eqn. 12) and δ_T (eqn. 13).

$$\mathrm{D}_\mathrm{T} = \mathrm{D}_\mathrm{ref} \times 10^{\left(\frac{\mathrm{T}_\mathrm{ref}-\mathrm{T}}{\mathrm{Z}_{\mathrm{T,D}}}\right)} \tag{12}$$

$$\delta_\mathrm{T} = \delta_\mathrm{ref} \times 10^{\left(\frac{\mathrm{T}_\mathrm{ref}-\mathrm{T}}{\mathrm{Z}_{\mathrm{T},\delta}}\right)} \tag{13}$$

where D_{ref} (s) is the decimal reduction time at the reference temperature, δ_{ref} (s) is the Weibull scale parameter at the reference temperature, $z_{T,D}$ and $z_{T,\delta}$ (°C) are the decimal temperature scaling parameters, and T_{ref} (°C) is the reference temperature which was pre-fixed at 60 °C. Initial analyses of variance found p not to be a function of temperature ($P > 0.05$). Because the modified Bigelow secondary model is typically incorporated into the log-linear model, and the

focus of this study was to quantify the effects of laboratory and regression methodologies, only one secondary model was examined, noting that there are several other common alternatives (Juneja and others 2003; Mattick and others 2001b; Stasiewicz and others 2008).

In addition to analyzing two primary inactivation models, three regression strategies were applied for estimating the model parameters: individual, aggregate, and global regression as described below. MATLAB non-linear fitting tools (nlinfit; version 2014; MathWorks, Natick, Massachusetts) were utilized in each case.

The individual regression method incorporated a two-step regression procedure separating the estimation of the primary and secondary model parameters. The primary model regression was performed on each individual series replication ($\log(N/N_0)$ vs. t), producing a D_T and δ_T parameter estimate for each replicate inactivation series. The shape parameter term within the Weibull model was confirmed via ANOVA ($P > 0.05$) not to be a function of temperature and was therefore fixed as the average value of the individual estimates generated during the initial primary model regression. A subsequent primary model regression using the fixed shape factor was performed to generate the δ_T parameter estimates used in the secondary model. The D_T and δ_T values within a temperature-methodology-laboratory-model combination were averaged and subsequently used within the second regression to estimate $z_{T,D}$ and $z_{T,\delta}$ for the log-linear and Weibull models, respectively.

The aggregate regression method also incorporated a two-step regression procedure separating the estimation for the primary and secondary model parameters. The primary model regression incorporated all time-survivor data (three replicates) within a temperature-methodology-laboratory source combination to generate single D_T and δ_T parameter estimates for each treatment, which were used within the second regression to estimate $z_{T,D}$ and $z_{T,\delta}$, as

described above. The shape parameter term within the Weibull model was confirmed via ANOVA analysis ($P < 0.05$) not to be a function of temperature and was therefore fixed as the average value of the p estimates generated during the initial primary model regression. A subsequent primary model regression using the fixed shape factor term was performed to generate the δ_T parameter estimates used in the secondary regression.

The global regression method involved estimating all parameters simultaneously in a one-step regression of all time-temperature-survivor data for a given methodology-laboratory data set. Application of global regression for the log-linear (eqns. 10 and 12) and the Weibull model (eqns. 11 and 13) directly yielded D_{ref} and $z_{T,D}$ as well as δ_{ref}, $z_{T,\delta}$, and p, respectively.

3.2.6 Statistical analyses

Model selection analyses were performed using the Akaike Information Criterion (AIC) (Motulsky and Christopoulos 2004) and the root mean square error (RMSE), where:

$$AIC = \mathrm{n}\, log\left(\frac{\sum\left(\log\left(\mathrm{N}/\mathrm{N_0}\right)_{\mathrm{predicted}} - \log\left(\mathrm{N}/\mathrm{N_0}\right)_{\mathrm{observed}}\right)^2}{\mathrm{n}}\right) + 2\mathrm{K} \qquad (14)$$

$$RMSE = \sqrt{\frac{\sum\left(\log\left(\mathrm{N}/\mathrm{N_0}\right)_{\mathrm{predicted}} - \log\left(\mathrm{N}/\mathrm{N_0}\right)_{\mathrm{observed}}\right)^2}{\mathrm{n} - \mathrm{p}}} \qquad (15)$$

and n is the number of samples, N and N_0 (CFU/g) are the *Salmonella* populations at times t and 0, respectively, p is the number of model parameters (i.e., 2 and 3 for the log-linear and Weibull models, respectively), and K is the number of model parameters plus 1. AIC values are relative

measures of model quality adjusted by the number of parameters in the model, in order to allow for comparisons between models of different forms and number of parameters. AIC values were compared using the equation below (Motulsky and Christopoulos 2004) to determine the percent likelihood of a given model being more correct.

$$Relative\ Likelihood\ of\ Model\ A\ over\ B = \frac{exp\left(\frac{(\mathrm{AIC_B} - \mathrm{AIC_A})}{2}\right)}{1 + exp\left(\frac{(\mathrm{AIC_B} - \mathrm{AIC_A})}{2}\right)} \qquad (16)$$

AIC values were calculated for every methodology-laboratory-model-regression combination and used to evaluate the relative likelihood of the log-linear and Weibull models being the better choice. Parameter estimates also were statistically compared using the Student's t-test ($\alpha = 0.05$) calculated on Microsoft Excel (version 2013; Microsoft, Redmond, Washington). Pairwise comparisons evaluated differences between laboratory source and methodology source. Conclusions on differences resulting from laboratory and method sources were based solely on comparisons between global regression parameter estimates.

3.3 Results

3.3.1 *Meat analysis*

The ground beef contained 3.35 ± 0.73% fat. Background microflora was not recovered on irradiated samples.

3.3.2 *Regression strategy analysis*

As expected, regression methodology had a large impact on the standard error of the parameter estimates (Tables 2 and 3). In general, the standard errors of the log-linear model

parameters ($D_{60°C}$, $z_{T,D}$) from two-step regressions (individual or aggregate) were on average ~12 times larger than those from the one-step regression. Similarly, the standard errors of the Weibull model parameters ($\delta_{60°C}$, $z_{T,\delta}$, p) from the two-step regression were on average ~2, ~8, and ~17 times larger, respectively, than those from the one-step regression. In most cases, the RMSEs of the two-step regressions were ~40% larger than the RMSEs of the corresponding global regressions; the exception was the RMSEs of the individual regression for the Weibull model, where the RMSEs from the two-step regressions were 64 to 510% larger than the corresponding global regression RMSEs. Larger standard errors for the parameter estimates and larger RMSEs indicated greater model uncertainty and poorer fit, respectively, of the two-step regression models applied to the full data set, compared to those from corresponding one-step regressions.

Table 2. Log-linear model global, aggregate, and individual regression parameter estimates (± std error)

Laboratory	Methodology	Regression	$D_{60°C}$* (s)	$z_{T,D}$ (°C)	RMSE (log CFU/g)	AIC
ERRC	**ERRC**	**Global**	**76.40 (0.29)[a]**	**6.30 (0.02)[a]**	**1.14**	**27.06**
ERRC	ERRC	Aggregate	74.95 (3.74)	6.25 (0.27)	1.10	
ERRC	ERRC	Individual	76.16 (3.48)	6.28 (0.25)	1.21	
MSU	**ERRC**	**Global**	**63.79 (0.15)[b]**	**6.27 (0.01)[a]**	**0.65**	**-64.19**
MSU	ERRC	Aggregate	64.59 (2.20)	6.29 (0.19)	0.83	
MSU	ERRC	Individual	64.07 (1.90)	6.28 (0.16)	0.70	
MSU	**MSU**	**Global**	**57.97 (0.29)[c]**	**5.02 (0.02)[b]**	**0.57**	**-71.80**
MSU	MSU	Aggregate	60.38 (3.52)	5.11 (0.21)	0.78	
MSU	MSU	Individual	59.80 (4.03)	5.09 (0.24)	0.62	
ERRC	**MSU**	**Global**	**53.01 (0.24)[d]**	**4.99 (0.01)[b]**	**0.65**	**-59.28**
ERRC	MSU	Aggregate	54.78 (2.55)	5.09 (0.17)	0.85	
ERRC	MSU	Individual	56.22 (2.57)	5.18 (0.17)	0.73	

* Within columns, parameter estimates sharing a common letter are not significantly different ($\alpha = 0.05$).

