IMPROVING THE SAFETY AND STABILITY OF ACID, ACIDIFIED AND WATER

ACTIVITY CONTROLLED FOODS PRODUCED BY SMALL-SCALE PROCESSORS

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IMPROVING THE SAFETY AND STABILITY OF ACID, ACIDIFIED AND WATER ACTIVITY CONTROLLED FOODS PRODUCED BY SMALL-SCALE PROCESSORS

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Products with pH below 4.6 or a_w below 0.91 are typically processed by less severe thermal treatments due to the lower public health concerns associated with these products. However, thermal processes and acidification steps should be evaluated and optimized, in order to guarantee commercial sterility and shelf stability, as well as reduce energy consumption and processing time. The effects of brine acetic acid concentration and packing conditions on the acidification rate of hard-boiled eggs were evaluated. Mixed models were used to predict accumulated lethality values given process conditions and heating times of pickled carrots, processed using a water bath. Mixed models were also used to assess and model the effect of process conditions on accumulated lethality values measured on the underside of the lid and vacuum formation, during a hot-fill-hold operation. Finally, the thermal tolerance (*D*- and *z*-values) and survival of *S. enterica* serovars Tennessee and Senftenberg were evaluated in milk caramel. Results demonstrated the importance of conducting acidification studies with proper pH measurements to determine safe conditions to manufacture commercially stable pickled eggs, produced satisfactory models (residual unexplained replicate-to-replicate variability of all constructed models was always < 3%)

of the effects of process conditions on accumulated lethality values, and showed interactions between serovar, temperature and a_w from the thermal inactivation studies of *S. enterica*. These results contribute to the establishment of science-based processing guidelines that ensure production of safe and stable products, with optimized processing temperatures and times, to enhance quality parameters of low pH and a_w products.

BIOGRAPHICAL SKETCH

Oscar Acosta obtained his BS in Food Technology (2004) and MBA (2009) from the University of Costa Rica. He has been a faculty member at the Food Technology Department of the University of Costa Rica since 2004 and will return to his position as Associate Professor after completion of his Ph.D. at Cornell.

DEDICATION

To my beautiful, smart and talented wife Jessie, and to my parents: Luis and Flora.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION AND RESEARCH OBJECTIVES

The food industry continues to improve as well as create processing technologies that allow production of higher quantities of safer, longer-lasting, more nutritious, and better consumer-accepted food products, while also trying to decrease costs and increase productivity. However, so-called traditional technologies such as thermal processing are still important processes used every day by the food industry. Unit operations such as sterilization and pasteurization are commonly applied to produce a wide variety of processed foods such as milk and other dairy products, juices, preserves, sauces, soups, canned vegetable or meat products, etc.

Besides the microbial load or bio-burden, the pH of the product and its water activity (a_w) have been identified as the most important critical factors in thermal processing (Tucker & Featherstone, 2011). It is widely recognized that thermal resistance of microorganisms decreases as the pH of their medium is lowered. This is especially evident in the case of spore-forming bacteria and their spores. In addition, most bacteria (particularly *Clostridium botulinum*) will not grow in media that has a pH lower than 4.6 (Larousse & Brown, 1997). The mechanisms by which pH reduction of food allows prevention of outgrowth of spores, reduce the heat resistance of microorganisms, and decrease or inhibit their growth rate, are detailed elsewhere (Derossi, Fiore, De Pilli, & Severini, 2011). Therefore, foods having a pH below 4.6 (acid) do not require as severe a thermal process regime as those with pH above 4.6 (low acid) to attain microbial

stability. Consequently, low acid foods are processed at temperatures above 100ºC (sterilized) while acid foods are processed at lower temperatures or pasteurized (Holdsworth & Simpson, 2007; Larousse & Brown, 1997). Acidification may therefore be considered as crucial as the thermal process for assuring the safety of pasteurized foods (Derossi, et al., 2011). Objective 1 of this project will address the effect of process conditions on the acidification rate of hard-boiled eggs, a complex food that acidifies slowly due to size and composition, thus representing one of the most challenging acidified food systems.

The first step when a thermal process is developed is to establish the processing targets based on a specific microorganism or group of microorganisms. Irrespective of the type of food, the goal is to achieve commercial sterility for the product, which depends on the types and numbers of organisms present, and on the intended storage conditions (Tucker & Featherstone, 2011). Many scientific publications (journal articles and books) have focused on the heat processing of low acid foods (pH > 4.6), both in setting guidelines and recommendations as well as trying to optimize and model processes. The reason is because the safety of such products has to be assured by the thermal treatment applied to the food (Larousse & Brown, 1997), due to the risk of growth of *C. botulinum* in the final product. These products are processed at higher pressures needed to achieve higher temperatures on the heating medium during thermal processing of the containers (Holdsworth & Simpson, 2007; Tucker & Featherstone, 2011).

Since thermal processes usually applied to acid foods are less severe (because they target vegetative cells of pathogenic bacteria and spoilage microorganisms), a limited amount of information is available on the scientific literature regarding modeling and optimization of process times. The US Food and Drug Administration has established specific Current Good Manufacturing Practices requirements for thermally processed low-acid foods packaged in hermetically sealed containers and acidified foods (CFR Title 21, Chapter 1, Subchapter B, Part 113 and 114, respectively) (GPO, 2013a, 2013b). Two ways to achieve pasteurization are the hot-fill-hold process and the water bath process. On the first one, the products are heated and held at a given temperature for a given number of minutes, and then filled into the container and immediately closed (rotation of the filled container serves to pasteurize the entirety of the internal surface, including the lid), without forced cooling for a given number of minutes. On the second process, the products are filled in the container and then thermally processed (Larousse & Brown, 1997). In objectives 2 and 3, this project will address the effects of process conditions on the accumulated lethality values measured during thermal processing of acidified vegetables and sucrose solutions by using the water bath and the hot-fill-hold processes.

Low a_w foods are naturally low in moisture or they are deliberately dried or formulated (by addition of salt or sugar, for example). While the minimum a_w at which microorganisms can grow is 0.60, it is generally accepted that 0.85 is the cut-off below which bacterial pathogens cannot grow, and most mycotoxin-producing molds cannot produce these secondary metabolites (Beuchat, et al., 2013).

Although low a_w foods have clear advantages with respect to controlling growth of foodborne pathogens, some microorganisms are able to survive drying processes, and it is usually difficult to eliminate pathogens from foods with low a_w by application of mild heat treatments. Moreover, pathogens can often persist longer in low a_w foods than in products with high a_w. Since the enhanced heat tolerance of microorganisms in low a_w foods is not easily predicted, it is imperative that good hygiene practices and food safety management systems are implemented, focusing particularly on preventing contamination with foodborne pathogens (Beuchat, et al., 2013), but also controlling for contamination by spoilage microorganisms.

US Federal regulations (CFR Title 21, Chapter 1, Subchapter B, Part 113. 3(e) (1) (ii)) (GPO, 2013a) state that commercial sterility can be achieved by the control of a_w and the application of heat. The heat is generally necessary at a_w levels above 0.85 to destroy vegetative cells of microorganisms of public health significance and spoilage microorganisms which can grow in a reduced a_w environment (FDA, 2010). However, there is no widely available scientific information on thermal processing guidelines that can be applied to these types of food products. Objective 4 of this project will address thermal tolerance and survival of two *Salmonella enterica* serovars in milk caramel (a_w 0.85 - 0.93) as the model food system due to its high fat, protein and sugar content.

The ultimate goal of this project is to provide science-based evidence for food processors and process authorities. It is envisioned that results will be used to establish processing guidelines and evaluate current processes for production of shelf-stable

foods. Organizations such as the New York State Food Venture Center at Cornell University, which provides comprehensive assistance to beginning and established food entrepreneurs, will benefit from these results. Ultimately, these organizations will continue to promote sustainable economic development of rural communities (NECFE, 2014). The following research objectives have been established for this project:

1. Evaluate the effect of brine acetic acid concentration and packing conditions (brine fill temperature, heat treatment to filled jars, and post-packing temperature) on the acidification rate of hard-boiled eggs.

2. Evaluate and model the effects of process conditions on the accumulated lethality values of thermally processed pickled vegetables, using carrots as a model food.

3. Evaluate and model the effects of process conditions on the accumulated lethality values measured on the underside of the lid and the vacuum formation, during a hot-fill-hold operation using sucrose solutions as a model food.

4. Evaluate the effect of a_w on the thermal tolerance and survival of two *Salmonella enterica* serovars in milk caramel, a product with high fat, protein and sugar content.

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CHAPTER 2

PICKLED EGG PRODUCTION: EFFECT OF BRINE ACETIC ACID CONCENTRATION AND PACKING CONDITIONS ON ACIDIFICATION RATE [1]

Abstract

US Federal Regulations require that acidified foods must reach a pH of 4.6 or lower within 24 h of packaging or be kept refrigerated until then. Processes and formulations should be designed to satisfy this requirement, unless proper studies demonstrate the safety of other conditions. Our objective was to determine the effect of brine acetic acid concentration and packing conditions on the acidification rate of hard-boiled eggs. Eggs were acidified (60/40 egg to brine ratio) at various conditions of brine temperature, heat treatment to filled jars, and post-packing temperature: a. 25ºC / none / 25ºC (cold fill), b. 25ºC / none / 2ºC (cold fill/refrigerated), c. 85ºC / none / 25ºC (hot fill), d. 25ºC, 100ºC for 16 min / 25ºC (water bath). Three brine concentrations were evaluated (7.5, 4.9, and 2.5% acetic acid) and egg pH values (whole, yolk, four points within egg) were measured from 4 to 144 h, with eggs equilibrating at pH 3.8, 4.0, and 4.3, respectively. Experiments were conducted in triplicate and effects were considered significant when $P < 0.05$. Multiple linear regression analysis was conducted to evaluate the effect on pH values at the center of the yolk. Regression analysis showed that brine concentration of 2.5% decreased the acidification rate, while packing conditions of the hot fill trial increased it. Inverse prediction was used to determine the time for the center of the yolk

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and the total yolk to reach a pH value of 4.6. These results demonstrate the importance of conducting acidification studies with proper pH measurements to determine safe conditions to manufacture commercially stable pickled eggs.

