EXPERIMENTAL VALIDATION OF A PREDICTIVE MODEL FOR *SALMONELLA* GROWTH IN RAW GROUND BEEF UNDER DYNAMIC TEMPERATURES

By

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ABSTRACT OF THE THESIS

Experimental Validation of a Predictive Model For *Salmonella* Growth In Raw Ground Beef Under Dynamic Temperatures

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When food is transported at ambient temperatures for extended periods of time, or when power is lost during natural disasters; foodborne pathogens can multiply. Current US Food and Drug Administration (FDA) Model Food Code guidelines state that food can be kept out of temperature control for up to 4 h or up to 6 h if the food product starts at an initial 41°F (5°C) and the temperature does not exceed 70°F (21°C). This project validates existing ComBase computer models for *Salmonella* spp. growth under changing temperature conditions in raw ground beef as model system, using scenarios that would exceed Food Code guidelines. This thesis is separated into a literature review describing FDA Model Food Code guidelines, *Salmonella* prevalence and concentration in ground beef, and dynamic models for bacterial growth (I) and experimental validation of ComBase computer models for *Salmonella* spp. growth in raw ground beef (II). The growth rate of a 5-strain cocktail of *Salmonella* spp. meat isolates was

inoculated in 20% fat ground beef at a concentration of 4-log CFU/g. Inoculated ground beef samples were temperature abused for different lengths of time and to different maximum temperatures. The temperature profiles represent loss of proper refrigeration, warming and then cooling following a linear temperature gradient. A total of 9 different conditions were studied. Results show that when maximum temperatures were low, there was generally good agreement between the ComBase models and experiments. When maximum temperatures were closer to the optimum growth temperature for *Salmonella* (37°C), predictive models were fail-safe. It appears that faster cooling times limit the growth of *Salmonella*, so rapidly cooling foods (e.g. in a freezer) after extended temperature abuse can work as a risk mitigation measure. Validation of these models will be useful to extension professionals advising consumers, restaurateurs transporting food in unrefrigerated vehicles, and retailers facing a power outage. These finding may also be useful to those seeking to improve the science base of the FDA Model Food Code.

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Chapter I – Literature Review

I.1 Time and temperature guidelines in the FDA food codes

The current Food and Drug Administration (FDA) guidelines covering time and temperature recommendations for potentially hazardous foods (i.e. foods that require temperature control for safety) are contained in the 2009 FDA food code (FDA, 2009). The 2009 code references a supplement to the 2005 FDA food code where the use of time alone as a public health control was first considered, this information was originally discussed in the 2000 Conference for Food Protection Conference in a position paper (FDA, 2005) (Figure I.1). Chapter 3, section 5 of the 2009 FDA food code describes the public health concern regarding limiting microbial growth by a means of time and temperature control. This section (3-501.19) titled, "Time as a Public Health Control," contains guidelines for holding food outside proper holding temperatures: above 40°F (4.4°C) for cold foods or below 135°F (57.2°C) for hot foods (FDA, 2009).

These guidelines form the basis of state and local regulations that supermarkets and restaurants must follow when holding or transporting foods. The guidelines are brief yet complex, with only two scenarios given for food held outside of temperature control. It is important to note how these guidelines were created and what assumptions may underlie any scientific basis.

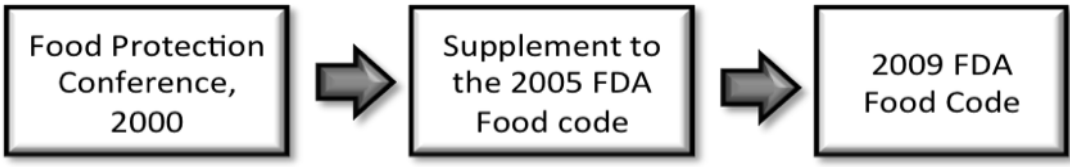
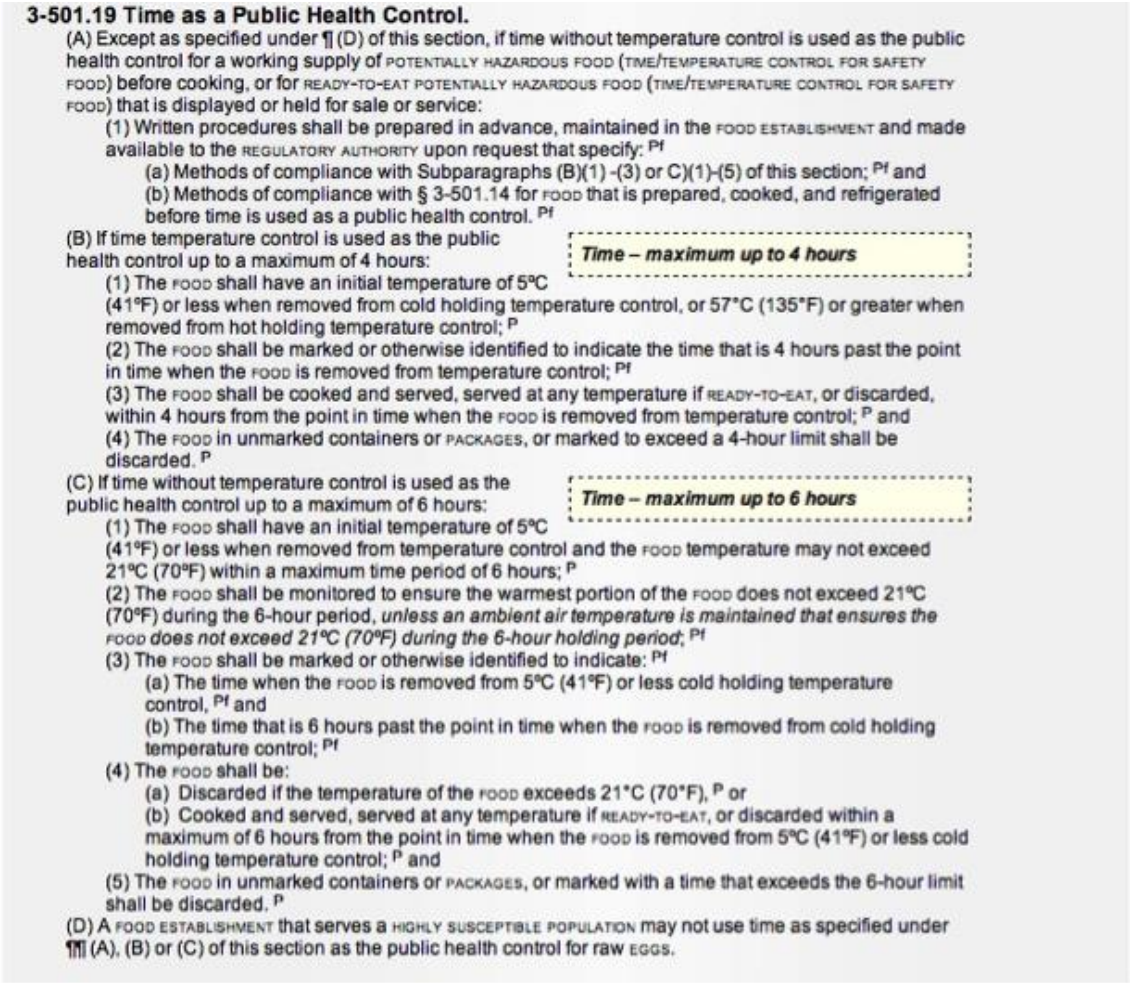


Figure I.1 Time and temperature guidelines in FDA food codes: The flow of information into the most current FDA food code concerning both time and temperature for ready-to-eat and potentially hazardous foods.

According to the FDA, potentially hazardous foods (i.e. foods that require temperature control for safety) are capable of supporting rapid microbial growth of organisms able to cause infections or produce toxins. Potentially hazardous food were once defined to be foods of animal or plant origins with a pH above 4.6 at 24°C (75°F) and a water activity (Aw) of or more than 0.85. A review conducted by the Institute of Food Technologists expert panel, under contract from FDA has suggested changes to that traditional definition (Busta, Bernard, Gravani, & Hall, 2003) to reflect a more nuanced understanding. Raw ground beef was chosen for this study because it meets all of the requirements of a potentially hazardous food and has been the subject of major recalls from contamination by foodborne illness-causing microbes (CDC, 2012, 2013a, 2013b; Dechet et al., 2006; Glynn, Bopp, & Dewitt, 1998; McLaughlin, Castrodale, Gardner, Ahmed, & Gessner, 2006; Roels et al., 1997).