Table 3. Weibull model global, aggregate, and individual regression parameter estimates (± std error)

Laboratory	Methodology	Regression	$\delta_{60°C}$* (s)	$z_{T,\delta}$ (°C)	p-value (unitless)	RMSE (log CFU/g)	AIC
ERRC	**ERRC**	**Global**	**34.61 (0.66)[a]**	**6.03 (0.01)[a]**	**0.67 (0.03)[a]**	**0.97**	**-1.94**
ERRC	ERRC	Aggregate	44.71 (1.85)	6.08 (0.21)	0.76 (0.03)	1.01	
ERRC	ERRC	Individual	39.84 (0.19)	5.33 (0.02)	0.51 (0.10)	1.73	
MSU	**ERRC**	**Global**	**33.97 (0.42)[a]**	**6.28 (0.004)[b]**	**0.71 (0.01)[a]**	**0.50**	**-104.28**
MSU	ERRC	Aggregate	33.18 (1.11)	6.31 (0.19)	0.70 (0.02)	0.51	
MSU	ERRC	Individual	38.81 (4.16)	5.60 (0.47)	1.06 (0.11)	3.49	
MSU	**MSU**	**Global**	**76.05 (0.57)[b]**	**5.03 (0.01)[c]**	**1.41 (0.01)[b]**	**0.50**	**-87.26**
MSU	MSU	Aggregate	82.48 (0.37)	5.03 (0.02)	1.59 (0.09)	0.53	
MSU	MSU	Individual	75.54 (3.02)	5.12 (0.15)	1.87 (0.45)	1.18	
ERRC	**MSU**	**Global**	**60.06 (0.63)[c]**	**4.93 (0.01)[d]**	**1.15 (0.01)[c]**	**0.64**	**-60.12**
ERRC	MSU	Aggregate	60.97 (2.25)	5.02 (0.13)	1.12 (0.07)	0.68	
ERRC	MSU	Individual	78.99 (0.86)	5.15 (0.04)	1.16 (0.22)	0.92	

* Within columns, parameter estimates sharing a common letter are not significantly different ($\alpha = 0.05$).

3.3.3 Model comparison

AIC analysis (Tables 2 and 3) indicated that the Weibull model was more likely correct (% likelihood > 95%) for all cases except the data set generated by ERRC laboratory using the MSU methodology. The likelihood that the Weibull model was more correct (eqn. 16) was greatest using the ERRC methodology (% likelihood > 99.9999%), regardless of the laboratory. The relative likelihood that the Weibull model was more correct using the MSU methodology for data generated at ERRC and MSU was ~57.9 and >99.9%, respectively.

3.3.4 Methodology comparison

MSU and ERRC methodologies yielded inoculation *Salmonella* populations of 6.78 ± 0.11 and 7.66 ± 0.24 log CFU/g, respectively.

Model parameters resulting from the MSU and ERRC methodologies were significantly different ($P < 0.05$; Tables 2 and 3) when compared across and within laboratories. When comparing the log-linear model parameters, the ERRC methodologies yielded more conservative results, where both $D_{60°C}$ and $z_{T,D}$ were ~25% larger than the corresponding MSU methodology parameters. Regressions for data pooled by methodology (i.e., aggregating data from two laboratories using the same methodology) yielded parameters similar to the regressions parameters of unpooled data of the same methodology; however, when data were pooled by laboratory source (i.e., aggregating data from two methodologies conducted at one laboratory), the resulting parameter estimates were not representative of the parameters yielded by the unpooled data analyses (results not shown). Observable differences in the rate of inactivation between the two methodologies were evident at 60 and 62°C (Figure 1).

The Weibull model parameters resulting from data generated using the ERRC and MSU methodologies indicated a characteristic difference between the two data sets, regardless of the laboratory source; not only were the $\delta_{60°C}$ values clustered separately (60-76 and ~34 s for MSU and ERRC methodologies, respectively), but the shape factor p was characteristically different. The p values from MSU methodologies ranged from 1.1-1.4, and the p values from ERRC methodologies were ~0.7. This difference in the shape of the response data (Figure 1) generated from ERRC and MSU methodologies suggests that methodology influenced the fundamental characteristics of the microbial population response during thermal treatment.

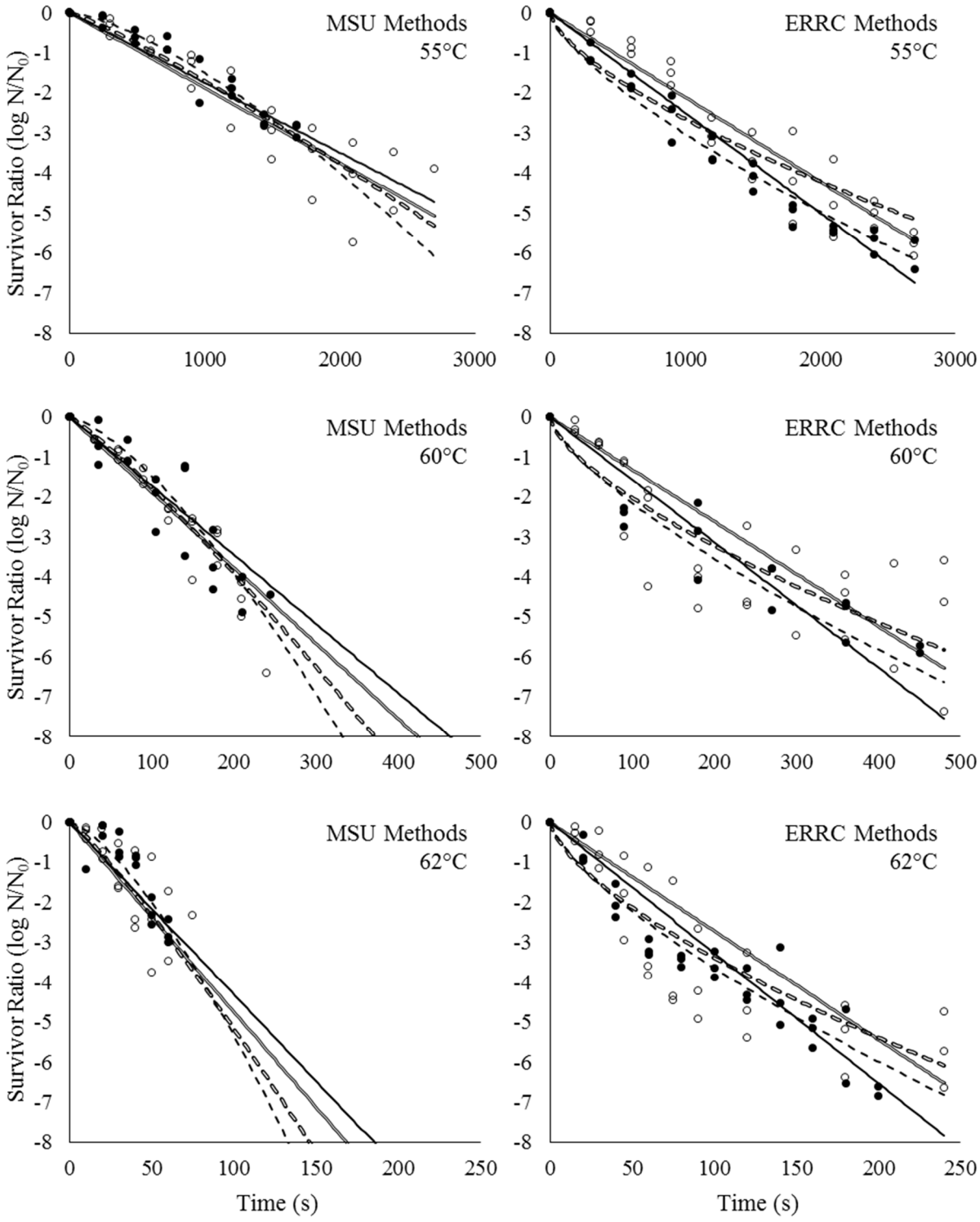

Figure 1. *Salmonella* inactivation curves by MSU (solid) and ERRC (hollow) laboratories, both using laboratory methodologies originating from MSU and ERRC laboratories. Log-linear (continuous line) and Weibull (dashed line) models were estimated using a global regression.

3.4 Discussion

The application of two substantively similar isothermal inactivation methodologies (i.e., identical *Salmonella* cocktail, identical food matrix, cultures suspended in broth during incubation at 37°C, small sample sizes treated isothermally, recovery of survivors using plate methods on non-selective media) yielded some significant differences in the resulting inactivation response, parameter estimates, and model uncertainty. Although there were some differences between the two methodologies (Table 1), it is unknown which of these differences caused the differences in outcomes. The basic experimental designs used by MSU and ERRC were equivalent: thermal resistance measured by enumerating survivors of the same eight-strain *Salmonella* cocktail inactivated in ground beef under isothermal conditions. Dynamic temperature profiles can impact the measurement of thermal resistance (Huang 2009; Janssen and others 2008); however, no previous report has explicitly quantified differences in the measured thermal resistance of *Salmonella* using isothermal methodologies compared across laboratories. The MSU and ERRC methodologies treated isothermal conditions differently. For example, the MSU methodology specified the temperature set-point of the water bath to be 0.5°C higher than the reported treatment temperature and initiated the isothermal time series when the samples were within 0.5°C of the treatment temperature, and the ERRC methodology initiated the isothermal time series when the samples were immediately submerged into the water bath. This difference between MSU and ERRC methodologies would be expected to result in a faster isothermal inactivation rate and a shouldering effect, respectively. Data collected using the MSU methodology resulted in a faster isothermal inactivation rate; however, the characteristic differences in $z_{T,D}$, $z_{T,\delta,}$ and p across lab methodologies were not expected to result from faster isothermal inactivation rates. In addition, data from ERRC methodologies exhibited a tailing

effect instead of the potentially expected shouldering effect. Therefore, it seems unlikely that the differences in isothermal conditions alone could have caused the observed differences in the inactivation response, parameter estimates, and model uncertainty.