1. Introduction

Eggs make important nutritional contributions to the American diet (Song & Kerver, 2000), and research carried out during the last decade has shown that egg consumption does not correspond with an increase in coronary heart disease (as once suggested), because there is no marked adverse effect on blood cholesterol concentrations (Gray & Griffin, 2009). Total egg production in the US during 2011 was estimated at 91.9 billion. 86% of the total was destined for table eggs (the rest were used as hatching eggs) (NASS, 2012). The majority of eggs destined for consumption are distributed to the food industry for use as ingredients in product formulation (Claire, et al., 2004). Common technologies applied for increasing shelf life and/or assuring the safety of eggs include pasteurization, freezing, and dehydration. Salting and alkaline pickling have long been used as methods to preserve eggs (mostly duck eggs) and obtain products with particular characteristics (Ganesan & Benjakul, 2010; Kaewmanee, Benjakul, & Visessanguan, 2009). Eggs are also commercialized hard-boiled and preserved by packaging in modified atmospheres (Claire, et al., 2004) or pickled.

Pickling is a longstanding method of preserving food. The process can be applied to various fruits and vegetables, as well as to foods of animal origin such as eggs and meats. According to federal regulations (21CFR114), a pickled product is an acidified

food, which is a low-acid food to which acid is added, its water activity is greater than 0.85 and it has a finished equilibrium pH of 4.6 or below (GPO, 2011b). The mechanisms by which pH reduction of food allows prevention of outgrowth of spores, reduce the heat resistance of microorganisms, and decrease or inhibit their growth rate, are mentioned by Derossi, Fiore, De Pilli, and Severini (2011).

Care must be taken so that the processing steps to produce pickled eggs are carried out correctly, in order to assure a safe, stable, and acceptable product. In this sense, variations in acid concentrations or processing times may affect the safety and stability of the product if process conditions are inadequate. Texture and flavor may be negatively affected if product is over processed. An example of inadequate process conditions of home-prepared pickled eggs is linked to a case of botulism (CDC, 2000), and at least one recall of pickled eggs has been reported, possibly due to contamination during manufacturing (FDA, 2006). On the other hand, even though freshly laid eggs are generally sterile (Jay, 2000), and the thermal treatment applied to harden the egg is usually severe enough to inactivate potentially harmful microorganisms (Grijspeerdt & Herman, 2003), improper handling before the acidification step (for instance, during the peeling operation) can contaminate the surface of the egg.

Federal regulations (21CFR114) indicate that to guarantee the safety of acidified foods, a thermal treatment should be applied for a sufficient time period to destroy the vegetative cells of microorganisms of public health significance and those of non-health significance capable of reproducing in the food under normal conditions of storage,

distribution, retail, or use. Regulations also state that acidified foods shall be manufactured, processed, and packaged so that a finished equilibrium pH value of 4.6 or lower is achieved within the time designated in the scheduled process (GPO, 2011b). Concerning this time period, regulations in 9CFR381 state that in an acidified low acid product, every component must have a pH of 4.6 or lower within 24 h after the completion of the thermal process unless data are available from the establishment's processing authority demonstrating that a longer time period is safe (GPO, 2011a). Otherwise, the acidification must take place under refrigerated conditions. Most resources specify that home prepared pickled eggs must be stored in the refrigerator and must not be left at room temperature other than during the period of time for serving (NCHFP, 2007; WSU, 2002).

Eggs differ from other products (such as most fruits and vegetables), due to composition and size, which can affect the rate of acidification. The composition and structure of the yolk, which has a high content of fat and protein (32.5% and 17.5%, respectively) (Potter & Hotchkiss, 1995), can pose a challenge to quick and complete acidification. Although some studies have addressed acidification rates of hard-boiled eggs (Acton & Johnson, 1973; Ball & Saffores, 1973; Richard & Cutter, 2011), none refer to the variations in acid penetration due to the effects of a variety of pickling acid strengths and processing conditions. Our objective was to determine the effect of brine acetic acid concentration and packing conditions (brine fill temperature, heat treatment to filled jars, and post-packing temperature) on the acidification rate of hard-boiled eggs.

2. Materials and methods

2.1 Preparation of eggs and brines.

Fresh, medium, grade A eggs were purchased from a local supermarket. Water was brought to a boil using an electric kettle (Groen TDB/6-10, Jackson, MS), and eggs were laid in a wire basket in a single layer. Once a rolling boil was achieved, the wire basket of eggs was placed in the water, so the eggs were completely submerged. The eggs were boiled for 10 min (timing started once the water returned to a boil). After cooking, eggs were immediately cooled in potable running water. Eggs were then stored at 2ºC until samples were prepared. Immediately before preparing samples, eggs were warmed to 25ºC in a water bath and peeled by hand. For each trial, three brines of different concentrations of acetic acid were prepared. The characteristics of each brine are as follows (initial acetic acid concentration in the brine / equilibrated target acetic acid concentration in the total product / equilibrated final pH value of the total product are given): 7.5% / 3% / 3.8, 4.9% / 2% / 4.0, and 2.5% / 1% / 4.3. The equilibrated target acetic acid concentration was calculated using a mass balance that included the mass and concentration of acetic acid in the brine and eggs before acidification. Distilled white vinegar (indicating 5% acetic acid, purchased from a local supermarket) was used straight for the 4.9% brine, diluted by half with distilled water for the 2.5% brine, or with added 99.7% glacial acetic acid (VWR Scientific Products, West Chester, PA) for the 7.5% brine (28.2 ml per 1000 ml vinegar). Sodium benzoate (J.T. Baker, Phillipsburg, NJ) was added to the 2.5% acetic acid brine in order to obtain a concentration of 0.05% preservative in the initial brine. The brine concentrations were confirmed by titration.

2.2 Preparation of samples.

Four separate trials (each one including the three previously mentioned brine concentrations) were carried out, as detailed in Table 2.1. For every trial, 946 ml glass jars with two-piece metal lids were used. Nine eggs were placed in each jar and the brines were added according to the conditions indicated in Table 2.1.

Table 2.1. Experimental design for acidification of hard-boiled eggs with acetic acid brines at 60/40 egg to brine ratio.

Trial	Brine temperature (ºC)	Heat treatment to filled jars	Post-packing temperature (ºC)	Brine concentration (% w/w acetic acid)
Cold fill	25	None	25	7.5
				4.9
				2.5
Cold fill / refrigerated	25	None	2	7.5
				4.9
				2.5
Hot fill	85	None	25	7.5
				4.9
				2.5
Water bath	25	100ºC / 16 min	25	7.5
				4.9
				2.5

Brines were added to obtain a 60/40 egg to brine ratio (by weight). This ratio was determined by a preliminary trial to be the greatest egg to brine ratio resulting in the top layer of eggs being completely covered with brine. For the cold fill and cold

fill/refrigerated trials, immediately after the brine was poured in the jars, the caps were tightened and the jars were tipped once in order to mix brine around the eggs. No heat treatment was applied and jars were stored at 25°C (cold fill) and 2°C (cold fill/refrigerated). For the hot fill trial, the brines were heated to 85°C and poured in the jars, which were immediately capped and tipped once. The jars were left to cool and stored at 25°C. For the water bath trial, the brines were poured in the jars, the caps were tightened and the jars were then preheated in a water bath at 43°C for 5 min. The jars were then submerged in a water bath at 100°C and held for 16 min. The jars were left to cool and then stored at 25°C. One of the eggs located in the middle layer in the jar was pierced in the center with a flexible thermocouple, and temperatures were registered at 10 sec intervals during the thermal treatment. Each experiment was carried out in triplicate, where each jar represented a separate sample. After 4, 24, 48, 72, 96, and 144 h, one egg was taken out from each jar, rinsed with distilled water, and dried with a paper towel. A corresponding amount of brine was removed to maintain the 60/40 egg to brine ratio in each jar and to measure the brine pH at each sampling time. Eggs were cut longitudinally in half, and pH values were measured in four sampling points of the egg as follows: the edge of white (point A), halfway between edge of the white and edge of the yolk in thickest part of the white (point B), the edge of yolk adjacent to the thickest part of white (point C), and the center of the yolk (point D). Afterwards, the yolk was ground, and pH was measured. Finally, ground yolk and white were combined and homogenized, and pH of the whole egg was measured. Hard-boiled eggs and eggs at the end of the pickling period processed following the conditions indicated in Table 2.1 (with the 7.5% acetic acid brine) were analyzed for firmness.

Eggs were cut longitudinally in half, and halves were placed with the cut side facing down so the puncture probe would enter the outer surface of the egg. Three points were measured, corresponding to points A, B, and the edge of the white, closest to the yolk (between points B and C). The largest value of force (N) obtained from those 3 points was chosen as the representative value for that trial. Measurements of texture were conducted in triplicate. Whole pickled eggs and brines were sampled at the end of the experiment (after 144 h) and titratable acidity analyses were performed. Measurements were conducted in triplicate.

2.3 Methods of analysis.

pH was measured using an Accumet Basic AB15 pH meter (Fischer Scientific, Pittsburgh, PA), equipped with a calibrated Orion 8220BNWP micro ROSS glass body pH electrode (Thermo Scientific, Beverly, MA). Titratable acidity was determined using a G20 compact titrator (Mettler Toledo, Schwerzenbach, Switzerland) and reported as percentage of acetic acid (w/w). Egg firmness was determined at room temperature using the TA.XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY). A puncture test was performed, with a puncture diameter of 3.32 mm, crosshead speed of 1 mm/s and a travel distance of 4 mm.

2.4 Statistical analysis.

A multiple linear regression analysis was carried out in order to evaluate the effects of storage time (after a logarithmic transformation), brine acetic acid concentration, packing conditions, and both their interactions with time, on pH values at the center of

the yolk (point D). A random effect of replicate (nested within brine concentration and packing conditions) was also added. Effects were considered significant when $P < 0.05$. Acidification rates correspond to change in pH per change in storage time (after a logarithmic transformation), thus lower and higher rates would correspond to more positive and negative slopes (respectively), because the linear relationship between pH and log transformed time during acidification is negative (pH decreases with time). Inverse prediction (which corresponds to the reverse process to linear regression) was used to predict the value of the independent variable (logarithmic transformation of process time) from a given value of the dependent variable (pH at point D). This process allowed determination of the time (hours) when pH 4.6 was reached at the center of the yolk (point D) and on the total yolk, for every trial at each one of the three tested brine concentrations. The 95% confidence intervals with respect to an expected response were calculated. For the texture measurements, means were compared using ANOVA. Differences were considered significant when $P < 0.05$. Analyses were performed using the statistical software JMP® Pro 9.0.2 (SAS Institute Inc., Cary, NC).