There are two main guidelines for holding foods out of temperature control. The Food Code stipulates that potentially hazardous foods can be held out of temperature control for 4 h and then cooked and/or consumed safely, or can be held out of temperature control for 6 h if the starting temperature is below 41°F (5°C) and does not exceed 70°F (21.1°C) at the end of 6 h. If the product reaches 70°F (21.1°C) between 4 h and 6 h time frame, it should be discarded (FDA, 2009) (Figure I.2). There are also guidelines for the storage of

hot foods; however, cold foods and temperature storage are the focus of this thesis.



3-501.19 Time as a Public Health Control.

(A) Except as specified under ¶ (D) of this section, if time without temperature control is used as the public health control for a working supply of POTENTIALLY HAZARDOUS FOOD (TIME/TEMPERATURE CONTROL FOR SAFETY FOOD) before cooking, or for READY-TO-EAT POTENTIALLY HAZARDOUS FOOD (TIME/TEMPERATURE CONTROL FOR SAFETY FOOD) that is displayed or held for sale or service:

(1) Written procedures shall be prepared in advance, maintained in the FOOD ESTABLISHMENT and made available to the REGULATORY AUTHORITY upon request that specify: Pf

(a) Methods of compliance with Subparagraphs (B)(1) -(3) or C)(1)-(5) of this section; Pf and

(b) Methods of compliance with § 3-501.14 for FOOD that is prepared, cooked, and refrigerated before time is used as a public health control. Pf

(B) If time temperature control is used as the public health control up to a maximum of 4 hours:

Time – maximum up to 4 hours

(1) The FOOD shall have an initial temperature of 5ºC (41ºF) or less when removed from cold holding temperature control, or 57°C (135°F) or greater when removed from hot holding temperature control; P

(2) The FOOD shall be marked or otherwise identified to indicate the time that is 4 hours past the point in time when the FOOD is removed from temperature control; Pf

(3) The FOOD shall be cooked and served, served at any temperature if READY-TO-EAT, or discarded, within 4 hours from the point in time when the FOOD is removed from temperature control; P and

(4) The FOOD in unmarked containers or PACKAGES, or marked to exceed a 4-hour limit shall be discarded. P

(C) If time without temperature control is used as the public health control up to a maximum of 6 hours:

Time – maximum up to 6 hours

(1) The FOOD shall have an initial temperature of 5ºC (41ºF) or less when removed from temperature control and the FOOD temperature may not exceed 21ºC (70ºF) within a maximum time period of 6 hours; P

(2) The FOOD shall be monitored to ensure the warmest portion of the FOOD does not exceed 21ºC (70ºF) during the 6-hour period, *unless an ambient air temperature is maintained that ensures the FOOD does not exceed 21ºC (70ºF) during the 6-hour holding period*; Pf

(3) The FOOD shall be marked or otherwise identified to indicate: Pf

(a) The time when the FOOD is removed from 5ºC (41ºF) or less cold holding temperature control, Pf and

(b) The time that is 6 hours past the point in time when the FOOD is removed from cold holding temperature control; Pf

(4) The FOOD shall be:

(a) Discarded if the temperature of the FOOD exceeds 21°C (70°F), P or

(b) Cooked and served, served at any temperature if READY-TO-EAT, or discarded within a maximum of 6 hours from the point in time when the FOOD is removed from 5ºC (41ºF) or less cold holding temperature control; P and

(5) The FOOD in unmarked containers or PACKAGES, or marked with a time that exceeds the 6-hour limit shall be discarded. P

(D) A FOOD ESTABLISHMENT that serves a HIGHLY SUSCEPTIBLE POPULATION may not use time as specified under ¶¶ (A), (B) or (C) of this section as the public health control for raw EGGS.

Figure I. 2 Section 3-501.19 of the 2009 FDA food model code: Screen shot of section 3-501.19 Time as a Public Health Control in the 2009 FDA food model code.

I.2. Criticism of the FDA food codes

The Conference for Food Protection position paper contained in the 2005 Food Code gives a detailed account of the science in support of the 4 h and 6 h guidelines, but without including any citations to the primary scientific literature. The primary microorganism of concern for holding cold food without temperature control identified in the position paper is *Listeria monocytogenes*. There is a zero tolerance of this organism in the food industry; however, there are conditions for which ready to eat (RTE) foods held outside of temperature control could permit growth of the organism. *L. monocytogenes* was the primary concern because of its ability to grow at refrigeration and room temperatures leaving foods initially brought out of temperature control at risk. Ensuring that microorganisms with a zero tolerance policy cannot grow under allowable temperature regulations is a conservative measure to ensure safety. *Salmonella* spp. were also evaluated in the position paper. The position paper indicated that *L. monocytogenes* could growth faster than *Salmonella* spp. at room and refrigeration temperatures, so it was assumed that limiting *L. monocytogenes* growth would also limit *Salmonella* spp. growth (FDA, 2005).

The position paper makes claims based on USDA Pathogen Modeling Program (PMP) model predictions. The initial predictions made in the position paper for *L. monocytogenes* do not correspond to the current version of PMP

(Huang, 2013). The 4 h time regulation was made assuming *L. monocytogenes* growth under optimal conditions at a pH 6.8, 0.5% NaCl, 0.0% nitrite and a constant temperature of 75°F (23.8°C), which would yield a 1-log increase in 4 h (FDA, 2005). This should represent a worst-case scenario since the models are based optimal laboratory conditions using broth cultures and therefore growth in a foodstuff may not match those same conditions. Current PMP models show the log increase to be a 0.42 CFU in 4 h assuming a typical lag time or 1.82 log CFU increase in 4 h assuming no lag time (Schaffner, 2013). Figure I.3 shows ComBase predictions from the conditions used in the FDA Food Model Code using the programs default lag times, comparing growth of *Salmonella* to *L. monocytogenes. Salmonella* has a log increase of 0.6; while, *L. monocytogenes* has a log increase of only 0.15. It has also been demonstrated the *Salmonella* spp. has a higher predicted growth rate than *L. monocytogenes* at temperatures above 17°C (62.6°F) using the ComBase Predictor Program (Schaffner, 2013).

Microorganisms grow differently in different foods. *Salmonella enterica* strains has been observed to have a higher growth rate on lettuce vs. *L. monocytogenes* (Sant'Ana, Franco, & Schaffner, 2012). *L. monocytogenes* shows little to no growth in ground beef at 10°C (Nissen, Alvseike, Bredholt, Holck, & Nesbakken, 2000). *Salmonella* spp. growth should also be considered when using