Aspects of the MSU methodology (inoculation with salt/phosphate solution and cylindrical thermal treatment container) have been previously reported to impact the thermal resistance of *Salmonella* (Chung and others 2007; Maurer 2001); however, previous research suggests that these factors should increase the reported thermal resistance, which was contradictory to the higher thermal inactivation rate using the MSU methodology. Different recovery media also may affect the thermal inactivation results; however, prior studies (Kang and Fung 2000; Leguerinel and others 2007) that examined the effects of varying levels of selective agents did not explicitly include the two recovery media included in this study, and neither are considered selective. Gurtler and Kornacki (2009) reported negligible differences between comparable recovery media used in this study in the effectiveness of recovery promoters after a single heating time. Overall, individual experimental methodology differences that appear minor might affect resulting inactivation responses and parameters, even though such effects have not been systematically evaluated or quantified.

With respect to the regression methodologies, multiple-step regression results were not consistently similar to the corresponding one-step global regression parameter estimates, and were accompanied by larger standard errors. Larger errors associated with the multiple step regressions reduce the certainty of prediction when the corresponding thermal inactivation models are used for an industry process validations. Despite similar $D_{60°C}$-values, the larger standard errors of the estimates for the two-step regressions reduce the inherent confidence in

inactivation predictions, which is typically under-reported or not considered when utilizing inactivation models.

The impact of the parameter differences observed across methods and models are difficult to discern without a case-study application of the resultant models. The following two paragraphs incorporate two thermal treatment scenarios, an isothermal and non-isothermal case, in order to emphasize the impact of regression methods and the overall inactivation methods/model selection, respectively.

As an example to illustrate the impact of regression methodologies, consider the time to achieve the USDA-FSIS-required 6.5 log reduction for *Salmonella* in ready-to-eat cooked meat products (USDA-FSIS 2001), assuming isothermal conditions at 60°C (Figure 2). Considering only the predicted lethality line, the time to achieve a 6.5 log reduction was ~385 s, regardless of regression methodology; however, the times for the upper confidence intervals, where 95% of the sampling means would be expected to receive at least a 6.5 log reduction, for the global, aggregate, and individual regressions were ~420, ~490, and ~500 s, respectively. The model parameters resulting from the two-step regressions required more than an additional minute of processing to achieve the same confidence in a 6.5 log reduction. Two-step regressions may be appropriate for preliminary estimations (Jewell 2012); the one-step regression was better overall for reducing uncertainty in parameter estimation (Jewell 2012; Martino and Marks 2007).

To illustrate the practical impact of overall methodology and inactivation model selection when predicting process lethality in a real-world application, the log-linear and Weibull models resulting from the global regressions were applied to a single case study from a previous pilot-scale challenge study (Tenorio-Bernal and others 201X). The case study data consisted of a

single time-temperature profile for the center point of a low-fat ground beef patty that was

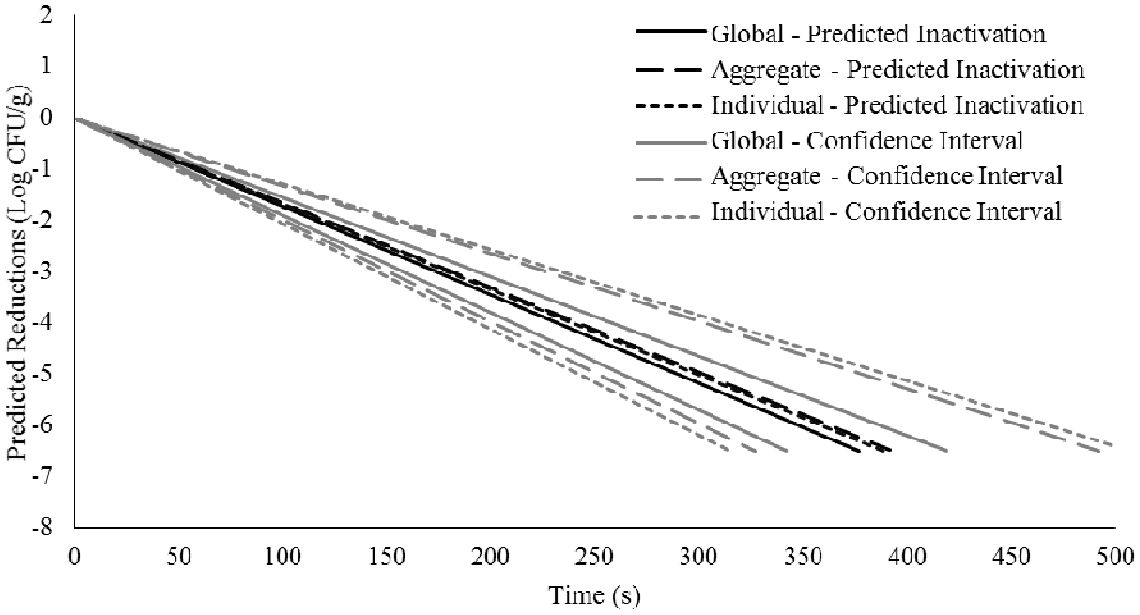

Figure 2. Predicted inactivation curves (black) and prediction intervals (gray) for isothermal *Salmonella* inactivation in low-fat ground beef at 60°C, with log-linear model parameters estimated using global (solid), aggregate (long dash), and individual (short dash) regressions on data generated by MSU using MSU methodologies.

inoculated with the same eight-strain *Salmonella* cocktail used in this study, then cooked in a moisture-air-impingement oven (JBT FoodTech, Sandusky, OH), after which surviving *Salmonella* at the patty core were enumerated to experimentally determine the process lethality (i.e., log reductions) (Tenorio-Bernal and others 201X). Eight models from the present study (by global regression of each methodology-laboratory-model combination) were applied to the dynamic core temperature data from that pilot-scale case study (Figure 3). The purpose of this analysis was not a statistical validation of the models, which is not possible with a single case study; rather, the purpose was to illustrate how the different model forms and parameters, which were influenced by the model-development methodologies, affect the relative predictions that

occur when applying these models to a dynamic data set representative of an actual commercial thermal process. Even though AIC indicated that the Weibull model was the more likely correct model in this study, the log-linear model more closely predicted the measured thermal inactivation of 5.4 log in the case study, regardless of the laboratory methodology used to estimate the model parameters. Although the Weibull model was considered the more likely correct model, based on analysis against the isothermal inactivation data, this example illustrates the importance of considering model robustness when applied to the domain of interest. For this specific case, the log-linear model appeared more robust (i.e., yielding more consistent predictions, regardless of the methodology and laboratory used to generate the data to estimate model parameters). Again, this case study is not a rigorous validation of the models, but is presented to illustrate the importance of considering model application and robustness as part of the model development and selection process.

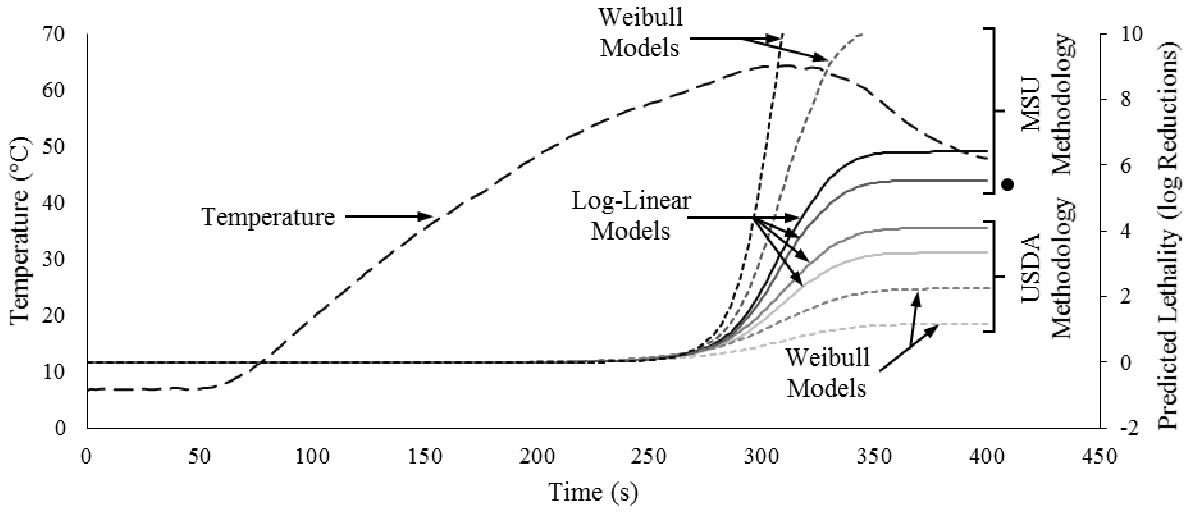

Figure 3 Predicted lethality curves for *Salmonella* in a low-fat ground beef patty cooked in a moist-air impingement oven, using log-linear (solid lines) and Weibull (short dashed lines) models estimated from data yielded by MSU and ERRC performing both MSU and ERRC methodologies (and global regressions). The reported experimental *Salmonella* lethality for this case study example was 5.4 log (●)(Tenorio-Bernal and others 201X).