3. Results and discussion

Figure 2.1 shows representative curves of variation of pH on different sampling points of the egg and brine, with storage time (conditions correspond to cold fill trial and brine acetic acid concentration of 7.5%). Points A and B, which correspond to egg white, show fast diffusion of acid. Since point D (center of the yolk) is shown to have the slowest acidification rate, it was chosen as the representative point to determine when the egg was thoroughly acidified, by use of multiple linear regression analysis.
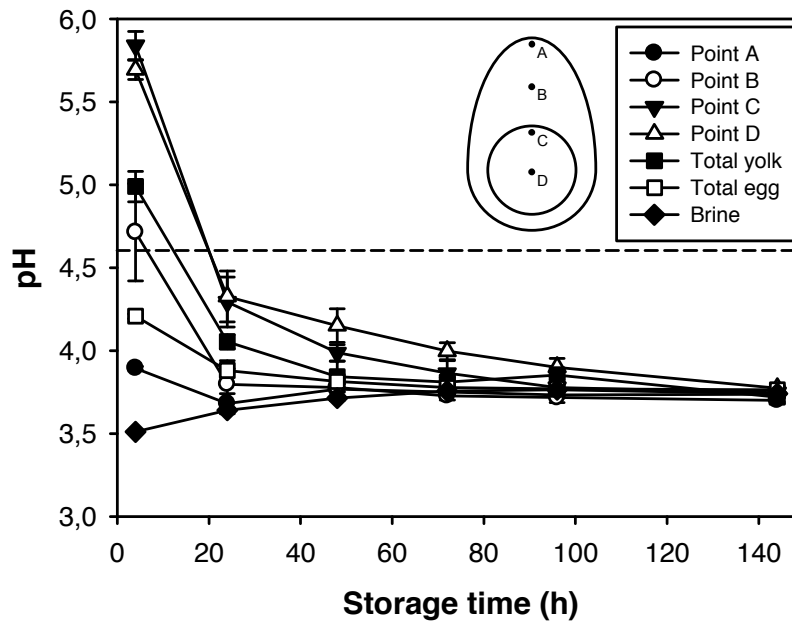
Figure 2.1. Variation of pH with storage time at 25ºC (cold fill trial, brine acetic acid concentration of 7.5%, 60/40 egg to brine ratio). Error bars represent standard deviation for n = 3. Dotted line marks the critical pH value of 4.6. Diagram of egg with sampling points is also shown.

Figures 2.2 and 2.3 show a representation of variation of pH in sampling point D, total yolk, total egg, and brine, as a function of storage time, for every trial, at each one of the three tested brine concentrations. Consistently, higher concentrations of acetic acid in the brine increase the acidification rate of the eggs. The total yolk measurements indicate it acidifies faster than point D, while measurements of the total egg show even faster acidification rates, due to the contribution from the egg white (which acidifies the fastest). Although the effect of process conditions is not evident in most cases, the cold fill/refrigerated conditions show longer acidification times for every sampling point and brine acetic acid concentration.

Figure 2.2. Variation of pH with storage time at sampling point D (●), total yolk (○), total egg (▼), and brine (△), for the cold fill (25°C) and cold fill/refrigerated trials (2°C), at the three tested brine acetic acid (AA) concentrations, 60/40 egg to brine ratio. Error bars represent standard deviation for n = 3. Dotted line marks the critical pH value of 4.6.
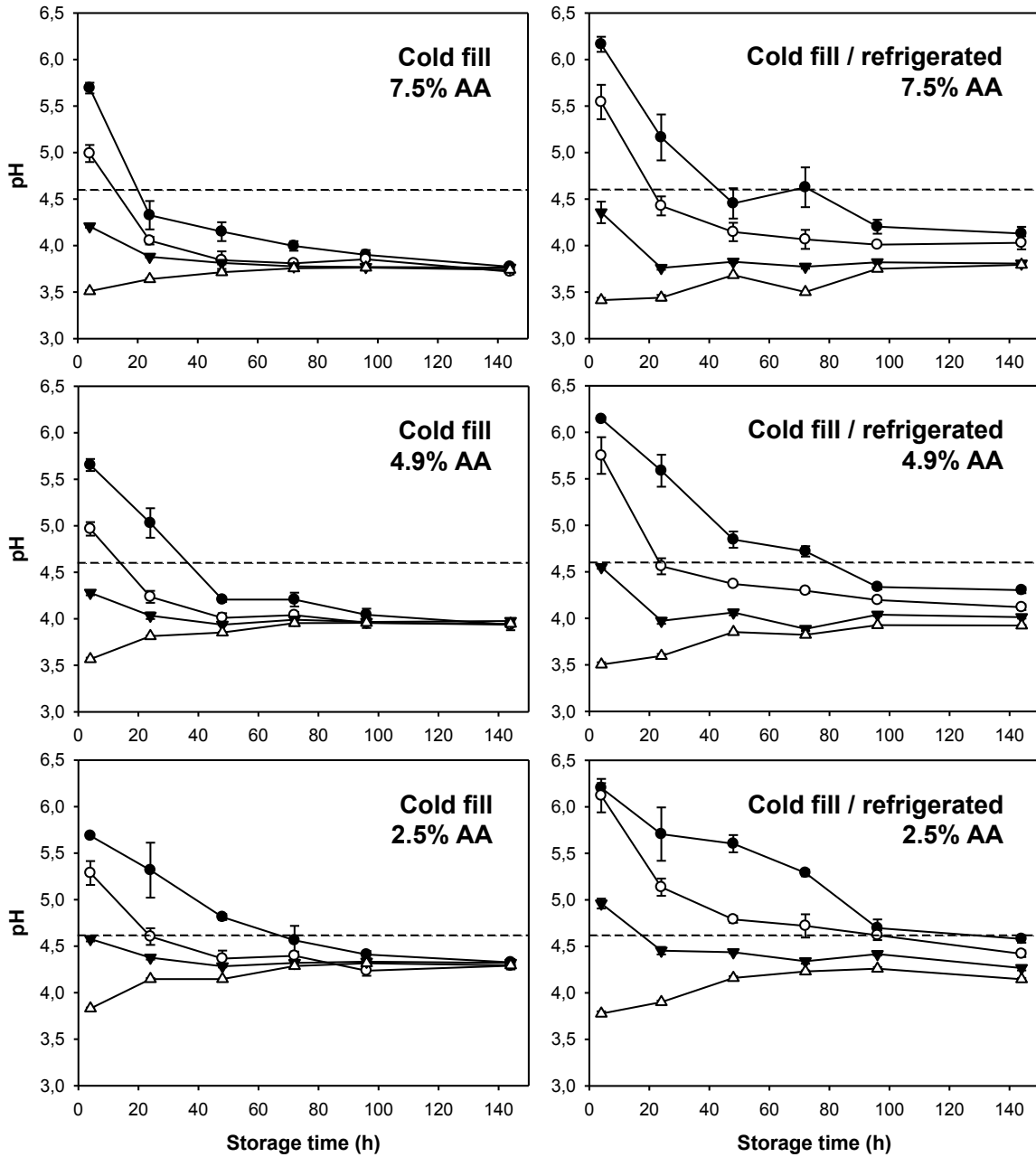
Figure 2.3. Variation of pH with storage time at 25°C at sampling point D (●), total yolk (○), total egg (▼), and brine (△), for the hot fill and water bath trials, at the three tested brine acetic acid (AA) concentrations, 60/40 egg to brine ratio. Error bars represent standard deviation for n = 3. Dotted line marks the critical pH value of 4.6.
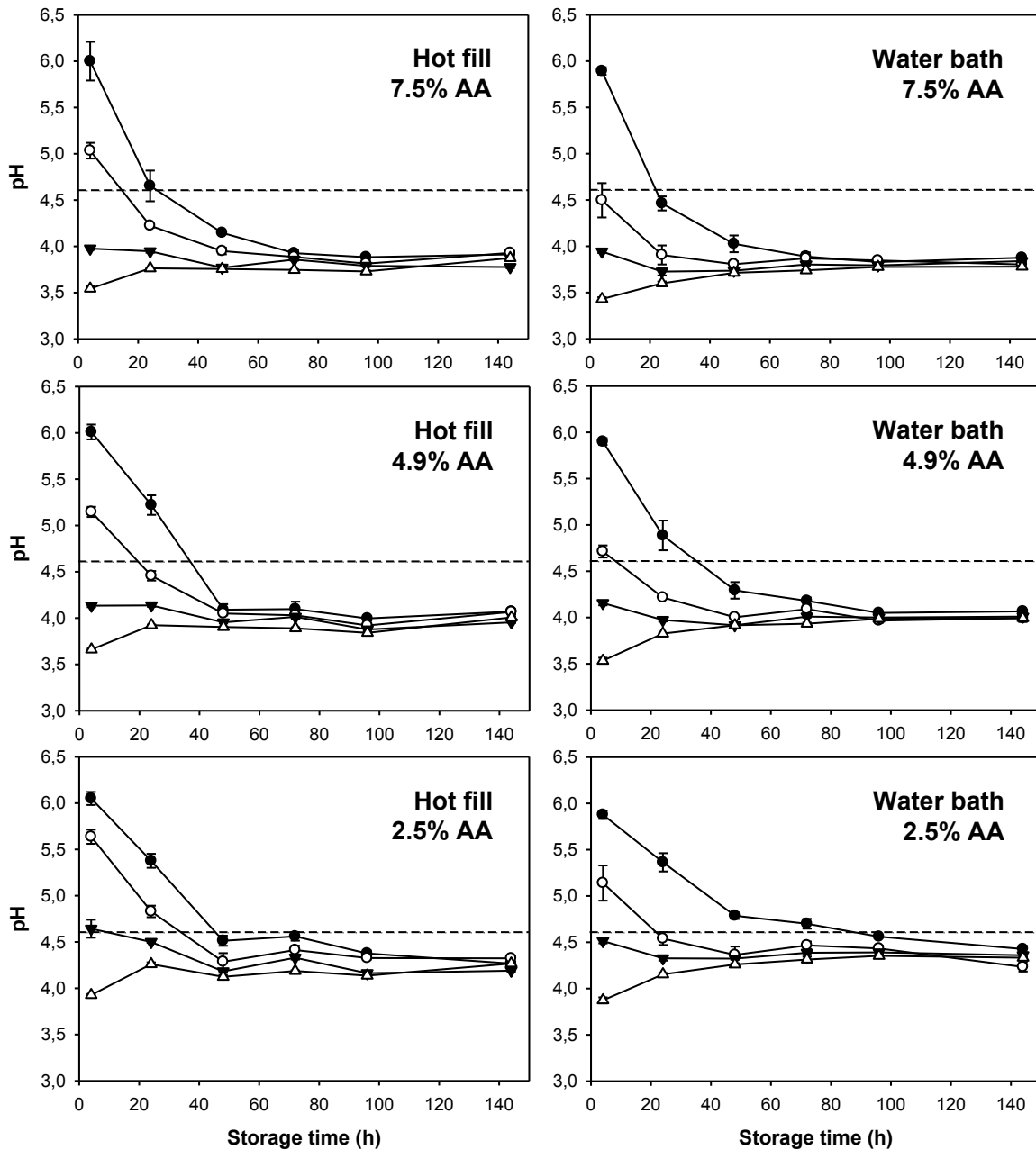
Federal regulations (21CFR114) specify that in order to measure pH on food samples such as pickled eggs, samples must be prepared by draining the solids and blending them to a uniform, workable paste (GPO, 2011b). This corresponds to the total egg sampling point shown in Figure 2.1, which acidifies at a faster rate than the center of the yolk (point D). Figures 2.2 and 2.3 show that for every trial condition, at initial brine concentrations of 7.5 and 4.9% acetic acid, after just 4 h of acidification, the whole egg samples have already achieved pH values under 4.6, while samples from the center of the yolk have pH values well over the critical value of 4.6. For eggs acidified with the 2.5% acetic acid brines (initial concentration), the pH of whole egg samples were near or over 4.6 in every case. Therefore, one could underestimate the time needed to reach a pH of 4.6, if the pH of the whole egg is used as indicator. The faster acidification rate in the whole egg (when compared to the center of the yolk or the whole yolk) was predictably observed in every brine acetic acid concentration and packing condition tested throughout the experiment.