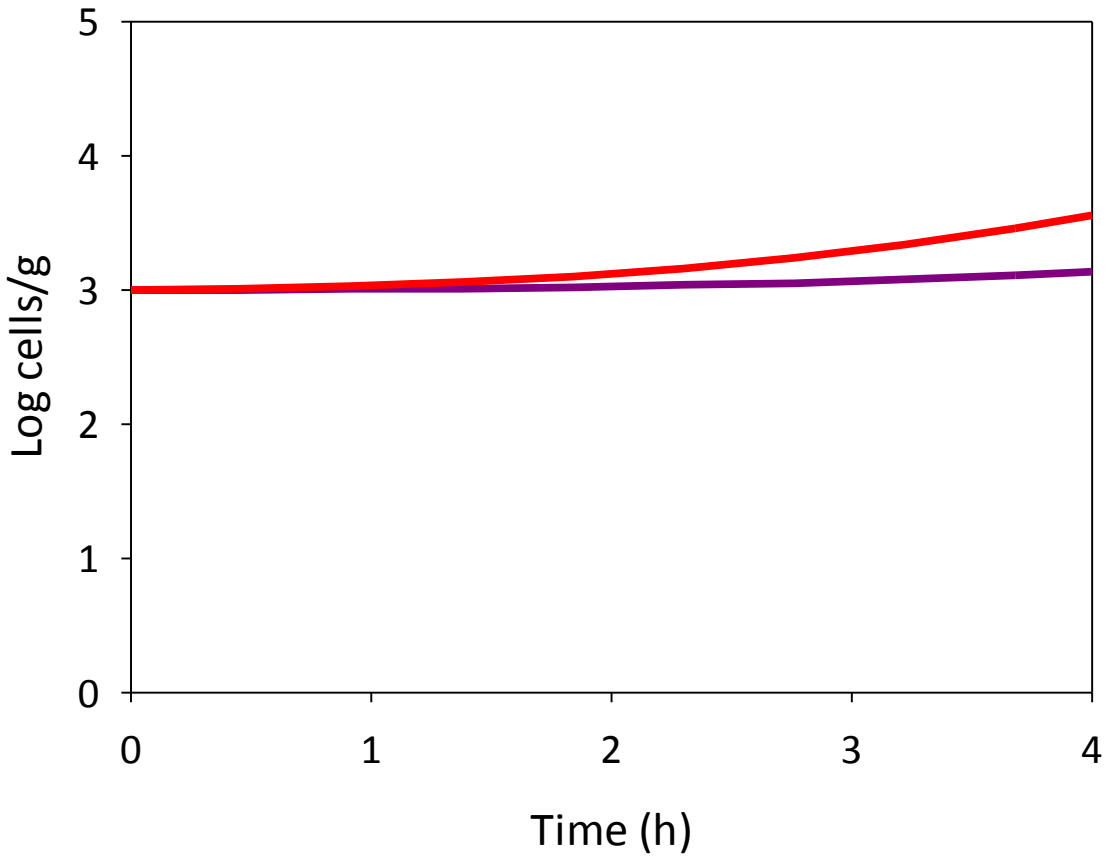


Figure I.3 ComBase prediction based on the original assumptions in the FDA Food Model Code: pH 6.8, 0.5% NaCl, 0.0% nitrite. The lag time has been left to default in the program: 0.019 for *Listeria monocytogenes* and 0.049 *Salmonella* The purple line represents Log cells/g from *L. monocytogenes* and the red line represents Log cells/g from *Salmonella*.

time as a public health control in the current Food Codes because it can grow over the temperature range of concern, and has a much lower median infectious dose when compared to *L. monocytogenes* (Schaffner, 2013).

I.3. *Salmonella* as an indicator organism

The USDA implemented the Pathogen Reduction/Hazard Analysis Critical Control Point in 1996 in a response to *Escherichia coli* O157:H7 outbreaks associated with ground beef (Schlosser et al., 2000; USDA, 1996). The program is a combination of microbial testing and with traditional sanitation inspections (USDA, 1996). Regular microbial testing began in January 1998 and was fully implemented by January 2000 from the largest facilities initially and then to smaller facilities (Schlosser et al., 2000).

Salmonella spp. was set as an indicator organism because of its common association in meat (USDA, 1996). Limits on *Salmonella* in meat and poultry were put into place to target enteric microbial contamination (Talbot, Gagnon, & Greenblatt, 2006). The Pathogen Reduction portion requires that *Salmonella* prevalence in ground beef must remain under the baseline level set to 7.5%, of positive identifications during a sliding sampling window (USDA, 1996). When the prevalence of Salmonella exceeds the baseline the facility must undergo further inspections, increase sampling, and report to the FSIS.

Salmonella enterica is a gram negative, rod shaped, facultative anaerobe from the Enterobacteriaceae family (Agbaje, Begum, Oyekunle, Ojo, & Adenubi, 2011). *Salmonella* taxonomy is constantly changing as new isolates are identified (Agbaje et al., 2011), but for the purposes of this paper this microorganism is simply identified as *Salmonella* spp. or simply *Salmonella* to cover general trends of isolates belonging to *S. enterica* unless otherwise noted. In humans, *Salmonella* can cause enteric fever (Typhoid), gastroenteritis (inflammation of the gastrointestinal tract), septicemia, and/or focal infections localized is specific areas (Agbaje et al., 2011). *Salmonella* infections commonly occur through the fecal-oral route, usually through food contamination with the exception of Typhoid strains which can be spread by an asymptomatic carrier.

I.4. Prevalence of *Salmonella*

Salmonella infections have increased with industrialization (Gomez, Motarjemi, Miyagawa, Käferstein, & Stöhr, 1997). Baseline surveys from the USDA Food Safety and Inspection Service recovered 1.0% samples positive for *Salmonella* in bulls and heifers in 1994 (USDA, 1994). The introduction of HACCP used the baseline of 7.5% positive tested samples of *Salmonella* in ground beef (USDA, 1996). A 1997 survey reported *Salmonella* in 38% of feed lots tested, with 5.5% of individual samples positive for the organism, and 4.8% of the individual samples representing serotypes capable of causing human salmonellonisis

(Fedorka-Cray, Dargatz, Thomas, & Gray, 1998). There are differences in the kinds of *Salmonella* species found in animal carcasses, but the most common are *Salmonella* species that result in disease in humans (Schlosser et al., 2000). A 2002 survey reported *Salmonella* spp. in 10.5% of recent arrival pens and 1.1% of individual samples tested (Sorensen et al., 2002). A difference between these studies may be due to the geographical location of the samples, or an overall reduction over time due to the PR/HACCP rule. The 1997 survey noted that *Salmonella* was less prevalent in northern states attributing to climate temperatures; however; this may not be the only variable since a later survey contained predominantly northern processors (Fedorka-Cray et al., 1998; Sorensen et al., 2002).

A 2002 retail study reported 3.5% contamination of *Salmonella* spp. in raw ground beef (Zhao, Doyle, Fedorka-Cray, Zhao, & Ladely, 2002). Other retail studies have reported similar findings in raw ground beef at 4.7% and 3.8%, respectively (Center for Veterinary Medicine, 2003; Samadpour et al., 2006). Retail studies have also isolated *Salmonella* in ground beef in as many as 6% of samples tested (White et al., 2001).

I.5. *Salmonella* Outbreaks

The largest outbreak of *Salmonella* in ground beef occurred in the North East region of the US in 2003 with reported cases in Maine, New Hampshire,

Vermont, Connecticut, Rhode Island, New York, Pennsylvania, and New Jersey (Dechet et al., 2006) with a total of 59 confirmed cases. CDC estimates that for every culture confirmed case of *Salmonella* there are an additional 38 unreported illnesses (Voetsch et al., 2004), which means this outbreak likely resulted in >2200 illnesses, most of which were not reported (Dechet et al., 2006). This outbreak lasted approximately 7 months, suggesting continual contamination from a reservoir of infected cattle or contamination of equipment in processing facilities. This was also the first multistate outbreak linked with the drug resistant strain *Salmonella enterica* serotype Typhimurium phage type DT104 increasing severity of illness (Dechet et al., 2006).

The spread of multidrug resistance is a cause for concern of *Salmonella* infections via foodborne illnesses. *S. enterica* Typhimurium D104 was found to be widespread in the United States and is commonly resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (Glynn et al., 1998). Research has reported little correlations of antibiotic resistance between *Salmonella* and generic *E. coli* when both were isolated from samples of ground beef (Zhao et al., 2002). Multidrug resistant strains of *Salmonella* are found in the retail end of meat products with more found in poultry than beef (White et al., 2001).

Hamburgers are more often linked to human salmonellosis than other food made from ground beef (Dechet et al., 2006). Meat grinding can be a potential source for contamination if equipment is not cleaned properly and can amplify initial contamination levels worsening outbreaks causing sustained contamination over time (Roels et al., 1997).

Since low concentrations (7.5% allowable positive samples in a sliding time-frame) of *Salmonella* are allowed in raw ground beef (USDA, 1996) improper cooking and temperature abuse has the potential to cause outbreaks. It is important for consumers to know and understand proper food handling and preparation. Outbreaks have been associated with food prepared in the home environment for potlucks (McLaughlin et al., 2006). Restaurateurs can be held to regulations and standards, while no control over the average consumer.