In their review, Doyle and Mazzotta (2000) described heat resistance of *Salmonella* as being influenced by strain, food product, pH, water activity, heating profile, etc. Within the context of the review, differences in D-values between experiments were attributed to difference in treatments, with an acknowledgement that laboratory methodologies may impact the reported values. The same disclaimer was made in other reviews of thermal resistance reviews (Doyle and others 2001; Stringer and others 2000) and for meta-analyses of thermal resistance studies (den Besten and Zwietering 2012; Farakos and Zwietering 2011; van Asselt and Zwietering 2006). Specifically, van Asselt and Zwietering (2006) conducted a meta-analysis of thermal resistance parameters based on a large collection of data sets encompassing different laboratory methodologies. They noted that the variations between studies were larger than many of the reported treatment effects within the studies. The present study is the first known to quantitatively evaluate these claims, directly testing the effects of relatively small differences in methodologies and differences across laboratories. Although previous work from MSU and ERRC have suggested differences in the measured thermal resistance for *Salmonella* spp. as large as ~300% in a comparable ground beef product at 60°C (Juneja and others 2001b; Mogollon and others 2009), results from this study yielded differences up to ~45% in $D_{60°C}$ values. Although the difference in parameters yielded from the two thermal inactivation methodologies investigated in this study are attributed to the sum of differences between the two methodologies, it is unknown which differences influenced the thermal resistance of *Salmonella,* or the relative magnitude of those effects. Because this study could not include all isothermal inactivation methodologies, uncertainty remains regarding the broad impact of laboratory and data handling methodologies on prior studies, meta-analyses, or applications. The present study has demonstrated that there is a finite and repeatable impact, and suggests that future research is

needed to evaluate and standardize isothermal inactivation laboratory methodologies in order to reduce uncertainty, and thereby improve the utility of reported inactivation data and parameters.

3.5 Conclusion

In conclusion, experimental details, data handling, and parameter estimation methods can significantly impact the interpretation of microbial inactivation studies, and the utilization of resulting models. Based on parallel isothermal inactivation studies, significant differences were observed between the characteristics and quality of data from the two separate laboratory methodologies. Two-step regressions of the data were inferior to a one-step regression and had a non-uniform bias on the parameter estimates for the log-linear and Weilbull models. Overall, the results of this study suggest that there is a large and typically unknown uncertainty when comparing or combining data from multiple studies across laboratories, which can limit the improvement of industrial thermal lethality processes, unless the influence of laboratory methodologies are characterized or laboratory methodologies are standardized.

4. EFFECTS OF INOCULATION PROCEDURES ON VARIABILITY AND REPEATABILITY OF *SALMONELLA* THERMAL RESISTANCE IN WHEAT FLOUR

4.1 Objective

The objective of this study was to evaluate the effects of cultivation and inoculation methodologies on the stability and thermal resistance of *Salmonella* in a low-moisture food (wheat flour), and the repeatability of those results, based on data generated in two independent laboratories. The cultivation and inoculation methodologies selected below were based on methodologies reported in the literature investigating *Salmonella* in low-moisture foods.

4.2 Materials and Methods

The overall experimental design consisted of evaluating five different inoculation methodologies, with a cross-laboratory comparison using two independent laboratories (Michigan State University (MSU) and Washington State University (WSU) under the direction of Drs. Bradley Marks and Juming Tang, respectively). Identical isothermal inactivation methodologies (80°C) were employed to quantify the thermal resistance of *Salmonella enterica* serovar Enteritidis phage type 30 (PT30) in wheat flour conditioned to ~0.45 a_w. A log-linear model was fit to the data generated by both laboratories (described below).

4.2.1 Wheat flour

Organic soft winter wheat flour was obtained from Eden Foods (Clinton Township, MI). Upon acquisition, initial a_w was measured using an a_w meter (Model 3TE, Decagon Devices, Pullman, WA), and particle size distribution was determined by sieve analysis. Tyler series

sieves (#30 through #200) were stacked with 100 g of wheat flour and shaken for 30 min with a motorized sieve shaker (H-4325, Humboldt Manufacturing, Elgin, IL). The flour remaining in each sieve after shaking was weighed and then used to calculate the geometric mean diameter of the wheat flour (ANSI/ASAE method S319.3, ANSI, Washington, DC). Wheat flour used in the isothermal inactivation treatment was run across a single sieve (Tyler series #120) prior to inoculation, to remove very fine particles more likely to become airborne after inoculation. *Salmonella* in the source wheat flour was periodically plating uninoculated samples onto tryptic soy agar (Difco, BD, Sparks, MD) supplemented with 0.6% (w/v) yeast extract (Difco, BD, Sparks, MD) (TSAYE) supplemented with ferric citrate (0.05%) (Sigma Aldrich, St. Louis, MO) and sodium thiosulfate (0.03%) (Sigma Aldrich, St. Louis, MO) (mTSA). The plates were incubated at 37°C for 48 h, and colonies with a characteristic black center were enumerated as *Salmonella*.

4.2.2 Bacterial strain and inoculation

Salmonella enterica serovar Enteritidis phage type 30 (SE PT30), previously obtained from Dr. Linda Harris (University of California, Davis), was used in this study because it was previously shown to be thermally resistant in low a_w systems (Abd and others 2012; Du and others 2010; Harris and others 2012). The culture was maintained at -80°C in tryptic soy broth (Difco, BD, Sparks, MD) supplemented with 20% (vol/vol) glycerol.

Five inoculation methodologies for low-moisture foods were evaluated, including several based on methodologies reported in the literature (Keller and others 2012; Komitopoulou and Penaloza 2009). The five inoculation methodologies (A, B, C, D, and E) are summarized as:

A. Broth cultivated, centrifuged, resuspended to obtain a high concentration liquid inoculum

B. TSAYE lawn cultivated, harvested, centrifuged, resuspended to obtain a high concentration liquid inoculum
C. TSAYE lawn cultivated, harvested, centrifuged, pelletized inoculum
D. TSAYE lawn cultivated, harvested directly into wheat flour
E. TSAYE lawn cultivated on filter papers for a dry inoculum

The details of each inoculation methodology are described below.

4.2.3 Inoculation methodology A: broth-based liquid inoculum (BLI)

SE PT30 was subjected to two consecutive transfers (24 h, each at 37°C) in 9 ml of TSBYE, and then transferred into 500 ml of TSBYE. After incubation (24 h, 37°C), the culture suspension (400 ml) was centrifuged for 15 min at 3,000 × *g*. The supernatant was discarded, and the remaining pellet was resuspended in 3 ml of 0.1% peptone water. Inoculum (1 ml) was then hand mixed into wheat flour (10 g) in a sterile plastic bag for 3 min. After hand mixing, the inoculated wheat flour was added to more wheat flour (90 g) and stomached (Masticator Basic, Neu-Tec Group Inc., Farmingdale, NY) for 3 min.

4.2.4 Inoculation methodology B: lawn-based liquid inoculum (LLI)

SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE, and then 1 ml was spread evenly over a plate (150 by 15 mm) of TSAYE to obtain uniform lawn. After incubation (24 h, 37°C), the bacterial lawn was harvested in 20 ml of sterile 0.1% peptone water, using a sterile plate spreader, and was collected into a sterile container. The suspension was centrifuged for 15 min at 3,000 × *g*. The supernatant was discarded, and the remaining pellet was resuspended in 3 ml of 0.1% peptone water. Inoculum (1 ml) was then hand

mixed into wheat flour (10 g) in a sterile plastic bag for 3 min. After hand mixing, this inoculated seed sample was added to a larger wheat flour sample (90 g) and stomached (Masticator Basic, Neu-Tec Group Inc., Farmingdale, NY) for 3 min.

4.2.5 Inoculation methodology C: lawn-based pelletized inoculum (LPI)

SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE, and then 1 ml was evenly spread on a plate (150 by 15 mm) of TSAYE to obtain uniform lawn. After incubation (24 h, 37°C), the bacterial lawn was harvested in 20 ml of sterile 0.1% peptone water, using a sterile plate spreader. The suspension was centrifuged for 15 min at 3,000 × *g*. The supernatant was discarded, and the remaining pellet was hand mixed into wheat flour (10 g) in a sterile plastic bag for 3 min. After hand mixing, this seed sample was added to a larger wheat flour sample (90 g) and stomached (Masticator Basic, Neu-Tec Group Inc., Farmingdale, NY) for 3 min.

4.2.6 Inoculation methodology D: direct-harvest method (DHM)

SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE, and then 1 ml was evenly spread on a plate (150 by 15 mm) of TSAYE to obtain uniform lawn. After incubation (24 h, 37°C), wheat flour (10 g) was added to the bacterial lawn and then agitated with a sterile plate spreader to incorporate *Salmonella* into the wheat flour. The inoculated wheat flour was then collected with a sterile spatula in a sterile plastic bag and hand mixed for 3 min. After hand mixing, this seed sample was added to a larger wheat flour sample (90 g) and stomached (Masticator Basic, Neu-Tec Group Inc., Farmingdale, NY) for 3 min.

4.2.7 Inoculation methodology E: dry-transfer method (DTM)

SE PT30 was subjected to two consecutive transfers (24 h each at 37°C) in 9 ml of TSBYE and then transferred to 18 ml of TSBYE. A culture suspension (5 ml per plate) was then added to two plates (150 by 15 mm) of TSAYE, each containing four filter membranes (0.45 µm pore, 5 cm diameter; Membrane Filter, Millipore Corporation, Billerica, MA) on the surface. After incubation (24 h, 37°C), the eight filter papers were removed from the TSAYE plates and air dried in a biosafety cabinet for 5 min. The eight filter papers were added to wheat flour (10 g) in a sterile plastic bag and hand mixed for 3 min. The filter papers were then discarded, and the inoculated wheat flour seed sample was added to a larger wheat flour sample (90 g) and stomached (Masticator Basic, Neu-Tec Group Inc., Farmingdale, NY) for 3 min.