Figures 2.2 and 2.3 also show the differences between acidification rates of eggs pickled in brines of different acid concentration. As will be further demonstrated by the multiple regression analysis, there are significant differences between the acidification rates of eggs pickled in brines of different acid concentration, and as expected, higher concentrations of acetic acid produce a faster acidification of the eggs. The uneven acidification of eggs is most likely dependent on the composition (and structure after the egg is hardened by boiling) of the egg white and yolk: the egg white has a water content of 88%, while the yolk has only 48%. The 12% solids of egg white are virtually all

protein, but the yolk has a high fat (32.5%) and protein (17.5%) content. Eggs contain about two parts white to one part yolk by weight (Potter & Hotchkiss, 1995). As has been previously reported (Acton & Johnson, 1973; Richard & Cutter, 2011), even though the white has a higher initial pH than the yolk, the acidification rate is consistently faster in the white. Acton and Johnson (1973) speculated that the penetration of acid to the yolk is dependent on two factors: (a) diffusion rate of acetic acid through the egg white, and (b) initial acid concentration in the pickling medium. Manufacturers of pickled eggs and similar products should determine a proper way of establishing when the target pH in their products has been achieved, in compliance with regulations. The point with the slowest acidification rate should be selected to monitor this change, and experiments should be conducted according to the product and process characteristics. Point D was consequently chosen to compare the acidification rates of pickled eggs processed with different brine acetic acid concentrations and packing conditions (brine fill temperature, heat treatment to filled jars, and post-packing temperature).

The multiple linear regression analysis conducted on pH values measured at point D (residual unexplained replicate-to-replicate variability <7%) showed that the lowest brine concentration (2.5% acetic acid) has a significantly lower acidification rate (more positive slope) when compared to the other two brine concentrations of 4.9 and 7.5% ($P < 0.05$). Packing conditions of the hot fill trial significantly increased the acidification rate (more negative slope) ($P < 0.05$). In terms of intercepts, brine concentration did not have a significant effect ($P > 0.05$), but packing conditions did: cold fill/refrigerated and

hot fill trials had significantly different (higher) intercepts ($P > 0.05$). These latter effects are likely caused by the influence of the first 4 h of acidification (which were not monitored).

Results from the multiple linear regression analysis indicate that the most important factors that affect the acidification rate of pickled eggs are the brine concentration and the brine's fill temperature. In the first case, the higher brine acetic acid concentrations (7.5 and 4.9%) decreased the egg pH at a faster rate, compared to the lowest concentration of 2.5% acetic acid. As for the brine fill temperature, results showed that the acidification rate increased with the packing conditions of the hot fill trial, which mainly differs because of its higher initial temperature. Not many studies have addressed factors that affect acidification rates of pickled eggs. Although Acton and Johnson (1973) indicate that the penetration of acid to the yolk is dependent on the initial acid concentration in the pickling medium, their results showed no apparent difference between acid intake rates of pickled eggs brined with 3 and 5% acetic acid vinegar solutions, although no statistically sound method of comparison was mentioned. It must also be taken into consideration that the brines used in this experiment contained between 40 and 45% sucrose, and 6% commercial pickling spice, which could have reduced the effect of the acid strength on the acidification rate. Although Ball and Saffores (1973) also studied pickling of eggs in solutions with different acid strengths (ranging from 1 to 6% acetic acid), they did not mention differences in acidification rates when comparing the different solutions. They only indicated that the

pH of egg white, yolk, and pickling solutions equilibrated after 6 to 7 days at room temperature.

A simpler analysis of the acidification data was performed by means of inverse prediction from the fitted linear relationship between pH and log time, which allowed calculating the time when pH 4.6 was reached at point D (center of the yolk) and in the total yolk, for every trial and every brine concentration tested. Table 2.2 shows the calculated times to reach a pH of 4.6 at the center of the yolk (point D) and in the total yolk, for every trial at each one of the three tested brine concentrations. Averages and 95% confidence intervals with respect to an expected response are shown, and results were obtained through inverse prediction from the fitted linear relationship between pH and log time. In accordance with results presented in Figures 2.2 and 2.3, the cold fill/refrigerated conditions show longer acidification times for every sampling point and brine acetic acid concentration. Higher concentrations of acetic acid in the brine appear to consistently decrease the times required for eggs to reach a pH of 4.6.

Results on Table 2.2 show the marked differences between the cold fill/refrigerated trial and the rest of the processing conditions (when comparing each one of the three brine concentrations), as well as the differences between brines in every trial: higher acetic acid concentrations mean a lower time to reach the targeted pH. The relatively high predicted time of 220.3 h (and the corresponding large confidence interval) for the 2.5% acetic acid brine at the cold fill/refrigerated trial is due to the fact that after 144 h (final sampling point of the experiment), the center of the yolk had barely reached a pH of 4.6.

Table 2.2. Calculated times for pickled eggs (60/40 egg to brine ratio) to reach a pH of 4.6 at the center of the yolk and in the total yolk, using inverse prediction from the fitted linear regressions.

Trial	Brine concentration (% w/w acetic acid)	Time (h)			
		Center of the yolk		Total yolk	
		Average	95% CI	Average	95% CI
Cold fill	7.5	23.5	19.7-27.5	8.4	6.1-10.8
	4.9	34.4	29.2-40.2	10.2	7.1-13.4
	2.5	75.9	61.6-96.8	32.1	25.9-39.3
Cold fill / refrigerated	7.5	56.5	47.2-68.5	24.3	20.0-28.8
	4.9	82.7	67.4-105.1	36.4	30.9-42.8
	2.5	220.3	154.9-359.2	89.1	74.8-109.1
Hot fill	7.5	30.6	25.8-35.9	10.5	7.4-13.8
	4.9	39.5	33.5-46.5	16.0	12.0-20.1
	2.5	66.2	54.5-82.4	44.7	37.5-53.5
Water bath	7.5	26.0	22.6-29.5	1.4	0.4-2.8
	4.9	38.1	33.3-43.6	5.1	2.7-8.0
	2.5	93.7	76.9-118.4	30.1	23.2-38.1

Pickled egg processors might not be able to purchase and use a microelectrode to determine pH in specific points of the yolk (such as the center). However, the pH of the ground whole yolk can be determined easily with standard pH probes, and blending of semisolid products to a paste consistency is deemed an acceptable method of sample preparation for pH analysis (GPO, 2011b). Data from Table 2.2 indicates that for pickled eggs manufactured following the described process conditions and formulation (60/40 egg to brine ratio), every process (except for the cold fill/refrigerated conditions) at brine acetic acid concentrations of 7.5 and 4.9% was able to produce pickled eggs that

reached a pH of 4.6 (measured on the total yolk) under 24 h. However, if acetic acid concentration of 2.5% is used in the brine, the product should be kept under refrigerated conditions until the whole egg yolk reaches a pH of 4.6 or lower (approximately 4 days), unless data are available from a processing authority demonstrating that a longer time period at room temperature to reach pH 4.6 is safe (GPO, 2011a).

Given that acidification of the center of the yolk occurs at a slower rate when compared to the whole yolk, data from Table 2.2 also shows that no process condition was able to produce pickled eggs that presented a pH value of 4.6 (measured in the center of the yolk) in less than 24 h (using the higher end of the 95% confidence interval as cut-off), and therefore the products should be refrigerated until the targeted pH value is reached. Acidification times of approximately 2.5, 3.5 and 9 days for brine acetic acid concentrations of 7.5, 4.9 and 2.5%, respectively, would be needed in order to achieve pH values of 4.6 or lower in the center of the yolk. Again, refrigeration might not be necessary if data are available from scientific studies or a processing authority showing that longer times to reach pH 4.6 at the center of the yolk are safe (GPO, 2011a). Results on the inactivation rate of *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* in pickled eggs processed without a final heat treatment have been reported (Richard & Cutter, 2011; Sullivan, Manns, Churey, Worobo, & Padilla-Zakour, 2013). Authors stated that pickling of eggs with acetic acid or acetic acid and salt, at refrigeration and/or room temperatures, effectively reduced pathogens by at least 5 log, when pathogens were inoculated on egg surface, thus

reinforcing the need for proper acidification studies to determine the most reliable conditions for reaching target pH and pathogen inactivation.