I.6. Risk assessment and risk reduction

No risk assessments for *Salmonella* in ground beef currently exist; however, several published *E. coli* 0157:H7 risk assessments in beef have been published. One of the earliest of such risk assessments concluded that the probability of *E. coli* O157:H7 infections causing Hemolytic Uremic Syndrome from ground beef is very low to the individual per meal ($3.7x10^{-6}$) and probability of death lower at $1.9X10^{-7}$ in the very young per meal (Cassin, Lammerding, Todd, Ross, & McColl, 1998). There are many ways to reduce the risk of foodborne

pathogens and proper storage temperature is one of the most effective means of control. Temperature is the key determining factor in microbial growth in ground beef packages (Cassin et al., 1998). In a quantitative risk assessment with *E. coli* O157:H7 in ground beef burgers, proper storage temperature at both retail and consumer levels reduced the risk of illness by 80%. Proper temperature was found to be more effective than reducing contamination than by urging consumers to cook ground beef more thoroughly (Cassin et al., 1998).

Irradiation has also been proposed as a means to reduce pathogen risk in ground beef. *Salmonella* in ground beef was less sensitive to gamma irradiation compared to *Campylobacter jejuni* or *E. coli* O157:H7 in a study that also considered irradiation temperature and fat content (Clavero, Monk, Beuchat, Doyle, & Brackett, 1994). Irradiation can be used for reduction of *Salmonella* in poultry and current guidelines permit irradiation as a means of reducing pathogens in ground beef.

Research has suggested that deliberately added lactic acid bacteria can compete with and reduce levels of pathogens at refrigeration temperatures without adverse affects to sensory properties (Smith, Mann, Harris, Miller, & Brashears, 2005). Lactic acid sprays have also been found to be effective in reducing *E. coli* O157:H7 and *Salmonella* contamination in ground beef when combined with pre-chilled treatments (carcasses held at 4°C for 24 h prior to

experiment) (Castillo et al., 2001). Organic acids have also shown to produce a reduction of *Salmonella* and *E. coli* O157:H7 with minimal sensory changes to ground beef (Harris, Miller, Loneragan, & Brashears, 2006; Pohlman, Stivarius, McElyea, Johnson, & Johnson, 2002).

Modified atmospheric packaging (MAP) can also be used to preserve meats. MAP improves shelf life, and helps maintain the bright pink color associated with ground beef. The low carbon monoxide (CO) concentration in the MAP helps maintain meat color, but does little to limiting bacterial growth (Nissen et al., 2000). Adding higher CO concentrations is controversial because it can maintain the pink fresh color can last beyond the shelf life (Nissen et al., 2000). High CO concentrations have been shown useful in controlling *L monocytogenes* and *Y. enterocolitica* growth (Nissen et al., 2000). Temperature control appears to be greater importance in limiting growth of *Salmonella*, when compared to MAP (Nissen et al., 2000).

Chapter II - PREDICITIVE MODELING AND EXPERIMENTAL VALIDATION OF *SALMONELLA* SPECIES IN A RAW GROUND BEEF

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II.1 Abstract

Temperature is a primary factor in controlling the growth of microorganisms in food. Current US Food and Drug Administration (FDA) Model Food Code guidelines state that food can be kept out of temperature control for up to 4 h or up to 6 h if the food product starts at an initial 41°F (5°C) temperature does not exceed 70°F (21°C) at 6 h. This project validates existing ComBase computer models for *Salmonella* spp. growth under changing temperature conditions modeling scenarios using raw ground beef as model system. A cocktail of *Salmonella* spp. isolated from different meat products (*Salmonella* Copenhagen, *Salmonella* Montevideo, *Salmonella* Typhimurium, *Salmonella* Saintpaul, and *Salmonella* Heidelberg) was used for all experiments. Inoculated samples were held in a programmable water bath at 4.4°C (40°F) and subjected to linear temperature changes to different final temperatures over different lengths of time, and then cooled back to 4.4°C (40°F). Maximum temperatures reached were 16, 27, or 37°C (98.6°F), and temperature rises took place over 4, 6, and 8 h with varying cooling times. Our experiments show that when maximum temperatures were lower (16°C or 27°C), there was generally good agreement between the ComBase models and experiments. For example, when temperature rises to 16°C or 27°C occurred over 8 h, experimental data were within 0.13-log CFU of model predictions. When maximum temperatures were

37°C, predictive models were fail-safe. In many of the conditions tested, a majority of growth happened after the temperature started the initial decline, but could still permit growth. Our experiments show FDA Model Food Code guidelines for holding food out of temperature control are quite conservative. Our research also shows that the ComBase models for *Salmonella* growth are accurate or fail-safe for dynamic temperature conditions as might be observed due to power loss due to natural disasters or during transport out of temperature control.

II.2 Introduction

Temperature is a primary factor in controlling the growth of microorganisms in food. When food is transported under warm conditions for an extended period of time, or when power is lost due to natural disasters, the safety of a food may be affected. Supermarkets and restaurants follow state or local regulations, which are in turn based on recommendations set in the FDA Model Food Code holding foods out of temperature control (FDA, 2005, 2009). The supplement to the 2005 FDA Model Food Code states that foods that require temperature control for safety can be kept out of temperature control for up to 4 h or up to 6 h if the food product starts at an initial 41°F (5°C) temperature does not exceed 70°F (21°C) (FDA, 2005). The scientific basis for these recommendations cites US Department of Agriculture (USDA) Agricultural Research Service (ARS) Pathogen Modeling Program (PMP) predictions for *Listeria monocytogenes* (FDA, 2005). Criticism of the scientific basis for these recommendations is discussed in detail elsewhere (Schaffner, 2013), but a brief summary of those criticisms follows: (1) The Model Food Code assumed *L. monocytogenes* to be the primary organism of concern. Depending on the assumptions and conditions, *Salmonella* (an enteric indicator for *Escherichia coli* OH157) may outgrow *L. monocytogenes* especially at higher temperatures. (2) The Model Food Code assumes that the food temperature changes

instantaneously once out of temperature control. In fact, the temperature of food changes gradually. The rate of temperature change is greatest when the food is placed in the new environment, and slows as the food and environmental temperatures converge. (3) The position paper is not clear regarding any assumptions about the organism's lag phase. This assumption of the existence of a lag time is a very critical one, since this can make a difference of more than an hour on the time scale or more than 1 log CFU on the growth scale. (4) The position paper assumed that no models could consider changing temperatures when predicting growth. This is not true today, as ComBase Predictor models allow dynamic temperature predictions in a relatively easy manner, where the user inputs the temperature profile over which growth is to be modeled.

Salmonella infections are a major concern, every 1 of 6 people are estimated to get sick from foodborne microbes with *Salmonella* being the second most common foodborne infectious agent behind norovirus (CDC, 2011). *Salmonella* infections commonly occur through the fecal-oral route, usually through food contamination with the exception of Typhoid strains, where an asymptomatic carrier may be to blame. Food products like meat, poultry, egg, fish, and fresh produce are common sources of salmonellosis. Ground beef is an ideal growth medium for *Salmonella* because it is a homogeneous product

containing no preservatives, leaving temperature as the primary means of preservation.

Previous literature shows growth of *Salmonella* in raw ground beef under increasing temperature (10°C-45°C) (Juneja, Melendres, Huang, Subbiah, & Thippareddi, 2009); however, dynamic profiles that incorporate a return to cooling temperatures have not been conducted. This would represent the growth that happens during refrigeration when food is returning back to proper holding temperatures. Dynamic growth assays (gradual temperature changes between 10°C and 30°C) have been performed with *Salmonella* in semi-skimmed milk showed that *Salmonella* growth corresponded most closely to the default lag times of a physiological state of α_0=0.045 (Bovill et al., 2000). The physiological state is the physical suitability of the cell to its environment (lag time) (Baranyi & Tamplin, 2004). In the ComBase predictor program, when this is left blank, the default lag time is used, which is based on a history of typical experiments commonly used for the particular model in use (Baranyi & Tamplin, 2004)

We seek to validate ComBase models to predict growth of *Salmonella* in ground beef under changing temperature conditions. We hypothesize that current growth models can be used to predict the growth of *Salmonella* in ground beef under changing temperature conditions that approximate power loss or consumer mishandling.