4.2.8 Sample conditioning

The inoculated wheat flour from each inoculation methodology was transferred to an a_w conditioning system to adjust the samples to a target a_w of 0.45. The conditioning system consisted of a equilibration chamber (69 cm x 51 cm x 51 cm) monitored by a custom computer-based control system comprised of relative humidity sensors inside the equilibration chamber, a desiccation column containing silica gel (VeriTemp, Encino, CA), a hydration column containing deionized water, solenoid valves, and air pumps, which maintained the chamber relative humidity within ±2%. Prior to thermal treatment, samples were conditioned for 4-6 days at the target 45% relative humidity for equilibration to the target a_w, which was subsequently confirmed using a a_w meter (n = 3) for each replication.

Inoculation homogeneity was evaluated by randomly removing ten ~1 g samples from an inoculated batch of flour (100 g) during the a_w conditioning period. *Salmonella* populations in

the samples were enumerated for each inoculation methodology, and homogeneity was reported as the standard deviation of the mean log population. Similarly, repeatability of inoculation was calculated as the standard deviation of the *Salmonella* population immediately before isothermal treatment ($n \geq 9$).

4.2.9 Isothermal treatment

The same isothermal inactivation methodology was used to obtain inactivation curves for *Salmonella*, regardless of the laboratory or inoculation methodology. Aluminum test cells (Chung and others 2008) were aseptically filled with inoculated and equilibrated flour (0.5 to 0.8 g, 4 mm thick) and immersed in a water bath (Neslab GP-400, Newington, NH) at 80.5°C. Come-up time was verified using an inoculated and equilibrated sample inside a test cell with a K-type thermocouple located at the center of the test cell. The come-up time (~90 s) for the sample core to reach within 0.5 of 80°C was used as time zero for the isothermal treatment. Thereafter samples were removed at ten uniform time intervals which were starting at time zero and immediately placed in an ice-water bath to stop the thermal inactivation ($T < 40$°C in ~20 s).

4.2.10 Recovery and enumeration

To enumerate *Salmonella* survivors, thermally treated wheat flour samples were aseptically transferred from the test cells to sterile plastic bags and diluted 10:1 in 0.1% peptone water. Appropriate serial dilutions were then plated in duplicate on mTSA. The plates were incubated (37°C for 48 h), and all black colonies were counted as *Salmonella* After converting the populations to log CFU/g, log reductions were calculated by subtracting the survivor counts from the population at time zero for the respective replicate.

4.2.11 Cross-laboratory comparison

Each inoculation methodology (A-E) and subsequent isothermal inactivation treatment was performed in triplicate at MSU. WSU independently repeated inoculation methodologies A, B, C, and D with subsequent isothermal inactivation treatment in triplicate, using the same materials and cultures. Materials obtained by MSU (sifted wheat flour and a_w conditioning chambers) were shared with WSU. Method E (DTM) was not repeated at WSU due to low initial inoculation levels.

4.2.12 Model regression and statistical analysis

In order to quantify *Salmonella* thermal resistance, the log-linear model was applied to the inactivation data sets, where:

$$log(\mathrm{N}) = log(\mathrm{N_0}) - \left(\frac{\mathrm{t}}{\mathrm{D_{80°C}}}\right) \quad (17)$$

and N and N_0 are the populations (CFU/g) at times t and 0, respectively, t is the time of the isothermal treatment (s) after the thermal come-up time, and $D_{80°C}$ is the decimal reduction time (s) at 80°C. Parameters for the log-linear model ($log(N_0)$ and $D_{80°C}$) were estimated via a single regression on the aggregated replicates for each inoculation treatment within a given laboratory, using MATLAB non-linear fitting tools (version 2014; MathWorks, Natick, Massachusetts). A non-linear regression was used to acquire symmetrical standard error estimates for $D_{80°C}$. Post-regression residual plots and normality of residuals analyses confirmed the appropriateness of the log-linear model. Parameter estimates and initial concentration levels were statistically compared

using the Student's t-test (α=0.05) calculated on Microsoft Excel (version 2013; Microsoft, Redmond, Washington), with pairwise comparisons evaluating differences between inoculation methodologies and data source. Inherent error associated with the inoculation methodologies was quantified by comparing standard errors for parameter estimates and root mean squared errors of the model fits to the population data sets (RMSE; eqn. 18).

$$RMSE = \sqrt{\frac{\sum\left(\log(\mathrm{N})_{\mathrm{predicted}} - \log(\mathrm{N})_{\mathrm{observed}}\right)^2}{\mathrm{n} - \mathrm{p}}} \quad (18)$$

where n is the number of samples, p is the number of parameters, and *N* (CFU/g) is the *Salmonella* population (CFU/g) at time *t*.

4.3 Results and Discussion

4.3.1 Wheat flour

Upon acquisition, the wheat flour had an a_w of ~0.46 and a geometric mean (± standard deviation) particle size of 144 ± 60 μm. The a_w values the inoculated wheat flour immediately before thermal inactivation at MSU and WSU was 0.440 ± 0.005 and 0.460 ± 0.009, respectively. The periodic sampling of uninoculated wheat flour all were below the LOD for *Salmonella*.

4.3.2 Inoculation repeatability

Inoculation homogeneity for methods A, B, C, D, and E were ± 0.24, 0.12, 0.42, 0.23, and 0.36 log CFU/g, respectively. The average post-conditioning *Salmonella* populations ranged from 4.93 to 8.71 log CFU/g, depending on methodology and laboratory (Table 4). Lawn-based

inoculation methodologies yielded higher concentrations of *Salmonella* during the equilibration/holding period (Keller and others 2012; Komitopoulou and Penaloza 2009; Uesugi and others 2006). Replication error of inoculation levels were consistent with the limited values previously reported (Farakos and others 2013; Keller and others 2012; Komitopoulou and Penaloza 2009; Uesugi and others 2006).

Table 4. *Salmonella* Enteritidis PT30 populations and resulting thermal resistance (D values) in wheat flour inoculated with different methodologies and subjected to 80ºC isothermal treatment.

Laboratory	Inoculation Methodology	Post-Conditioning Population[1,2] (log CFU/g)	Post-Come-Up Time Population[1,2] (log CFU/g)	$D_{80°C}$[2,3] (s)	RMSE (log CFU/g)
MSU	A (BLI)	6.18 (0.23)^{e}	3.86 (0.19)^{e}	504.9 (4.4)^{a}	0.204
MSU	B (LLI)	7.39 (0.50)^{d}	6.17 (0.31)^{b,c}	250.9 (2.6)^{b}	0.766
MSU	C (LPI)	8.29 (0.42)^{b}	7.81 (0.50)^{a}	285.9 (2.1)^{c}	0.385
MSU	D (DHM)	8.71 (0.24)^{a}	7.54 (0.51)^{a}	226.7 (1.7)^{d}	0.568
MSU	E (DTM)	6.33 (0.70)^{e}	4.79 (1.07)^{d}	N/A	N/A
WSU	A (BLI)	4.93 (0.16)^{f}	N/A[4]	N/A[4]	N/A[4]
WSU	B (LLI)	7.83 (0.31)^{c}	5.72 (0.26)^{c,d}	256.4 (2.4)^{b}	0.349
WSU	C (LPI)	7.85 (0.17)^{c}	7.28 (0.45)^{a,b}	293.6 (5.5)^{c}	0.571
WSU	D (DHM)	7.66 (0.43)^{c,d}	8.00 (0.39)^{a}	318.9 (7.5)^{e}	0.596
WSU	E (DTM)	N/A[5]	N/A[5]	N/A[5]	N/A[5]

[1] Values are reported as the mean (±standard deviation) of plate counts
[2] Within columns, values sharing a common letter are not significantly different ($\alpha = 0.05$).
[3] Values are reported as the parameter estimate (±standard error) yielded from regression analyses
[4] Values could not be estimated due to low *Salmonella* population levels
[5] Inoculation method E was not replicated at WSU

Although significant differences ($P < 0.05$) were observed between the MSU and WSU inoculation levels for methods A, C, and D, the differences were only 0.44 log CFU/g for methodologies B and C and ~1 log CFU/g for methodologies A and D. Because this experiment was replicated in only two laboratories, there is uncertainty in the reproducibility of the initial

population, population replication error, and population homogeneity within a sample; therefore, a larger group of laboratories is needed to replicate the inoculation methodologies in order to reduce uncertainty in these estimates of variability. Examples suggesting the reproducibility of inoculation for low-moisture foods are limited; however, studies using the Danyluk et al (2005) almond inoculation methodology as a template for almond kernel inoculation reported initial mean *Salmonella* populations of 7.8 to 8.9 log CFU/g (Abd and others 2012; Danyluk and others 2005; Du and others 2010; Harris and others 2012; Jeong and others 2012; Uesugi and others 2006).

4.3.3 Thermal resistance

Inoculation methodology significantly impacted the thermal resistance of *Salmonella* (Table 4 and Figure 4). Inoculation method A (BLI) yielded the highest D-value (~505 s), but also yielded the largest decrease in population during equilibration (~4 log). Consequently, due to the low post-conditioning population levels, WSU was unable to reliably estimate thermal resistance. The higher thermal resistance of broth-based cultures contradicts trends presented by Keller et al. (2012); however, they investigated the effects of growth media on thermal resistance of *Salmonella* in a different food matrix (peanut butter emulsion) (Keller and others 2012).