Table 2.3 shows the equilibrated target acetic acid concentration in the total product, as well as the measured acetic acid concentration in eggs and brines at the end of the pickling experiments, for every trial and initial acetic acid concentration in the brine. Mean values and standard deviations for triplicate measurements are shown. In most cases brines show significantly higher titratable acidity values than whole eggs, which indicates an incomplete equilibration of acid concentration in the product. Results of measured titratable acidity (% acetic acid) in the eggs and brines at the end of the pickling experiment (Table 2.3) show that more than 144 h are needed in order to observe a complete equilibration of acid concentration in the product, although this time is not considered as important as the time required to reach the critical pH of 4.6 (or lower) in the center of the yolk. Predictably, most values of measured titratable acidity in eggs and brines are lower than the equilibrated target acetic acid concentration in the total product, which is likely due to neutralization of acid by compounds present mainly in the egg whites (Potter & Hotchkiss, 1995).

Besides the concentration of acid in the brine and packing conditions, other process parameters should be considered when assessing the acidification rate of hard-boiled eggs. For instance, the ratio of eggs and brine will affect the acidification rate, because more acid will be available to enter the egg if the proportion of brine is higher. The addition of other ingredients (such as salt or spices) could also affect the acidification

rate. Acton and Johnson (1973) found that during preparation of the pickle solution, a significant reduction of the acetic acid concentration occurred (20 to 23% decrease), possibly due to the absorption and neutralization of acid by the ingredients added to the vinegar solutions (which include mustard, coriander, cinnamon, ginger, allspice, dill seed, black pepper, bay leaves, cloves, chilies and mace).

Table 2.3. Equilibrated target acetic acid concentration in the total product (60/40 egg to brine ratio) and measured acetic acid concentration in whole eggs and brines at the end of the experiment (144 h). Mean values ± standard deviation for n = 3 are shown.

Trial	Titratable acidity (% w/w acetic acid)			
	Initial in the brine	Equilibrated target in the total product	Measured in whole egg	Measured in brine
Cold fill	7.5	3.0	2.62 ± 0.04	2.98 ± 0.03
	4.9	2.0	1.65 ± 0.01	1.80 ± 0.01
	2.5	1.0	0.720 ± 0.009	0.759 ± 0.006
Cold fill / refrigerated	7.5	3.0	2.70 ± 0.04	3.01 ± 0.04
	4.9	2.0	1.75 ± 0.03	1.87 ± 0.02
	2.5	1.0	0.82 ± 0.01	0.788 ± 0.005
Hot fill	7.5	3.0	2.68 ± 0.03	2.97 ± 0.04
	4.9	2.0	1.77 ± 0.03	1.86 ± 0.03
	2.5	1.0	0.86 ± 0.03	0.776 ± 0.007
Water bath	7.5	3.0	2.59 ± 0.05	2.80 ± 0.03
	4.9	2.0	1.66 ± 0.02	1.73 ± 0.01
	2.5	1.0	0.730 ± 0.006	0.717 ± 0.003

Figure 2.4 shows a characteristic temperature – time profile obtained from the thermal treatment applied to pickled eggs processed according to the conditions of the water bath trial. Jars were immersed in boiling water at minute 5 and removed at minute 21. Accumulated lethality values shown in Figure 2.4 were calculated every 10 min from the end of the immersion time. Accumulated lethalities (in minutes) were calculated using the reference values established for ensuring the commercial sterility of acidified foods, with a z-value = 8.9°C and reference temperature 93.3°C (Padilla-Zakour, 2009). If pickled eggs were processed following the conditions described in this experiment, the thermal treatment corresponding to the water bath trial (100°C for 16 min) would yield an appropriate lethality value to assure shelf-stability of products with a final equilibrated pH of 4.2 (or below). The observed lethality is well above one that corresponds to a process of 2.5 min at a reference temperature of 93.3°C, the minimum required for a maximum equilibrium pH of 4.2 (Padilla-Zakour, 2009).

A significant (P < 0.05) textural change was detected when hard-boiled eggs (0.6 ± 0.1 N) were compared to eggs at the end of the pickling period (1.0 ± 0.2 N). However, results showed that there were no significant differences in texture of egg samples subject to different process conditions at the end of the pickling period (P > 0.05). These results indicate that texture is overall affected by the pickling process, but not by the method employed.
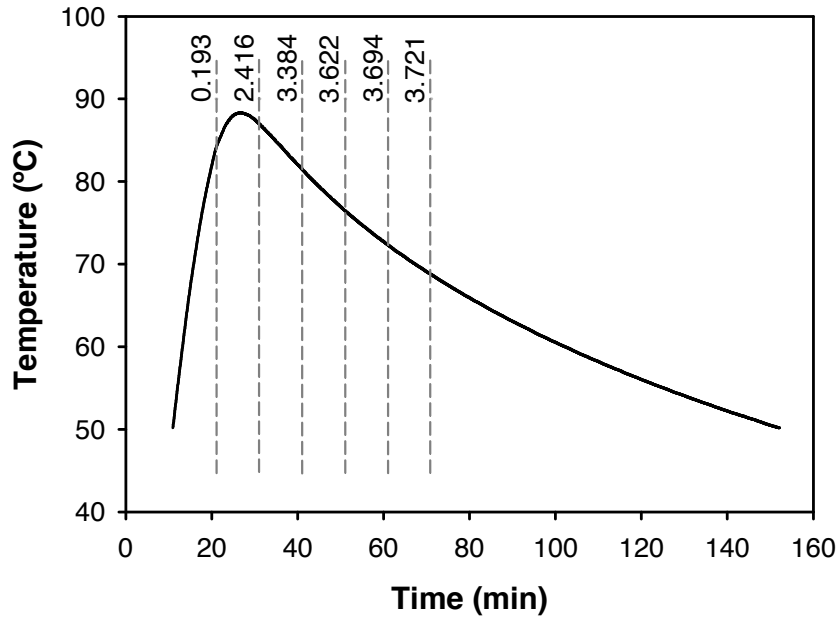
Figure 2.4. Heating profile of pickled eggs processed in boiling water bath. Jars were immersed in boiling water at minute 5, and removed at minute 21. The accumulated lethality values (calculated using a z-value of 8.9ºC and the reference temperature 93.3ºC) achieved are shown, calculated every 10 min from the end of the immersion time.

4. Conclusions

The results discussed in the present study demonstrate the importance of conducting acidification studies with proper pH measurements to determine safe conditions to manufacture commercially stable pickled eggs. These results should be analyzed in context with validation and challenge studies that address the survival of pathogenic microorganisms in pickled egg systems, with the ultimate goal of establishing science-based recommendations that can be used by the food industry.

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CHAPTER 3

MODELING THE EFFECTS OF PROCESS CONDITIONS ON THE ACCUMULATED LETHALITY VALUES OF THERMALLY PROCESSED PICKLED CARROTS

Abstract

Shelf-stable pickled products are thermally processed to ensure safety and stability. Carrots packed in glass jars and processed in a boiling water bath were chosen to construct models to predict accumulated lethality values given process conditions and heating times. Mixed models with a logarithmic transformation of accumulated lethality as response showed that the effect of blanching prior to filling did not significantly impact the response ($P > 0.05$), while the effects of log process time, jar size, carrots to brine ratio, carrot spear diameter, brine temperature, and concentration of sucrose in the brine and interactions among these variables significantly affected the response ($P < 0.001$), as evaluated in different experimental designs. The residual unexplained replicate-to-replicate variability of all constructed models was always < 3% and every trial was conducted in triplicate. Process authorities can use these models to establish processing guidelines or evaluate current processes for production of shelf-stable pickled carrots or similar foods (as demonstrated by the validation experiments) with pH values from < 3.9 to 4.4. This study also demonstrated that it is feasible to use this experimental setup to evaluate the impact of changes in processing conditions on the accumulated lethality values reached through thermal processing of similar foods. Overall, these results contribute to the establishment of science-based processing

guidelines that will ensure production of safe and stable products with optimized heating times to enhance quality parameters.

1. Introduction

According to US federal regulations (CFR Title 21, Chapter 1, Subchapter B, Part 114), a pickled product is an acidified food (a low-acid food to which acid is added) whose water activity is above 0.85 and has a finished equilibrium pH of 4.6 or below (GPO, 2013). The low pH allows acid and acidified foods to receive only a mild thermal processing to achieve shelf-stability (Tucker & Featherstone, 2011), since it has long been accepted that spores of *Clostridium botulinum* do not germinate and grow (and consequently produce toxin) at or below pH 4.6. Therefore, shelf-stable foods of pH < 4.6 are not required to be processed to inactivate *C. botulinum* spores (Anderson, et al., 2011), but the thermal treatment is designed to kill the less heat resistant molds, yeasts, vegetative cells of bacteria, to inactivate enzymes and cook the product (if necessary) (Tucker & Featherstone, 2011).

Thermal processing guidelines for these food products are often available from trade organizations or research associations such as the Grocery Manufacturers Association (http://www.gmaonline.org/), Pickle Packers International (http://www.ilovepickles.org/) and Campden BRI (http://www.campdenbri.co.uk/). However, given the lower public health concerns associated with acid and acidified foods, and the less severe thermal processes applied to these products (when compared to low-acid foods), research regarding the effect of process conditions on the lethality values obtained during thermal

processing of acid and acidified foods is limited. Furthermore, there has been commensurably less attention given to producing, collecting and organizing the microbial control literature applicable to acid and acidified foods (Pflug, 2003). It should also be considered that while the thermal process must achieve the target lethality (that all the regions of the product are processed at a high enough temperature for a long enough time), the action of heat also destroys nutrients and affects the texture of the product. Hence these undesirable effects need to be minimized (Awuah, Ramaswamy, & Economides, 2007; Banga, Balsa-Canto, Moles, & Alonso, 2003) while simultaneously reducing energy consumption, time, and other valuable resources.