II.3. Methods

II.3.b. Materials

A cocktail of 5 *Salmonella* strains isolated from meat sources were provided by Dr. Vijay Juneja, USDA ARS Eastern Regional Research Center, and were used for al. experiments (Table 2). Strains were made resistant to rifampicin in order to differentiate them from the native flora present in ground beef. This was performed by inoculating isolated colonies of *Salmonella* in tryptic soy broth plus rifampicin (TSB + rif) at 50 μg/ml.

Ground beef (20% fat) used in these experiments was collected from local supermarkets no more than 1-day prior to use.

Tryptic soy broth (TSB) and TSB + rif was used to culture overnight broths of *Salmonella*. Tryptic soy agar + rifampicin at 50 μg/ml (TSA + rif) and xylose lysine tergitol 4 agar (XLT4) media were used to enumerate *Salmonella* spp. Negative controls were used to check for counts of the native flora on tryptic soy agar (TSA), rifampicin resistance (TSA + rif), and initial *Salmonella* presence (XLT4). A 5% peptone water solution was used for dilution buffer and washing cells to remove any residual growth media prior to inoculation.

Table II.1 Experimental Strains: Culture collection of *Salmonella* strains obtained from the USDA used in this project.

Strains	Name	Strain ID	Source
S21	*Salmonella* Typhimurium Copenhagen	8457	Pork
S24	*Salmonella* Montevideo	FSIS 051	Beef
S25	*Salmonella* Typhimurium	FSIS 026	Beef
S26	*Salmonella* Saint-Paul	FSIS 039	Beef
S40	*Salmonella* Heidelberg	F5038BG1	Stuffed ham

II.3.c. Inoculation

Salmonella strains were grown separately in TSB media at 37°C for 8 h and then held at refrigeration until use. Cultures were combined to a total volume of 1ml, centrifuged for 5 min at 5*g*, the supernatant was removed, and pellets were suspended in 5% peptone water. The process was repeated for a total of 3 times. The culture was diluted 1:1000 and 1ml was inoculated into 300g of ground beef, to give a starting concentration of about 4 log CFU/g. Ground beef was kneaded for 10 min and separated into 5g samples using sterile gloves. Kneading the samples by hand did change the composition of the ground beef; however, hand mixing is preferred to avoid possible inhalation from using a mechanical mixer. Preliminary experiments showed that this procedure provides even distribution of *Salmonella* cells throughout the ground beef. Inoculated ground beef samples were held under refrigeration (4.44°C) until initiation of the water bath program of approximately 8 h.

II.3.d. Temperature Profiles

Samples were held in a programmable water bath (Chiller Recirculating Water Bath RTE 17 and RTE 221, Thermo-NESLAB, Portsmouth, NH) following a linear profile starting at 40°F (4.44°C) and taking 4, 6, or 8 h to reach 60°F (15.55°C), 80°F (26.66°C), or 100°F (37.77°C). The samples were submerged in a rack that allowed water to flow between, for this reason the samples were

assumed to be the temperature of the waterbath. Samples were then cooled to 40°F (4.44°C) at a corresponding rate. Subsequent experiments tested faster cooling times, returning the samples to 4.44°C within 2 or 4 h. Constant temperature control experiments for growth of *Salmonella* in ground beef at 37°C were also performed.

II.3.e. Growth assay

Samples were tested at hourly intervals. Samples were homogenized in a lab stomacher (Stomacher Lab Blender 400, Cooke Laboratory Products, Alexandria, VA) at a 1:50 dilution of 5% peptone buffer for 2 min. Samples were plated in duplicate and experiments repeated for nearly all temperature profiles. *Salmonella* species were enumerated by spread plating using TSA + rif and XLT4 media. Negative controls were spread plated onto TSA, TSA + rifampicin, and XLT4 at the beginning, middle, and end of both heating and cooling sections of the temperature program. Plates were incubated at 37°C overnight and colonies enumerated.

II.3.f. Predictive Modeling

The online ComBase predictive modeling program was used to generate predictions for all experiments (Baranyi & Tamplin, 2004). The experiments (24 in total) included: 8 h heating series with 3 different cooling conditions (8 h, 4 h, and 2 h) reaching to 3 different maximum rising temperature (37.7°C, 26.6°C, and

15.5°C); 6 h heating series with 3 different cooling conditions (6 h, 4 h, and 2 h) reaching to 3 different maximum rising temperature (37.7°C, 26.6°C, and 15.5°C); and 4 h heating series reaching to 2 different cooling conditions (4 h and 2 h) with 3 different maximum rising temperature (37.7°C, 26.6°C, and 15.5°C). ComBase predictions were made for *Salmonella*, under changing temperature with the specific profiles used, at pH 5.7, assuming 0.5% NaCl, and either the default or zero lag times. Since ComBase does not allow *Salmonella* growth rate predictions below 7°C, any temperature below 7 °C was changed to 7°C when making predictions.

II.4. Results

II.4.a Experimental Growth Curves and Predictions

ComBase predictions for *Salmonella* growth in ground beef at constant temperature (37 °C) matched experimental results (data not shown).

Initial experiments showed that using XLT4 media for enumeration required a higher inoculum by approximately 1 log. For those purposes TSA+rif was used exclusively for all experiments.

Figure II.1a shows the most permissive temperature abusive profiles with the longest time spans of abuse. The right panel of Figure II.1a shows corresponding temperature profiles. The experimental data agree well with the model predictions for the two lower temperature profiles (those reaching 15.5°C

(60°F) and 26.6°C (80°F)). The model is fail-safe for the experiments where temperature profiles reach 37.7°C (100°F). In this case there is as much as an approximate 2-log difference between the experimental data and the model predictions. The model predicts a 4-log increase in the concentration of *Salmonella*, but the experimental data shows only a 2-3 log increase.

Figure II.1.b (left panel) shows the *Salmonella* growth profiles for 8 h heating followed by 4 h cooling. The corresponding temperature profiles are shown in the right panel of Figure II.1.b. As with Figure II.1.a (the 8 h heating and 8 h cooling), the experimental data agree well with the model predictions for the two lower temperature profiles (those reaching 15.5°C (60°F) and 26.6°C (80°F)). The model is fail-safe for the experiments where temperature profiles reach 37.7°C (100°F). In this case there is an approximate 1-log difference between the experimental data and the model predictions.

Figure II.1.c (left panel) shows the *Salmonella* growth profiles for 8 h heating followed by 2 h cooling. The corresponding temperature profiles are shown in the right panel of Figure II.1.c. As with two prior experiments (8 h heating and cooling; 8 h heating and 4 h cooling), the experimental data agree well with the model predictions for the two lower temperature profiles (those reaching 15.5°C (60°F) and 26.6°C (80°F)). The model is fail-safe for the experiments where temperature profiles reach 37.7°C (100°F). In this case there

is an approximate 2-log difference between the experimental data and the model predictions.

Figures II.2 show the *Salmonella* growth profiles 6 h heating series with a 6 h cool (a), 4 h cool (b), and 2 h cool (c) in the left hand panels and the temperature profiles in the right hand panels. In the 6 h heating and cooling experiment (Figure II.2.a), the prediction and experimental data show good agreement for the two lower temperature profiles (those reaching 15.5°C (60°F) and 26.6°C (80°F)). The experiment with the highest temperature profile, reaching 37.7°C (100°F) after 6 h, shows good agreement with prediction through 6 h, the heating portion of the profile. The prediction becomes increasingly fail-safe during the cooling portion of the profile, ending up with a difference of an approximate 1-log CFU at the completion of the experiment. Figure II.2.b shows a similar trend, where the data show good agreement with prediction through 6 h, the heating portion of the profile, with the prediction becoming fail-safe during the cooling portion of the profile, ending up with a difference of an approximate 0.5 log CFU at the completion of the experiment. This trend is not seen in Figure II.2.c for 6 h heating followed by 2 h cooling.