Cross-laboratory comparisons of D-values yielded differences within 3, 3, and 41% for lawn-based inoculation methods B, C, and D, respectively (Table 4). The larger cross-laboratory difference in estimated D-values for inoculation method D (DHM) may have resulted, in part, from nutrient uptake from the solid medium when the inoculum was incorporated into the wheat flour with a plate spreader. The level of incidental nutrient/media removal with the harvested *Salmonella* depended heavily on the individual performing the procedure, and could have altered the bacterial response in the wheat flour during thermal inactivation; therefore, based on this

observation and resulting difference in D-values, method D was not considered sufficiently repeatable.

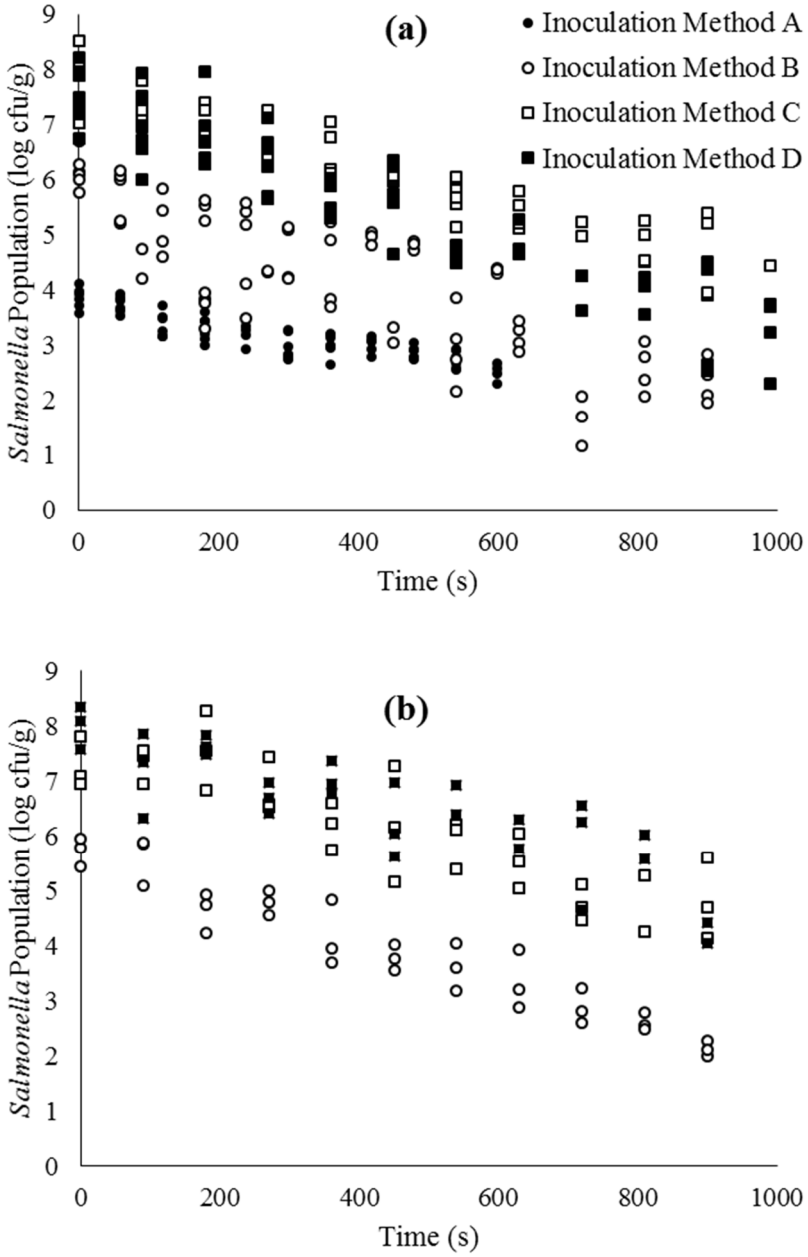

Figure 4. *Salmonella* Enteritidis PT30 survivors during isothermal treatment (80ºC) of inoculated wheat flour, from: (a) MSU and (b) WSU laboratories, using inoculation methods A, B, C, and D (as described in the text). Inoculation method D was not performed by WSU.

Inoculation methods B (LLI) and C (LPI) yielded thermal resistance parameter estimates that were statistically different across inoculation methodologies and laboratories ($P<0.05$); however, the nominal differences between the parameter estimates using the same inoculation methodology across the two laboratories were less than 3%. Inoculation method C consistently yielded higher D-values than method B (at both laboratories) despite an identical *Salmonella* cocktail, heat treatment, a_w, and food matrix. The addition of water to the wheat flour from the re-suspended *Salmonella* inoculum used in method B may result in a *Salmonella* population less able to survive in the low a_w environment. This small difference in inoculation methodology resulted in ~35 s (~14%) difference in D-values. Though inoculation methods B and C are considered to be repeatable, further research including replication across additional independent laboratories is needed in order to more fully characterize the repeatability of results generated by each inoculation methodology.

The method of inoculation clearly influences the thermal resistance of *Salmonella*. Prior to this study, the literature investigating the effect of different inoculation methods on *Salmonella* survival (Blessington and others 2013; Komitopoulou and Penaloza 2009; Uesugi and others 2006) and thermal resistance (Keller and others 2012) in low-moisture foods was limited to two inoculation methods each and two specific products (nuts and peanut butter). For most low-moisture products, the impact of inoculation methodologies on thermal resistance has not been evaluated (do Nascimento and others 2012; Farakos and others 2014a; Feng and others 2007; Jung and Beuchat 1999; Nummer and others 2012); for the few products with evaluated inoculation methodologies (almond kernels, peanut butter, and flour), there are other inoculation methodologies used in the literature that have not been investigated, in terms of repeatability and inherent uncertainty (He and others 2013; Izurieta and Komitopoulou 2012; Li and others 2014;

Ma and others 2009; Shachar and Yaron 2006; Villa-Rojas and others 2013). Of the five inoculation methods investigated in this study, only methods B (LLI) and C (LPI) produced results that were deemed sufficiently stable and repeatable to be used reliably when comparing or aggregating data across multiple studies or laboratories. Inoculation methods B and C, which were very similar, yielded $D_{80°C}$ values that differed by ~14%. This result suggested that, beyond having unknown stability and repeatability, other un-evaluated inoculation methodologies for low-moisture foods may have important effects on the reported thermal resistance of *Salmonella*.

In addition to the inoculation method, the isothermal inactivation method also may influence observed thermal resistance (Chung and others 2007; Sorqvist 1989; Zimmermann and others 2013), adding additional uncertainty when comparing results. Zimmermann and others (2013) reported that "test methods are at least as important in determining thermal processes as the micro-organisms and media used." Unfortunately, the inoculation and isothermal inactivation methods used in studies quantifying the thermal resistance of *Salmonella* are highly variable. Consequently, combining results from multiple laboratory sources (e.g., in reviews, meta-analyses, industry processes) may propagate errors due to variability across methods. For example, in their analysis of D-values for *Salmonella*, van Asselt and Zwietering (2006) observed that most factors reported to influence thermal resistance are irrelevant compared to the large variability in reported D-values in the literature (van Asselt and Zwietering 2006). While error is inherent to every experiment, variability between future studies can be reduced by incorporating pre-evaluated experimental methodologies with known reproducibility and minimal artifacts influencing the resulting thermal resistance. Future experiments can use the

recommended inoculation methodologies evaluated by this study to reduce variability within and across future studies involving similar low-moisture products.

4.4 Conclusion

Overall, the method of inoculation significantly influenced repeatability, survival, and measured thermal resistance of *Salmonella* during isothermal treatment in wheat flour. Of the five inoculation methods investigated, only methods B (LLI) and C (LPI) yielded a stable *Salmonella* population in wheat flour prior to isothermal treatment and consistent D-value estimates resulting from the tests repeated in two individual laboratories. Despite similar inoculation methods, the $D_{80°C}$ value for *Salmonella* was significantly larger with the inoculation method C (~285 s) than with inoculation method B (~250 s). Overall, the results of this study suggest that inoculation methodology can meaningfully impact the accuracy, precision, and overall results of experiments quantifying the thermal resistance of *Salmonella* in low-moisture products; therefore, variability between studies could be reduced with improved standardization of inoculation methodologies.

5. OVERALL CONCLUSIONS AND RECOMMENDATIONS

5.1 Effects of Experimental Methods on *Salmonella* Thermal Resistance

This thesis does not purport to uniquely quantify the true *Salmonella* thermal resistance in the tested materials, nor identify all sources of error and bias in developing inactivation models. Furthermore, the results of this thesis alone are not sufficiently conclusive to yield standard practices for *Salmonella* inactivation methods. However, the aggregated findings in this thesis are evidence that survival/inactivation kinetics reported in the literature are the inseparable product of experimental treatments, methods, and the biological response of *Salmonella*.

The first objective of this thesis was to evaluate the effects of experimental methods on *Salmonella* thermal resistance. Chapter 3 evaluated the effects of different thermal inactivation methods in ground beef, and Chapter 4 evaluated the specific effects of inoculation methods in wheat flour. In both chapters, a substantial impact on the subsequent thermal resistance was observed. Inoculation methods influenced the survival and inactivation kinetics of *Salmonella* in low-moisture foods, which supports results of prior research (Keller and others 2012; Li and others 2014). Importantly, the results of this study identified two inoculation methods that yielded populations with apparently repeatable thermal resistances. Because *Salmonella* research is dependent on the artificial contamination of food matrices, the results of this study provide valuable insight for future research.