Mixed models will be used in this study to establish processing guidelines for production of shelf-stable pickled foods, as well as to evaluate current processing conditions of similar products. Carrots were selected as the model food based on their high relative firmness and the tissue's homogeneity when compared to similar vegetables, as well as the flexibility to achieve the targeted dimensions in the pieces. This study will use the measure of accumulated lethality (F value) as the response variable as it is the best indicator to assess the safety and stability of thermally processed, shelf-stable foods. The purpose of this study was to evaluate and model the effects of several process conditions on the accumulated lethality values of thermally processed pickled vegetables, using carrots as a model food, as well as to recommend general processing guidelines for production of shelf-stable pickled foods, based on the results obtained from the evaluation of the process conditions.

2. Materials and methods

2.1 Experimental setup

Carrots were purchased from a local supermarket and were stored at 4ºC. Before being thermally processed, they were warmed in running water at 25ºC, washed and peeled. Carrots were cut according to the experimental design (varying diameter and length), and if required by the experiment, were blanched (immersed in boiling water for 5 min, cooled in an ice and water mix for 2 min, immersed in water at 25ºC for 2 min, drained and towel-dried). Carrot spears were weighed and placed in 237 or 473 mL (8 or 16 fl oz) Mason jars (Jarden Home Brands, Daleville, IN) (height × inner diameter: 9.8 × 6.0 and 12.1 × 7.6 cm for the 237 and 473 mL jars, respectively). A brine consisting of 50% distilled water and 50% distilled white vinegar (5% acidity, purchased from a local supermarket) was weighed and added to each jar. The ratio of solids to liquid and the temperature of the brine were dictated by the experimental design.

Needle, type T thermocouples (Ecklund-Harrison Technologies Inc., Fort Myers, FL) were fixed to the jars' metal lids, and inserted through the center of the carrot piece located in the middle of the jar. The depth of the temperature probe was dictated by the experimental design. In order to pre-heat the exterior of the glass jars, filled jars were placed in a wire basket (7 or 5 jars, if 237 or 473 mL jars were used, respectively) and placed in a water bath at 49 ± 1ºC for 1 min. Afterwards, the basket and jars were placed in boiling water in an electric kettle (model TDB/6-10, Groen, Jackson, MS) and the time it took for the water to return to a boil was recorded. The temperatures were recorded every 10 sec using a CALPlex temperature logger and CALSoft32 thermal

processing software (TechniCAL, Inc., New Orleans, LA) as well as a Fluke Hydra

Series II data acquisition unit and Hydra Logger version 3.0 (Fluke Corporation, Everett,

WA) for preliminary tests, while a CALPlex temperature logger and CALSoft5 thermal

processing software (TechniCAL, Inc., New Orleans, LA) were used for the rest of the

experiments. Jars were taken out of the kettle when their corresponding thermocouples

measured temperatures of 60.0, 65.6, 71.1, 76.7, 82.2, 87.8 and 93.3°C (140 to 200°F

at 10°F intervals) for the 237 mL jars, and 60.0, 68.3, 76.7, 85.0, 93.3°C (140 to 200°F

at 15°F intervals) for the 473 mL jars and left to cool at room temperature (24 ± 2°C)

until temperatures inside the jars reached 60°C. Times were recorded when individual

jars were removed from the kettle.

2.2 Data analysis

For each experiment, mixed models were constructed using a logarithmic

transformation of accumulated lethality as response. Based on time and temperature

data, accumulated lethality (F) was calculated according to the following equation:

$$F = \int_0^{t_f} 10^{\left(\frac{T - T_{ref}}{z}\right)} dt$$

where T corresponds to the temperature, T_{ref} to the reference temperature (93.3°C), z to

the thermal resistance (8.9°C), and t to the time. Only temperatures above 60°C were

used for the calculation of the accumulated lethality values. The fixed main effects

tested were the logarithmic transformation of process time (corresponding to the time

between the return of the water to a boil and the time when jars were removed from the

kettle) and the process conditions, in addition to the interactions between the process

conditions. A random effect of replicate nested within the process conditions was

added. Every combination of processing conditions (trial) was replicated three times, each replicate corresponding to a separate trial consisting of 5 or 7 jars, depending on their size. The order of the trials was randomized within experiments. Significant terms of the model were selected through backwards elimination. Terms were considered significant at $P < 0.001$. Analyses were performed using the statistical software JMP® Pro 9.0.2 (SAS Institute Inc., Cary, NC).

2.3 Preliminary experiments

The effects of five process conditions were evaluated: (a) jar size (237 or 473 mL), (b) carrots to brine ratio (65:35 or 35:65), (c) carrot spear diameter (17 or 20% length of spear), (d) brine temperature (25 or 75ºC) and (e) blanching or not blanching prior to filling. Table 3.1 shows the detailed experimental conditions for the combinations of the first three variables. The last two variables did not make the rest of the conditions differ, so more detail was deemed unnecessary. Thermocouples used during these experiments were selected according to preliminary coldspot-location trials. Therefore, thermocouple length varied according to jar size and carrots to brine ratio: 54 mm (237 mL / 65:35), 72 mm (237 mL / 35:65) and 65 mm (473 mL / 65:35). Four independent experiments were run (Table 3.2), and three 2^3 factorial designs were constructed by combining experiments B, C and D with experiment A, respectively. Therefore, each one of the three factorials tested the effect of brine temperature and the effect of blanching or not blanching prior to filling, in addition to the effect of jar size (first factorial), carrots to brine ratio (second) and carrot spear diameter (third), and their interactions. In order to overcome the issue that parts of the three factorial designs were

run at different moments, the effect of order of the trial within the experiment was tested. This was achieved by adding a fixed and continuous effect of order to the model, which was nested within the different process factors tested in each of the three factorials (jar size, carrots to brine ratio and carrot spear diameter). The reasoning behind this setup is that if the effect of order is not significant within experiments, the effect would also be negligible between experiments. Therefore, the approach of combining four experiments to create three factorial designs is valid.

Table 3.1. Experimental conditions for the combinations of three variables evaluated during the pickled carrot thermal processing trials.

Jar size (mL)	Carrots to brine ratio	Carrot spear diameter (% length of spear)
237 (Length of carrot spears: 7 cm)	65:35 (130 g of carrots and 70 g of brine)	17 (Center piece: 9 g, rest of pieces between 5 and 10 g)
		20 (Center piece: 12 g, rest of pieces between 15 and 25 g)
	35:65 (70 g of carrots and 130 g of brine)	17 (Center piece: 9 g, rest of pieces between 5 and 10 g)
		20 (Center piece: 12 g, rest of pieces between 5 and 15 g)
473 (Length of carrot spears: 9 cm)	65:35 (270 g of carrots and 145 g of brine)	17 (Center piece: 19 g, rest of pieces between 10 and 20 g)
		20 (Center piece: 25 g, rest of pieces between 30 and 50 g)
	35:65 (145 g of carrots and 270 g of brine)	17 (Center piece: 19 g, rest of pieces between 10 and 20 g)
		20 (Center piece: 25 g, rest of pieces between 10 and 30 g)

Table 3.2. Process conditions evaluated in the four preliminary experiments used to test their effects on the accumulated lethality values of thermally processed pickled carrots.

Experiment	Jar size (mL)	Carrots to brine ratio	Carrot spear diameter (% length of spear)	Brine temperature (°C)	Blanching or not prior to filling
A	237	65:35	20	25	Blanched
					Unblanched
				75	Blanched
					Unblanched
B	473	65:35	20	25	Blanched
					Unblanched
				75	Blanched
					Unblanched
C	237	35:65	20	25	Blanched
					Unblanched
				75	Blanched
					Unblanched
D	237	65:35	17	25	Blanched
					Unblanched
				75	Blanched
					Unblanched

2.4 Effect of process conditions

The effects of four process conditions were evaluated: (a) jar size (237 or 473 mL), (b) carrots to brine ratio (65:35 or 35:65), (c) carrot spear diameter (17 or 20% length of spear) and (d) brine temperature (25 or 75°C). The experimental conditions were identical to those used for the preliminary tests (Table 3.1). Thermocouples used during this test were selected according to preliminary coldspot-location trials. Therefore,

thermocouple length varied according to jar size and carrots to brine ratio: 54 mm (237 mL / 65:35), 72 mm (237 mL / 35:65), 65 mm (473 mL / 65:35) and 81 mm (473 mL / 35:65). A 2^4 factorial design was constructed from two separate experiments differing by carrots to brine ratio (65:35 versus 35:65). The issue of segmenting the full factorial in two experiments was approached similarly to the preliminary tests in that the model included a fixed and continuous effect of order of the trial within the experiment, which was nested within the carrots to brine ratio factor.

2.5 Validation experiments

Four validation experiments were carried out. Each experiment consisted of 7 or 5 jars per trial (if 237 or 473 mL jars were used, respectively), and was replicated three times (three independent trials). The first two experiments correspond to the following process conditions: jar size 237 mL, carrots to brine ratio 65:35, carrot spear diameter 17% length of spear, brine temperature 25ºC, process time 7 min. They were carried out using carrots (experiment 1) and green beans (experiment 2). Green beans were previously blanched: immersed in boiling water for 5 min, cooled in an ice and water mix for 2 min, immersed in water at 25ºC for 2 min, drained and towel-dried. The other two experiments correspond to process conditions: jar size 473 mL, carrots to brine ratio 65:35, carrot spear diameter 20% length of spear, brine temperature 75ºC, process time 11 min. They were carried out using carrots (experiment 3) and cucumbers (experiment 4). All process conditions were randomly selected for the validation trials. The lengths and diameters of carrots, green beans and cucumbers correspond to those indicated in Table 3.1 (green beans and cucumbers were prepared as close as possible to the

40

indicated conditions). Trials were run according to conditions mentioned in section 2.1, with the exception that the wire basket and all jars were taken out of the kettle when the chosen process time was achieved. The logarithmic transformation of the accumulated lethality value achieved by each jar was calculated, which corresponds to the observed values. A model with the logarithmic transformation of the accumulated lethality value as the response and trial as a random effect assessed significant differences between trials within the same experiment. The predicted values of the logarithmic transformation of the accumulated lethality values at the tested conditions of the validation experiments were obtained from the model built from the experiment described in section 2.4, after the non-significant terms were eliminated. 95% confidence intervals were calculated for the observed and predicted values.