Figure II.3.a shows the experimental results and predictions for the 4 h heating, 4 h cooling experiments and Figure II.3.b shows the same for 4 h heating followed by 2 h cooling experiments. As seen in the other results above, the

prediction and experimental data show good agreement for the two lower temperature profiles (those reaching 15.5°C (60°F) and 26.6°C (80°F)). The experiment with the highest temperature profile, reaching 37.7°C (100°F) after 4 h, shows good agreement with prediction through 4 h, the heating portion of the profile, with the predictions becoming fail-safe during the cooling portion of the experiment. The fail-safe differences are generally less than 1 log CFU.

II.4.b Data summaries

Figure II.4 summarizes the difference in log increase by changes in cooling times. As noted above, predicted log increases (black and white bars) generally exceeded experimentally determined log increases (colored bars), with greater differences for greater log increases. Most of the experimental growth increases occur in the temperature increase phase of the experiment for the profiles that reached 37.7°C (100°F), and this becomes more so as cooling time shortens. This trend is even more pronounced in the experiments where profiles reached 26.6°C (80°F). The 15.5°F (60°F) experimental data and predictions are not shown in Figure II.4, due to the very small magnitude of the expected and actual increases.

Figure II.5 provides a summary of the observed log increase plotted against the corresponding predicted log increase data. The plot is bisected by the diagonal line-of-equivalence, and points which lie on this line represents exact agreement between experiment and prediction. Points above the line-of-

equivalence represent fail-safe predictions, where the model predicts more growth than that observed experimentally. Points below the line-of-equivalence represent fail-dangerous prediction, where the experiments show higher growth than the model predicts. It is clear from Figure II.5 that temperature profiles reaching a maximum of 37.7°C (100°F) (open circles) are overwhelmingly fail-safe, with the exception of a single point where the observed log increase was slightly greater than 1 log CFU. Temperature profiles reaching a maximum of 26.6°C (80°F) (closed squares) tend to be fail-dangerous, but in almost every case the observed log increase was less than 1 log CFU. Temperature profiles reaching a maximum of 15.5°C (60°F) (open triangles) tend to be fail-dangerous, but all increases are less than 0.5 log CFU, a value considered by some expert microbiologists (NACMCF, 2010) as representing true growth, rather than expected variation in bacterial counts.

Figure II.1.a

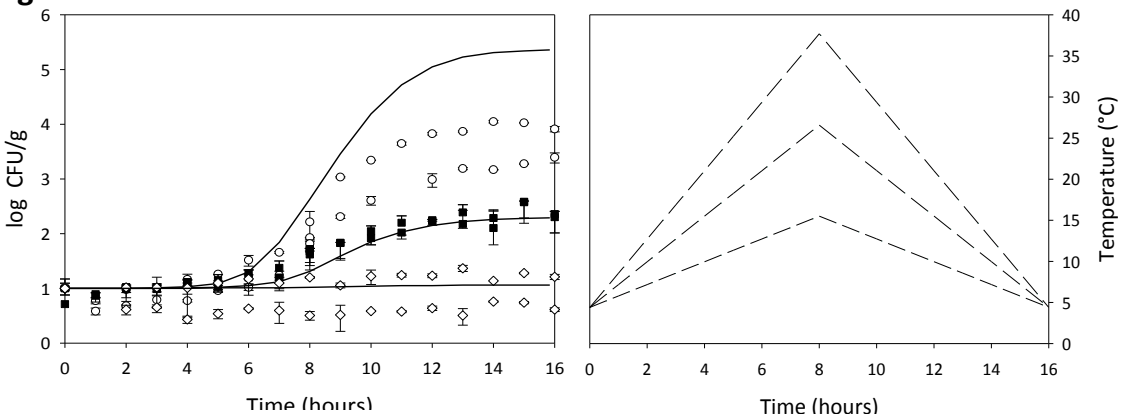


Figure II.1.b

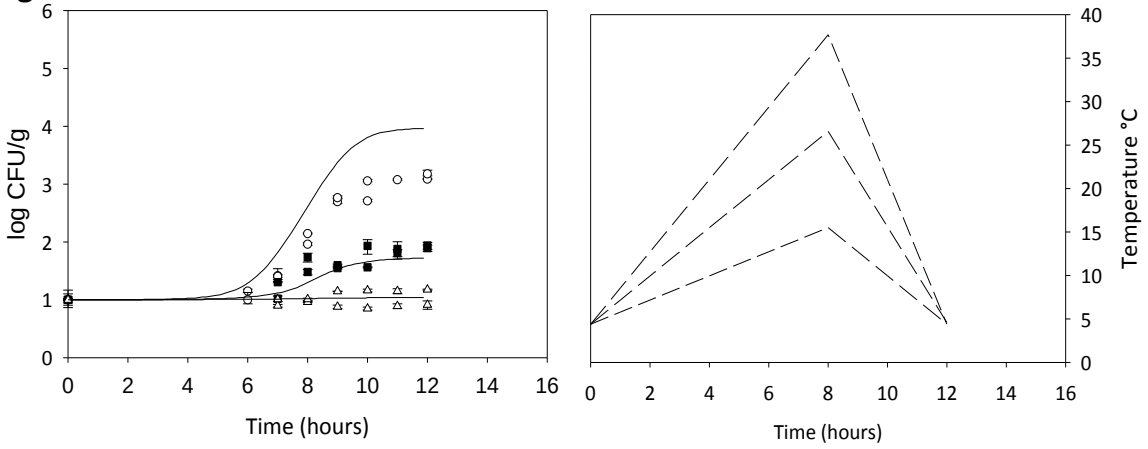
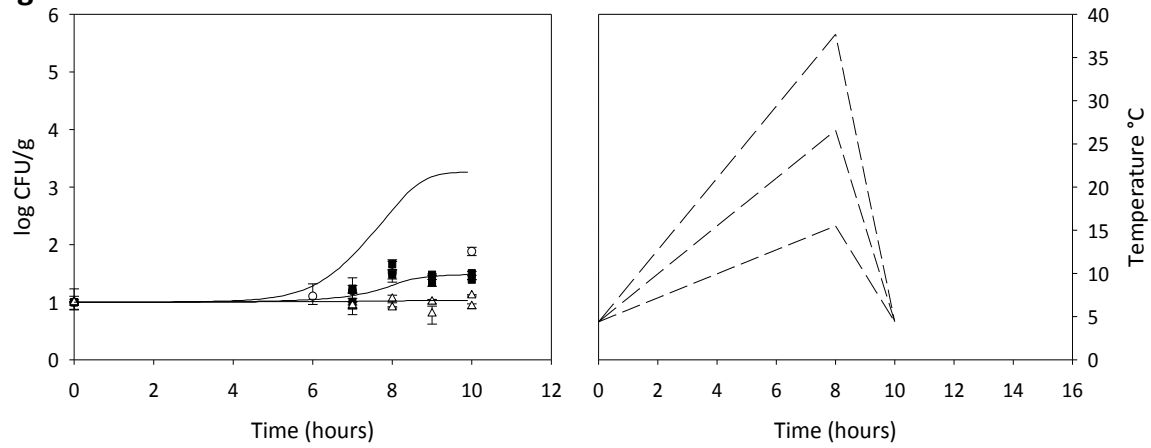


Figure II.1.c



Figures II.1. a-c Growth profiles of the 8 h heating series: with a 8 h cool (a), 4 h cool (b), and 2 h cool (c). The log CFU/g vs. time are on the left hand side with the corresponding temperature profiles on the right. All CFU data was normalized to 1 for ease of comparison. Open circles represent a maximum temperature of 37.7°C (100°F), closed squares represent a maximum temperature of 26.6°C (80°F), and open diamonds represent a maximum

temperature of 15.5°C (60°F). Solid lines represent the corresponding ComBase predictions and the dashed lines are the temperature profiles