The second objective of this thesis was to evaluate the effects of different thermal inactivation methods (inoculation, treatment, recovery, etc.) on subsequent estimates of thermal resistance (via inactivation model parameters). Although numerous other studies have reported the effects of thermal inactivation methods on microbial kinetics, this is the first study known to

directly compare the observed thermal resistance resulting from two different methodologies compared across two independent laboratories.

The application of two substantively similar *Salmonella* inactivation methods in ground beef yielded significantly different results. Unlike the study presented in Chapter 3, it is unknown which specific differences in the experimental methods significantly affected the results. However, given the lack of prior direct quantification of the impact of experimental methods on observed thermal resistance, the focus of this study was to quantify these differences, rather than to identify the specific causes at this point. Although the effects of all experimental thermal inactivation methods on observed thermal resistance are still poorly understood, the results presented in Chapters 3 and 4 demonstrate the potential of systematic error introduced by using different methods.

5.2 Regression and Model Selection

Regardless of model selection, one-step regressions provided a superior estimation of model parameters and the associated errors. This result reinforces findings from previous studies (Jewell 2012; Martino and Marks 2007). Multi-step regressions yielded similar model parameters but with larger model and parameter errors. The larger errors associated with multi-step regressions would adversely impact the utility of the model when applied to industrial processes, because of the larger resulting prediction intervals.

Because *Salmonella* inactivation kinetics are impacted by experimental methodologies, the choice of the best inactivation model could be biased by the inactivation methods, as well as *Salmonella* strain, food matrix, and the process treatment. Despite differences in the inactivation kinetics of *Salmonella* presented in Chapter 3, the Weibull model was the more likely correct model when fitted to data from each lab-method combination, except for the data generated by

MSU using MSU methods. However, when the resultant log-linear and Weibull models were applied to a single case study from a previous pilot-scale challenge study (Tenorio-Bernal and others 201X), the log-linear model more closely predicted measured thermal inactivation, regardless of the laboratory method used to estimate the model parameters. This case study emphasizes the importance of model validation procedures and experiments, which are not commonly reported in the literature.

Overall, the use of multi-step regressions yielded an inferior model for predicting process lethality, so that one-step regressions should be used whenever applicable. Because the results of this thesis indicate that experimental methods impact *Salmonella* inactivation kinetics, it is important that future experiments include model selection procedures. In order to further improve the utility of experiments, it is strongly recommended that prior and future studies publish raw inactivation data (and submit them to ComBase) in addition to the final model parameters.

5.3 Future Work

5.3.1 Evaluation of Salmonella inactivation methods

Because the results of this thesis indicate that experimental methods significantly impact *Salmonella* inactivation kinetics, it is critical that a broad evaluation of experimental methods is conducted in order to better understand the impact on subsequent data and models. Furthermore, standardization of experimental and regression methods should be considered to reduce cross-study variations. A future study could include a coalition of laboratories with prior thermal inactivation experience investigating the variability across and within isothermal inactivation methods. The first phase of the study would be to evaluate the current state of isothermal inactivation methods, where each laboratory would be given identical *Salmonella* strains and

food materials, and would execute an identical experimental design, but using whatever methods have been previously used in each individual laboratory. The second phase would be to repeat the first phase, but with each laboratory utilizing identical isothermal inactivation methods. Subsequent results would give insight into the variability in reported *Salmonella* thermal resistances caused by variability amongst methods and variability inherent to a single method, respectively.

5.3.2 Predictive model validations

There is a need to validate existing models reported from prior research, both by replicating experiments on a laboratory-scale and evaluating model performance when applied to a pilot- or commercial-scale process. Unfortunately, there is a lack of validation practices reported in the literature; therefore, there is a need to increase awareness of the importance of model validation experiments. Previously published thermal inactivation models have been used for the development of guidelines and industry analyses; however, the robustness and reliability of these models are relatively unknown. Because thermal inactivation methods impact *Salmonella* resistance, it unknown whether the models are appropriate for identical, similar, or different food products. The focus of future studies needs to shift from generating study-specific models to include the validation of prior and future lethality models.

5.3.3 Quantifying sources of error in pilot-scale experiments

The results from pilot-scale experiments have a direct utility for industrial processes; however, errors associated with scaled-up processes generally are poorly understood. While laboratory-scale experiments develop critical understanding of scientific principles, there is a

need to be a better understand how these principles translate into an industrial process. Therefore, future research is needed to identify and quantify sources of error in pilot-scale processes.

Specifically, a future study would use paired samples for evaluation of laboratory- and pilot-scale treatment. Using the same batch of inoculated food, parallel samples would be used to develop (via laboratory-scale experimentation) and validate (via pilot-scale processing) the predictive model. The resulting comparison should minimize uncertainty from product variation and limit post-hoc bias.

APPENDICES

Appendix A –ERRC and MSU Generated Data (Chapter 3)

This appendix includes the raw data used in the study discussed in Chapter 3.

Table 5. *Salmonella* data generated by ERRC using ERRC isothermal inactivation methods

	55°C		60°C		62°C	
Rep	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**
REP 1	0	8.40	0	8.38	0	8.51
	300	8.20	30	8.06	15	8.02
	600	7.36	60	7.74	30	7.34
	900	6.58	90	7.22	45	5.56
	1200	5.16	120	6.34	60	4.89
	1500	4.68	180	3.60	75	4.15
	1800	3.12	240	3.67	90	3.58
	2100	2.77	300	2.92	120	3.11
	2400	3.01	360	2.81	180	2.11
	2700	2.32	420	2.08	240	1.85
			480	1.00		
REP 2	0	8.41	0	6.40	0	8.34
	300	7.91	30	6.00	15	8.23
	600	7.55	60	5.75	30	7.52
	900	6.91	90	3.40	45	6.57
	1200	5.80	120	2.15	60	4.52
	1500	5.44	180	2.38	75	3.87
	1800	5.45	240	3.66	90	4.12
	2100	4.76	300	3.05	120	3.63
	2400	3.71	360	2.43	180	3.15
	2700	2.92	420	2.72	240	3.58
			480	2.79		
REP 3	0	8.41	0	8.39	0	8.42
	300	8.06	30	8.03	15	8.12
	600	7.45	60	7.74	30	7.43
	900	6.74	90	7.31	45	6.06
	1200	5.48	120	6.24	60	4.71
	1500	5.06	180	3.99	75	4.01
	1800	4.29	240	3.67	90	3.85
	2100	3.76	360	2.99	120	3.37
	2400	3.36	480	2.62	180	2.63
	2700	2.62	600	2.40	240	2.71
			720	1.90		

Table 6. *Salmonella* data generated by ERRC using MSU isothermal inactivation methods

	55°C		60°C		62°C	
Rep	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**
REP 1	0	7.15	0	6.68	0	3.83
	300	6.88	30	6.13	10	3.40
	600	6.14	60	5.88	20	2.90
	900	5.24	90	5.11	30	2.18
	1200	4.26	120	4.35	40	1.40
	1500	3.49	150	4.05	50	1.40
	1800	2.48	180	3.83		
	2100	1.40	210	2.54		
REP 2	0	7.03	0	6.72	0	5.16
	300	6.89	30	6.16	10	5.03
	600	6.36	60	5.86	20	4.42
	900	5.97	90	5.03	30	3.57
	1200	5.13	120	4.44	40	2.51
	1500	4.09	150	4.18	50	1.40
	1800	3.62	180	3.81	60	1.70
	2100	3.01	210	2.18		
	2400	2.10				
REP 3	0	7.09	0	6.70	0	4.50
	300	6.88	30	6.15	10.2	4.21
	600	6.25	60	5.87	19.8	3.66
	900	5.61	90	5.07	30	2.87
	1200	4.69	120	4.40	40.2	1.95
	1500	3.79	150	4.11	49.8	1.40
	1800	3.05	180	3.82	60	0.85
	2100	2.20	210	2.36		
	2400	1.05				

Table 7. *Salmonella* data generated by MSU using ERRC isothermal inactivation methods

	55°C		60°C		62°C	
Rep	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**
REP 1	0	7.56	0	7.37	0	7.79
	300	6.83	90	4.98	20	7.47
	600	6.05	180	4.52	40	5.72
	900	5.50	270	3.58	60	4.56
	1200	4.47	360	2.72	80	4.17
	1500	3.81	450	1.65	100	3.90
	1800	2.77			120	3.33
	2100	2.26			140	3.25
	2400	2.13			160	2.87
	2700	1.88			180	3.11
					200	1.18
REP 2	0	7.59	0	7.37	0	7.77
	300	6.37	90	5.10	20	6.87
	600	5.76	180	5.22	40	6.24
	900	4.34	270	3.59	60	4.85
	1200	3.94	360	2.65	80	4.36
	1500	3.13	450	1.48	100	4.53
	1800	2.26			120	4.12
	2100	2.18			140	2.69
	2400	1.95			160	2.61
	2700	1.18				
REP 3	0	7.53	0	7.98	0	8.01
	300	6.36	90	5.24	20	7.05
	600	5.64	180	3.91	40	5.65
	900	5.12	270	3.15	60	4.72
	1200	3.86	360	2.35	80	4.68
	1500	3.46			100	4.37
	1800	2.62			120	3.68
	2100	2.02			140	4.89
	2400	1.48			160	2.35
					180	1.48
					200	1.18