2.6 Effect of position of thermocouple

A 2^4 factorial design was constructed which tested the effects of: (a) jar size (237 or 473 mL), (b) carrot spear diameter (17 or 20% length of spear), (c) brine temperature (25 or 75ºC) and (d) position of thermocouple (coldspot or middle of the carrot piece –non coldspot–). Carrots to brine ratio was fixed at 35:65, and the experimental conditions were identical to those used for the preliminary tests (Table 3.1). Thermocouples used during this test were selected according to preliminary coldspot-location trials, as well as according to the length of the carrot spears. Therefore, thermocouple length varied according to jar size and position of thermocouple: 54 mm (237 mL / middle of carrot), 72 mm (237 mL / coldspot), 65 mm (473 mL / middle of carrot) and 81 mm (473 mL / coldspot). The factorial design was constructed from two separate experiments differing

by the position of the thermocouple (coldspot versus middle of the carrot piece). The issue of segmenting the full factorial in two experiments was approached in a way that was similar to the preliminary tests in that the model included a fixed and continuous effect of order of the trial within the experiment, which was nested within the position of thermocouple factor.

2.7 Effect of concentration of sucrose in the brine

The experimental conditions selected to test the effect of concentration of sucrose in the brine correspond to: jar size 237 mL, carrots to brine ratio 65:35, carrot spear diameter 17% length of spear and brine temperature 75ºC (details shown in Table 3.1). The thermocouples used during this test correspond to 54 mm, and were chosen according to preliminary coldspot-location trials. The effect of a logarithmic transformation of concentration of sucrose (expressed as ºBrix) was added to the model as a continuous fixed effect, in addition to its interaction with the logarithmic transformation of process time. Tested concentrations of sucrose correspond to 20, 24, 30, 35, 40, 50 and 60ºBrix, which comprise typical concentrations used for pickling vegetables. However, it must be noted that since concentrations increased during the heating of the brine due to the loss of water, the values that were used in the model correspond to those that were measured immediately before placing the lids on the jars. ºBrix were measured using a digital Abbe refractometer (Leica Inc., Buffalo, NY). The volume of the brine was kept constant at 70 mL and thus its mass increased with increasing concentration of sucrose. The random effect of replicate was not nested within any process condition.

3. Results and discussion

3.1 Preliminary experiments

The fixed and continuous effect of order of the trial within the experiment, nested within the different process factors tested in each of the three preliminary factorial designs of jar size, carrots to brine ratio and carrot spear diameter did not present a significant effect (P = 0.9051, 0.6820 and 0.9215, respectively). Therefore, the method of combining four experiments to create three factorial designs was deemed valid. Table 3.3 shows the results from the three preliminary models used to evaluate the effects of the five process conditions on the logarithmic transformation of accumulated lethality. Neither the main effect of blanching or not blanching prior to filling nor its interactions with the other factors had a significant effect on the response variable (P > 0.001). Although blanching can result in undesirable softening of vegetable tissues (Reyes de Corcuera, Cavalieri, & Powers, 2004) due to loss of turgor pressure and occluded air, thermal degradation of middle lamella pectins and other cell wall polysaccharides, and starch gelatinization (Stanley, Bourne, Stone, & Wismer, 1995), it has been reported that low-temperature blanching can actually improve texture and increase firmness of thermally processed vegetables such as carrots and green beans (Lin & Schyvens, 1995; Vu, et al., 2004). There are numerous studies in the literature that have attempted to explain thermal texture degradation in carrots (Peng, Tang, Barrett, Sablani, & Powers, 2014; Smout, Sila, Vu, Van Loey, & Hendrickx, 2005).

Table 3.3. Results from the models for logarithmic transformation of accumulated lethality, according to the three 2^3 preliminary factorial designs. Dummy variables coded 0 and 1 were used for nominal factors.

Factorial	Amount of residual variance explained by the model	Parameter estimates			
		Term	Estimate	Standard error	$P > |t|$
Jar size	98.82%	Intercept	- 4.92	0.07	<0.0001
		Log process time	2.81	0.03	<0.0001
		Jar size (473 mL = 0)	- 0.30	0.06	<0.0001
		Blanching (Unblanched = 0)	0.01	0.06	0.8583
		Brine temperature (25ºC = 0)	- 0.23	0.06	0.0009
Carrots to brine ratio	98.08%	Intercept	- 3.24	0.07	<0.0001
		Log process time	2.65	0.03	<0.0001
		Carrots to brine ratio (65:35 = 0)	- 1.31	0.08	<0.0001
		Blanching (Unblanched = 0)	- 0.11	0.05	0.0533
		Brine temperature (25ºC = 0)	- 0.90	0.08	<0.0001
		Carrots to brine ratio × Brine temperature	0.6	0.1	<0.0001
Carrot spear diameter	98.82%	Intercept	- 4.28	0.06	<0.0001
		Log process time	2.82	0.03	<0.0001
		Carrot spear diameter (20% = 0)	- 0.61	0.05	<0.0001
		Blanching (Unblanched = 0)	0.02	0.05	0.7786
		Brine temperature (25ºC = 0)	- 0.32	0.05	<0.0001

While no measurements of firmness or any other texture property of the vegetable pieces were conducted in this experiment, the non-significant effect of blanching or not blanching prior to filling on the accumulated lethality values indicated that textural properties of the raw and blanched carrots were similar enough to result in comparable heat transfer properties. Therefore, this process condition was not evaluated in the following trials, and carrots were left unblanched. Since the effects of jar size, carrots to brine ratio, carrot spear diameter and brine temperature significantly affected the response variable ($P < 0.001$), they were all included in the following experiment, as part of a full factorial design.

3.2 Effect of process conditions

The fixed and continuous effect of order of the trial within the experiment, nested within the carrots to brine ratio factor did not present a significant effect ($P = 0.5693$). As with the preliminary trials, the method of combining two experiments to create a 2^4 factorial designs was deemed valid. Table 3.4 shows the results from the model that was used to evaluate the effects of four process conditions on the logarithmic transformation of accumulated lethality. The amount of residual variance explained by the model is 98.38%. As expected, longer processing times produced higher accumulated lethality values, following a power trend. Also, as expected, smaller jars (237 mL), lower carrots to brine ratios (35:65), smaller carrot spear diameters (17% length of spear), and higher brine temperatures (75ºC) all increased the measured accumulated lethality. The reasons behind these higher accumulated lethality values mainly correspond to differences in initial temperature of the product and jar (affected by the brine's

temperature); mass of the jar and product (affected by the jar's size); density, specific

heat, and thermal conductivity of the product (affected by the carrots to brine ratio);

radius and volume of the jar and carrot pieces (affected by both the jar's size and

diameter of the carrot spears); and influence of the natural convection process of heat

transfer (affected by the carrots to brine ratio). The effect of these properties on heat

flow in thermally processed packaged foods has been described in the literature

(Holdsworth & Simpson, 2007).

Table 3.4. Results from the model for logarithmic transformation of accumulated
lethality, according to a 2^4 factorial design. Dummy variables coded 0 and 1 were used
for nominal factors.

Term	Estimate	Standard error	$P > |t|$
Intercept	- 4.99	0.06	<0.0001
Log process time	2.54	0.02	<0.0001
Jar size (237 mL = 0)	0.54	0.07	<0.0001
Carrots to brine ratio (35:65 = 0)	0.69	0.07	<0.0001
Jar size × Carrots to brine ratio	- 0.38	0.08	<0.0001
Carrot spear diameter (17% = 0)	0.73	0.05	<0.0001
Jar size × Carrot spear diameter	- 0.30	0.06	<0.0001
Carrots to brine ratio × Carrot spear diameter	- 0.47	0.06	<0.0001
Brine temperature (75ºC = 0)	0.51	0.06	<0.0001
Jar size × Brine temperature	- 0.32	0.09	0.0005
Carrots to brine ratio × Brine temperature	- 0.44	0.09	<0.0001
Jar size × Carrots to brine ratio × Brine temperature	0.7	0.1	<0.0001

Table 3.5 shows the least squares means for the combinations of terms included in the three significant interactions from the model (one three-way and two two-way interactions). Least squares means are values predicted by the model for levels of a categorical effect where the other model factors are set to neutral values (the sample mean for log process time, and the average of the coefficients for the nominal effects not involved in the corresponding interaction). Because least squares means are predictions at fixed (neutral) values of the other factors, comparisons are able to be made (SAS Institute Inc., 2014). A *post hoc* multiple comparison with a Tukey correction was used to compare the combinations in this study. Results are presented in Table 3.5. For example, in the case of the three-way interaction, the lowest lethality value (0.40 min) corresponds to the combination of big jars (473 mL), high carrots to brine ratios (65:35), and low brine temperatures (25ºC), while the highest lethality (1.04 min) corresponds to the combination of the opposite values of the nominal terms. In general, intermediate lethality values from other combinations of the terms included in this interaction are not significantly different from each other (Tukey's test, $P > 0.05$).

Table 3.5. Least squares means for the combinations of nominal factors included in the three significant interactions from the model for logarithmic transformation of accumulated lethality, according to a 2^4 factorial design.

Interaction	Least squares mean ± Standard error [a]
Jar size × Carrots to brine ratio × Brine temperature	
Jar size 237 mL / Carrots to brine ratio 35:65 / Brine temperature 75ºC	0.03 ± 0.04 [A]
Jar size 237 mL / Carrots to brine ratio 65:35 / Brine temperature 75ºC	- 0.33 ± 0.04 [B]
Jar size 473 mL / Carrots to brine ratio 35:65 / Brine temperature 75ºC	- 0.38 ± 0.04 [BC]
Jar size 473 mL / Carrots to brine ratio 65:35 / Brine temperature 75ºC	- 0.40 ± 0.05 [BC]
Jar size 237 mL / Carrots to brine ratio 35:65 / Brine temperature 25ºC	- 0.45 ± 0.04 [BC]
Jar size 473 mL / Carrots to brine ratio 35:65 / Brine temperature 25ºC	- 0.45 ± 0.04 [BC]
Jar size 237 mL / Carrots to brine ratio 65:35 / Brine temperature 25ºC	- 0.52 ± 0.04 [C]
Jar size 473 mL / Carrots to brine ratio 65:35 / Brine temperature 25ºC	- 0.91 ± 0.04 [D]
Carrots to brine ratio × Carrot spear diameter	
Carrots to brine ratio 65:35 / Carrot spear diameter 17%	- 0.25 ± 0.03 [A]
Carrots to brine ratio 35:65 / Carrot spear diameter 17%	- 0.26 ± 0.03 [AB]
Carrots to brine ratio 35:65 / Carrot spear diameter 20%	- 0.37 ± 0.03 [B]
Carrots to brine ratio 65:35 / Carrot spear diameter 20%	- 0.83 ± 0.03 [C]
Jar size × Carrot spear diameter	
Jar size 237 mL / Carrot spear diameter 17%	- 0.22 ± 0.03 [A]
Jar size 473 mL / Carrot spear diameter 17%	- 0.29 ± 0.03 [A]
Jar size 237 mL / Carrot spear diameter 20%	- 0.41 ± 0.03 [B]
Jar size 473 mL / Carrot spear diameter 20%	- 0.78 ± 0.03 [C]

[a] Values in the same interaction section not sharing a common superscript letter represent significantly different values (*P* < 0.05) based on *post hoc* multiple comparisons with a Tukey correction.