Figure II.2.a

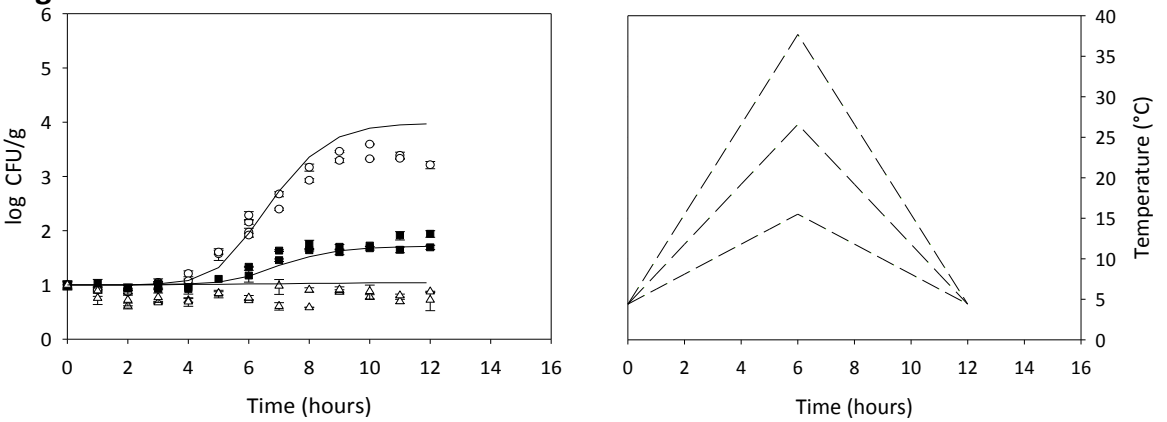


Figure II.2.b

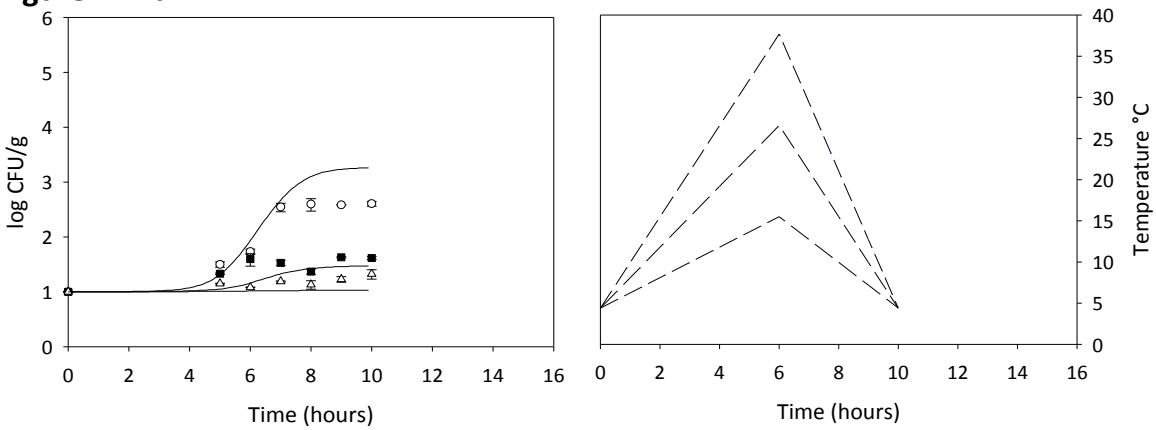
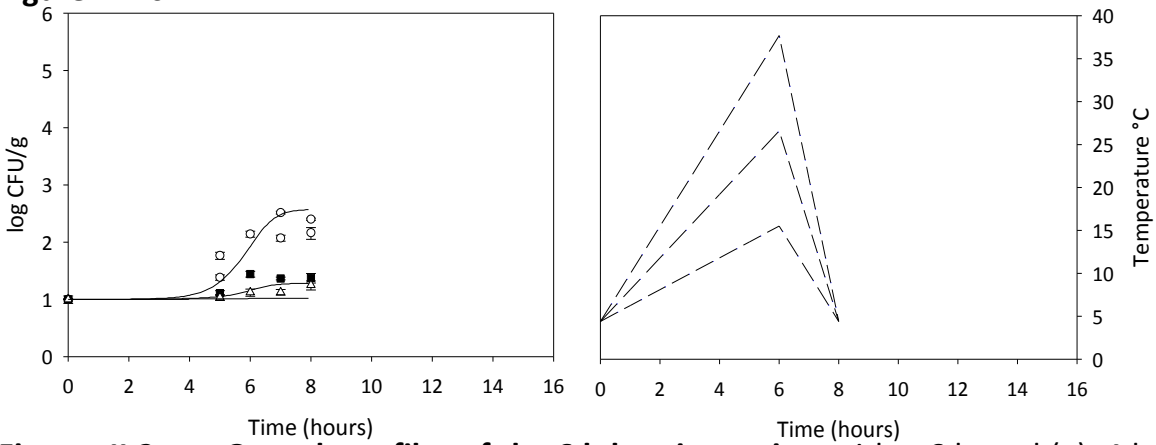


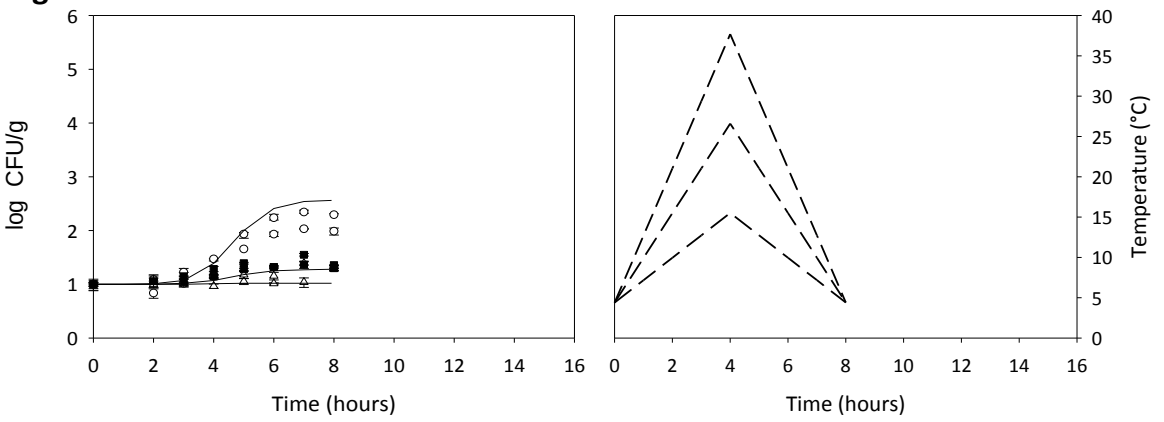
Figure II.2.c



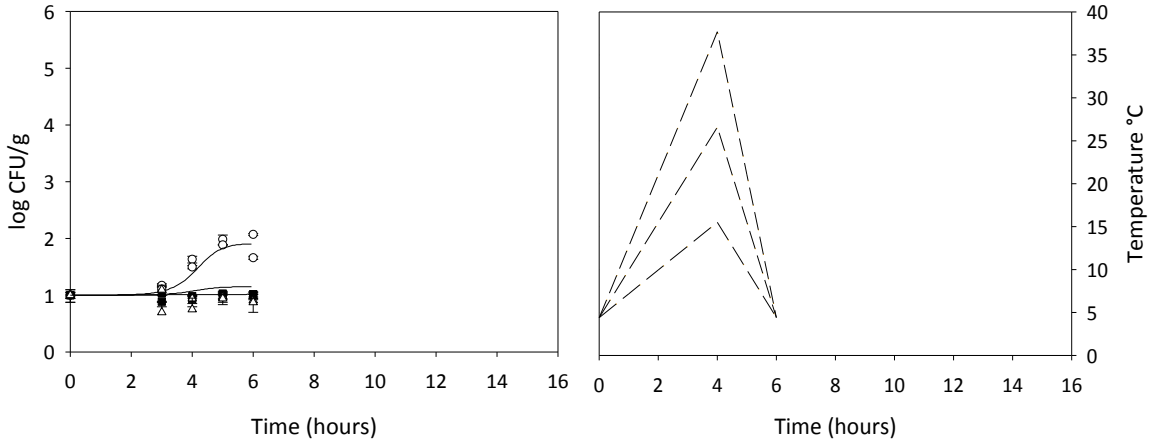
Figures II.2. a-c Growth profiles of the 6 h heating series: with a 6 h cool (a), 4 h cool (b), and 2 h cool (c). The log CFU/g vs. time are on the left hand side with the corresponding temperature profiles on the right. All CFU data was normalized to 1 for ease of comparison. Open circles represent 37.7°C (100°F), closed squares represent 26.6°C (80°F), and open diamonds represent 15.5°C

(60°F). Solid lines represent the ComBase predictions and the dashed lines are the temperature profiles.