Table 8. *Salmonella* data generated by MSU using MSU isothermal inactivation methods

	55°C		60°C		62°C	
Rep	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**	**Time**	**Log CFU/g**
REP 1	0	7.31	0	6.84	0	4.95
	240	6.95	35	6.12	10	5.60
	480	6.71	70	6.28	20	4.87
	720	6.39	105	5.27	30	4.72
	1200	5.26	140	5.57	40	4.13
	1440	4.55	175	4.00	50	2.40
	1680	4.20	210	2.83	60	2.10
			245	2.40		
REP 2	0	6.98	0	6.01	0	6.06
	240	6.91	35	5.95	10	4.89
	480	6.56	70	4.92	20	5.71
	720	6.40	105	4.13	30	5.32
	960	5.82	140	2.54	40	4.98
	1200	5.34	175	1.70	50	4.18
	1440	4.44			60	3.08
	1680	4.17				
REP 3	0	6.69	0	6.57	0	4.71
	240	6.60	35	5.38	10	4.80
	480	5.94	70	5.45	20	4.64
	720	5.77	105	3.70	30	3.85
	960	4.44	140	5.36	40	3.84
	1200	4.82	175	2.81	50	2.40
	1440	3.88	210	1.70	60	2.30
	1680	3.89				

Appendix B –Homogeneity Data (Chapter 4)

This appendix includes the data used to estimate the homogeneity of inoculation in Chapter 4.

Table 9. Homogeneity of five *Salmonella* inoculation methods in wheat flour

Inoculation Method	Sample	Log CFU/g	Inoculation Method	Sample	Log CFU/g
A	1	5.07	D	1	7.94
	2	5.29		2	7.92
	3	4.91		3	8.49
	4	4.91		4	8.58
	5	5.35		5	8.32
	6	5.28		6	8.09
	7	4.86		7	8.11
	8	4.69		8	8.16
	9	4.83		9	8.06
	Average	5.02		Average	8.19
	Std. Deviation	0.25		Std. Deviation	0.23
B	1	7.38	E	1	5.24
	2	7.25		2	4.62
	3	7.10		3	4.26
	4	7.39		4	5.18
	5	7.33		5	5.30
	6	7.37		6	4.64
	7	7.19		7	5.30
	8	7.08		8	4.93
	9	7.18		9	5.29
				10	5.04
	Average	7.25		Average	4.98
	Std. Deviation	0.12		Std. Deviation	0.36
C	1	8.94			
	2	8.43			
	3	8.75			
	4	8.33			
	5	7.95			
	6	7.95			
	7	7.78			
	8	8.09			
	9	8.02			
	10	7.66			
	Average	8.19			
	Std. Deviation	0.42			

Appendix C –WSU and MSU Generated Data (Chapter 4)

This appendix includes data generated by WSU and MSU using five inoculation methods prior to isothermal inactivation.

Table 10. *Salmonella* data generated by WSU using inoculation methods B, C, and D

Method B		Method C		Method D	
Time	Log CFU/g	Time	Log CFU/g	Time	Log CFU/g
0	5.77	0	7.09	0	8.08
0	5.94	0	6.95	0	8.34
0	5.44	0	7.80	0	7.57
90	5.09	90	6.95	90	7.34
90	5.85	90	7.45	90	7.86
90	5.87	90	7.55	90	6.32
180	4.74	180	7.56	180	7.63
180	4.93	180	6.84	180	7.47
180	4.22	180	8.26	180	7.83
270	4.55	270	6.52	270	6.41
270	5.00	270	6.56	270	6.96
270	4.78	270	7.44	270	6.68
360	3.95	360	6.22	360	7.36
360	4.82	360	5.74	360	6.94
360	3.69	360	6.59	360	6.75
450	4.02	450	5.16	450	6.96
450	3.75	450	6.16	450	5.61
450	3.55	450	7.28	450	6.03
540	4.03	540	5.40	540	6.38
540	3.59	540	6.20	540	6.93
540	3.19	540	6.11	630	6.30
630	3.92	630	5.05	630	5.75
630	2.88	630	6.04	720	6.56
630	3.19	630	5.53	720	6.24
720	3.23	720	4.45	720	4.64
720	2.81	720	5.10	810	5.57
720	2.60	720	4.69	810	6.01
810	2.54	810	4.24	900	4.41
810	2.48	810	5.28	900	4.03
810	2.79	900	4.12		
900	2.00	900	5.61		
900	2.28	900	4.70		
900	2.11				

Table 11. *Salmonella* data generated by MSU using inoculation methods A, B, C, D, and E

Method A		Method B		Method C		Method D		Method E	
Time	Log CFU/g	Time	Log CFU/g	Time	Log CFU/g	Time	Log CFU/g	Time	Log CFU/g
0	3.85	0	6.14	0	7.46	0	6.74	0	5.42
0	4.11	0	6.71	0	7.91	0	7.20	0	5.49
0	3.98	0	6.28	0	8.02	0	7.28	0	4.93
0	3.93	0	5.77	0	8.51	0	7.49	0	3.74
0	3.57	0	6.10	0	7.91	0	7.89	0	4.32
0	3.73	0	6.00	0	7.04	0	7.97	0	3.83
60	3.82	60	6.00	90	7.47	0	8.21	0	3.89
60	3.88	60	6.08	90	7.79	90	6.00	60	4.64
60	3.93	60	6.16	90	7.52	90	6.56	60	4.61
60	3.68	60	5.20	90	7.14	90	6.72	60	2.60
60	3.55	60	5.26	90	7.27	90	6.96	60	3.08
60	3.64	90	4.22	90	6.99	90	7.46	60	2.49
120	3.18	90	4.74	90	6.69	90	7.93	120	4.56
120	3.49	120	5.44	180	6.83	180	6.29	120	3.48
120	3.74	120	5.85	180	7.39	180	6.39	120	4.38
120	3.51	120	4.60	180	7.26	180	6.67	120	2.66
120	3.26	120	4.89	270	6.50	180	6.71	120	1.90
120	3.18	180	5.54	270	6.43	180	6.98	180	3.87
180	3.11	180	5.26	270	7.26	180	7.95	180	3.75
180	3.00	180	5.63	270	6.25	270	5.64	180	3.90
180	3.60	180	3.85	360	6.19	270	5.69	180	2.19
180	3.45	180	3.30	360	7.04	270	6.26	180	1.54
180	3.45	180	3.78	360	6.78	270	6.57	240	3.38
180	3.22	180	3.95	360	6.12	270	6.67	240	4.77
240	2.93	240	5.18	450	5.97	270	7.12	300	3.69
240	3.19	240	5.43	450	5.98	360	5.31	300	3.95
240	3.35	240	5.57	450	6.03	360	5.49	300	3.30
240	3.28	240	4.11	450	6.19	360	5.90	300	1.30
300	2.81	240	3.50	450	6.08	360	6.03	360	3.41
300	2.74	270	4.33	540	5.15	450	4.65	360	3.59
300	2.98	270	4.35	540	5.96	450	5.59	360	0.70
300	3.25	300	5.09	540	5.82	450	5.74	420	3.99
300	3.28	300	5.14	540	5.57	450	6.23	420	3.16
300	2.85	300	4.23	540	6.05	450	6.35	420	3.10
300	2.78	300	4.22	540	5.67	540	4.49	420	3.17
360	2.96	360	5.24	630	5.12	540	4.55	480	2.88
360	3.00	360	5.32	630	5.54	540	4.64	480	2.66
360	3.20	360	4.90	630	5.79	540	4.81	480	3.66
360	2.98	360	3.85	630	5.21	630	4.66	480	1.18

Table 11. (cont'd)

360	3.22	360	3.70	720	4.99	630	4.75	480	0.70
360	2.64	420	5.04	720	5.24	630	5.28	540	3.72
360	3.14	420	4.99	810	5.00	720	3.62	540	2.77
420	2.79	420	4.82	810	5.26	720	4.25	540	1.30
420	3.17	450	3.33	810	4.51	720	4.26	600	2.57
420	3.10	450	3.04	810	4.54	810	3.57	600	2.42
420	3.08	480	4.89	900	3.90	810	4.08		
420	2.94	480	4.74	900	5.22	810	4.23		
480	2.76	480	4.84	900	5.40	900	2.54		
480	2.80	540	4.72	900	3.96	900	2.65		
480	2.90	540	3.13	990	4.45	900	4.37		
480	2.94	540	3.88			900	4.51		
480	3.05	540	2.18			990	2.30		
480	2.77	540	2.74			990	3.23		
540	2.93	600	4.39			990	3.71		
540	2.56	600	4.30			990	3.75		
540	2.92	600	4.38						
540	2.68	630	3.04						
540	2.80	630	2.89						
540	2.57	630	3.28						
600	2.59	630	3.45						
600	2.68	720	2.08						
600	2.30	720	1.70						
600	2.49	720	1.18						
		810	2.38						
		810	2.08						
		810	3.07						
		810	2.79						
		900	2.48						
		900	2.85						
		900	2.10						
		900	1.95						

Appendix D – Photographs of Inactivation Methods Materials (Chapter 4)

Figure 5. Closed and open aluminum test cells filled with wheat flour that were used for thermal inactivation studies

Figure 6. Equilibrium chamber conditioning wheat flour samples to 0.45 a_w

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