Figures II.3.a



Figures II.3.b



Figures II.3.a-b Growth profiles of the 4 h heating series: with a 4 h cool (a) and 2 h cool (b). The log CFU/g vs. time are on the left hand side with the corresponding temperature profiles on the right. All CFU data was normalized to 1 for ease of comparison. Open circles represent 37.7°C (100°F), closed squares represent 26.6°C (80°F), and open triangles represent 15.5°C (60°F). Solid lines represent the ComBase predictions and the dashed lines are the temperature profiles.

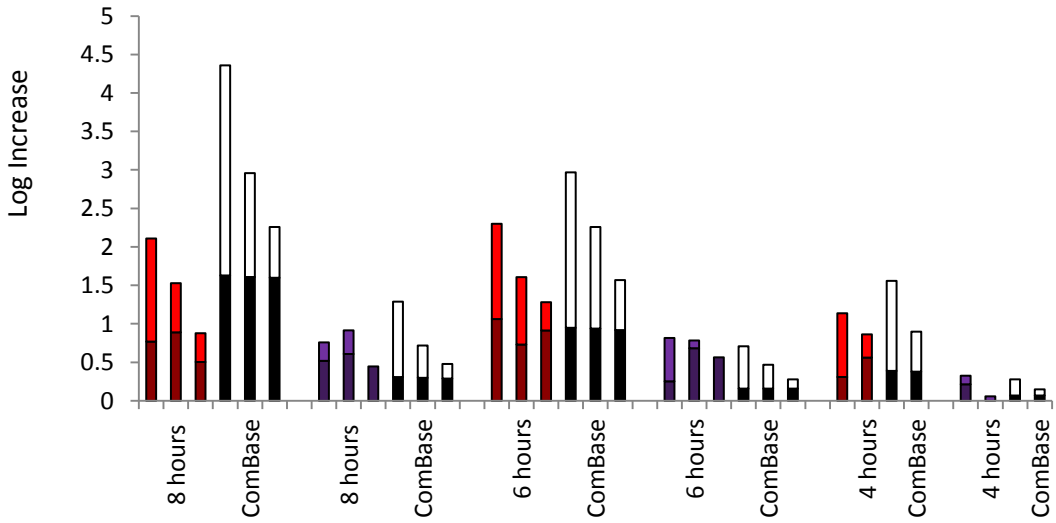


Figure II.4 Relative log increase of experimental and predicted data for *Salmonella* growth in ground beef vs. ComBase predictions: Data is organized by the heating time with longest to shortest cooling times from left to right. Predictions are shown to the right of the corresponding experimental data in black and white. Columns shaded red represents profiles that reached 37.7°C (100°F) and purple shaded columns represent profiles that reached 26.6°C (80°F). The shading change in each column shows the point in the temperature profile where the cooling cycle begins.

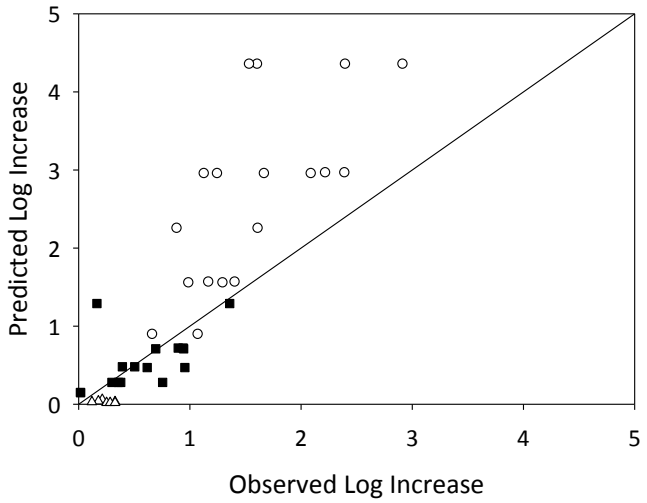


Figure II.5 ComBase predicted log increase vs. observed log increase for *Salmonella* growth in ground beef: Open circles represent temperature profiles reaching a maximum of 37.7°C (100°F), closed squares represent temperature profiles reaching a maximum of 26.6°C (80°F), and open triangles represent temperature profiles reaching a maximum of 15.5°C (60°F). Points that fall in the top left half of the graph are fail-safe and points on the bottom right half are fail-dangerous.

II.5. Discussion and Conclusion

Under the most permissive conditions for growth of *Salmonella* in ground beef, ComBase predictions were fail-safe. In developing or validating models, a balance must be struck in finding a model that is accurate vs. one that is appropriately fail-safe. A more accurate model may work for *Salmonella* growth in ground beef, but may be different for *Salmonella* growth in milk or eggs. When growth reaches narrow log increase shows the inaccuracy of comparing ComBase to CFUs. Measuring microbial growth via CFU is simply not a precise measurement and can prove to be difficult when the log increase is less than 1 log.

Approximations were made in creating the temperature profiles by forcing linear temperature gradients when these changes occur on a sigmoid curve (Schaffner, 2013). For a temperature change on a sigmoid curve, more variables would have to be taken into consideration including the size and shape of foods that are warming. Approximations would have to be made in a step-wise function to program these kinds of temperature changes into the programmable waterbath and for predictions made in ComBase. Since this is the first kind of experimental system to look at growth in these kinds of dynamic patterns a simpler approach was taken and linear gradient profiles were chosen.

More molecular based approaches could be useful to validating these models, but there are pros and cons to these methods. If industry standards are measured in CFU's, a molecular measurement will be more sensitive and not very practical to compare to current data. Molecular based methods will not yield a qualitative result like a CFU; however, can still be useful. It is important to use similar tools to validate models that industry and the FDA use to detect for the presence so that adequate comparisons can be made. Molecular based methods may not be able to detect viable cells but cells themselves. The slight inactivation that occurred in the 60°F data may not be seen in DNA based methods because the cells are still present in the sample, just not actively growing. RNA based methods might still be useful in detecting minimal growth, but may prove to difficult to measure in a complex food source. Low abusive temperature profiles may be most economically significant for determining when to discard food.

The FDA Model Food Code sets guidelines for holding food out of temperature control for 4 and 6 h time periods. Those guidelines required food to be discarded upon reaching 70°F (21 °C). Our experiments show these guidelines are quite conservative. For example, when ground beef containing *Salmonella* is allowed to rise in temperature over a 4 h time period from 40°F (4.44°C) to 60°F (15.55°C) or 80°F (26.66°C) both the predicted increases and the experimentally observed increases are very slight (less than 0.5 log CFU). Those

increases change only slightly even when the ground beef containing *Salmonella* is allowed another 4 h to cool back to 40°F (4.44°C). When ground beef containing *Salmonella* is allowed to rise in temperature over a 4 h time period to temperatures as high as 100°F (37.77°C), the predicted and observed increases are less than 0.5 log CFU. When cooling takes place over 4 h, the increase exceeds 1 log CFU, but not when cooling takes place over 2 h, when an increase of 0.9 log CFU is predicted. Similarly, when ground beef containing *Salmonella* is allowed to rise in temperature over a 6 h time period from 40°F (4.44°C) to 60°F (15.55°C) or 80°F (26.66°C), log increased are less than 1 log CFU. These increases remain less than 1 log CFU even when cooled over another 4 h back to 40°F (4.44°C).

In summary, our experiments show FDA Model Food Code guidelines for holding food out of temperature control are quite conservative for this particular model system. Our research also shows that the ComBase models for *Salmonella* growth are accurate or fail-safe for dynamic temperature conditions as might be observed due to power loss due to natural disasters or during transport out of temperature control.

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