These results can be used by food processors and process authorities to establish processing guidelines for production of shelf-stable pickled foods with pH values from < 3.9 to 4.4, corresponding to accumulated lethality values between 0.1 and 10 min (reference temperature 93.3ºC, thermal resistance 8.9ºC) (Pflug, 2003). Additionally, they can be used to evaluate current processing conditions of similar products. Although results are limited to carrots and to the tested ranges of levels of process conditions, the model could be applied to vegetables with similar physical properties within the tested ranges of levels. Alternatively, the relatively simple methodology of this experiment as well as the low amount of residual variance unexplained by the model show that this methodology is valid and can be used in the future to further evaluate the impact of various processing conditions on the accumulated lethality values achieved during thermal processing.

3.3 Validation experiments

For validation experiments carried out with carrots and cucumbers, 0% of the total variance was explained by differences among trials. For the experiment using green beans, differences among trials explained 23% of the total variance. Nonetheless, no significant differences were found between trials ($P > 0.001$), and individual measured values of log transformed accumulated lethalities were averaged. Figure 3.1 presents results from the four validation experiments that were conducted, including the predicted and observed log transformed accumulated lethality values (averages from three trials) as well as their respective 95% confidence intervals. Overall, the model seems to under predict the log transformed accumulated lethality values. From the food safety and

stability standpoint, this under-prediction is preferable compared to an over-prediction. Results showed larger differences between observed and predicted log transformed accumulated lethality values in green beans and cucumbers. This is likely caused by structural differences of the vegetable pieces. The effect is more marked in green beans, which in turn are a more heterogeneous product, hence the wider confidence interval shown in Figure 3.1A. Also, the larger disparities of green beans and cucumbers can be due to differences in their thermal properties when compared to carrot pieces (mainly density, specific heat, and thermal conductivity of the product).
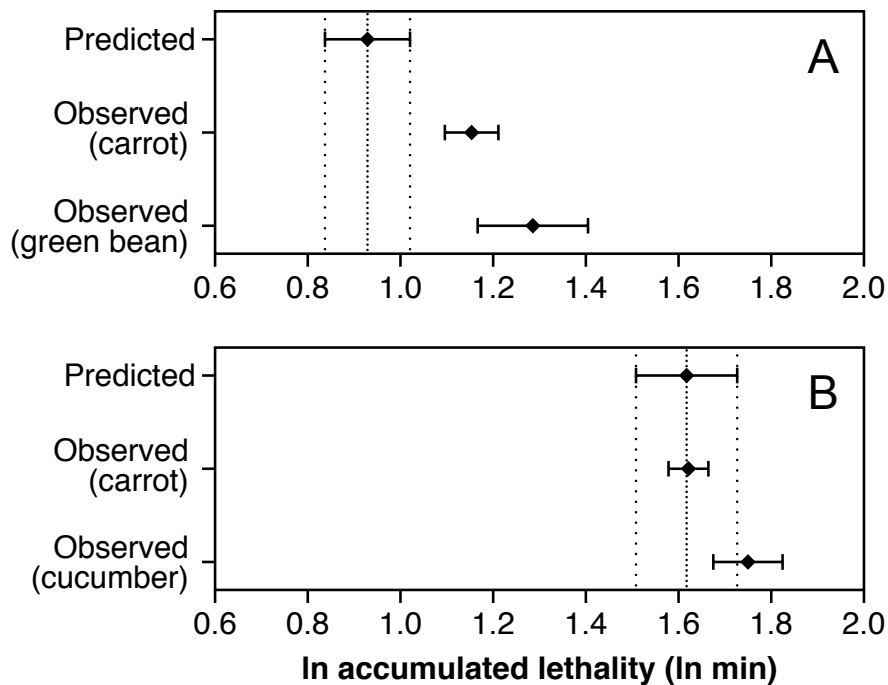

Figure 3.1. Average log transformed accumulated lethality values estimated from the validation experiments. Error bars represent 95% confidence intervals (n = 3). Process conditions (jar size, carrots to brine ratio, carrot spear diameter, brine temperature, process time): (A) 237 mL, 65:35, 17% length of spear, 25ºC, 7 min. (B) 473 mL, 65:35, 20% length of spear, 75ºC, 11 min.

3.4 Effect of position of thermocouple

The fixed and continuous effect of order of the trial within the experiment, nested within the position of thermocouple factor did not present a significant effect (P = 0.7646). Table 3.6 shows the results from the model used to evaluate the effect of the position of the temperature probe in the carrot piece located in the middle of the jar, as well as three other process conditions on the logarithmic transformation of accumulated lethality. The amount of residual variance explained by the model is 98.42%. The carrots to brine ratio of 35:65 was fixed in this test so that there was a higher amount of liquid in the jars and therefore the effect of natural convection during heat transfer increased. It was expected that trials with thermocouples positioned in the coldspot of the container (lower section of the carrot spear) would heat slower than those with the thermocouple in the middle of the carrot piece. Thus it was anticipated that the coldspot trials would present overall lower lethalities than non-coldspot trials. Analysis of the model's coefficient for the effect of position of thermocouple (Table 3.6) confirmed this, showing a significant difference in accumulated lethality of 1.6 min when coldspot trials were compared to non-coldspot trials.

In order to evaluate the relative relevance of the four main effects included in this experimental design, likelihood ratios were used to compare reduced or restricted models with the full or unrestricted model. Four reduced models were tested, each without one of the four main effects and their corresponding interactions. The full model corresponds to the one shown in Table 3.6, which includes all significant terms (main

effects of jar size, carrot spear diameter, brine temperature, and position of thermocouple, and their significant interactions).

Table 3.6. Results from the model for logarithmic transformation of accumulated lethality, according to a 2^4 factorial design which includes the effect of position of thermocouple. Dummy variables coded 0 and 1 were used for nominal factors.

Term	Estimate	Standard error	$P > |t|$
Intercept	- 4.26	0.06	<0.0001
Log process time	2.44	0.02	<0.0001
Jar size (237 mL = 0)	0.43	0.06	<0.0001
Position of thermocouple (middle of the carrot piece = 0)	0.49	0.03	<0.0001
Carrot spear diameter (17% = 0)	0.38	0.06	<0.0001
Jar size × Carrot spear diameter	- 0.55	0.08	<0.0001
Brine temperature (75ºC = 0)	0.26	0.06	<0.0001
Jar size × Brine temperature	- 0.03	0.08	0.6938
Carrot spear diameter × Brine temperature	- 0.29	0.09	0.0014
Jar size × Carrot spear diameter × Brine temperature	0.5	0.1	0.0001

When compared to the full model, the likelihood ratios showed that the model without the effect of the thermocouple position had a worse fit than the models without the effects of jar size, brine temperature, and carrot spear diameter. Furthermore, the least squares means were analyzed to assess the impact of the four main effects or the combination of effects in interactions on the response variable. In terms of difference in values of log transformed accumulated lethality, the overall difference between coldspot trials and non-coldspot trials was higher than any other comparison of main effects or

combination of factors within interactions. The only exception was within two combinations: smaller jars (237 mL), higher brine temperatures (75ºC) and any carrot spear diameter (17 or 20% length of spear), compared to larger jars (473 mL), lower brine temperatures (25ºC) and larger carrot spear diameters (20% length of spear). These results show that even in low-temperature processes applied to pickled foods, the correct position of the temperature probe for data collection is vital for accurate measurements (Larousse & Brown, 1997). Furthermore, even small variations on its location can significantly affect the magnitude of the accumulated lethality values collected. This factor alone could have a larger impact than the variation of other process conditions.

3.5 Effect of concentration of sucrose in the brine

Table 3.7 shows the results from evaluating the effect of a logarithmic transformation of concentration of sucrose in the brine (expressed as ºBrix) on the logarithmic transformation of accumulated lethality. The amount of residual variance explained by the final model is 97.61%. As in the previous experiments, the effect of process time on the resulting accumulated lethality follows a power trend. At the average log sucrose concentration, longer processing times produce higher accumulated lethality values and the positive trend becomes stronger with an increase in log sucrose concentration, as indicated by the interaction term. Inversely, the effect of concentration of sucrose in the brine on the resulting accumulated lethality also followed a power trend. However, this time higher sucrose concentrations produced lower accumulated lethality values at the

average log process time, and the interaction term indicates that this negative trend diminishes with an increase in log process time.

Table 3.7. Results from the model used to test the effect of concentration of sucrose (20 - 60°Brix) in the brine on the logarithmic transformation of accumulated lethality. Both log process time and log sucrose concentration (°Brix) were centered.

Term	Estimate	Standard error	$P > |t|$
Intercept	5.6	0.3	<0.0001
Log process time	2.66	0.04	<0.0001
Log °Brix	- 3.35	0.09	<0.0001
(Log process time - 2.31) × (Log °Brix - 3.60)	1.2	0.1	<0.0001

The observed effect of the concentration of sucrose on the accumulated lethality values is most likely due to changes in the mass of the product. The volume of the brine was kept constant at 70 mL, so higher concentrations of sucrose cause higher mass in the jars. Also, the addition of sucrose to the brine leads to variations in brine density, viscosity, thermal conductivity, and specific heat, which can affect the natural convection process of heat transfer (density variations due to changes in temperature being the driving force for the liquid motion) (Datta & Teixeira, 1988; Earle, 2004). Although natural convection tends to push the slowest heating region or coldspot to the bottom of the container (Ghani, Farid, Chen, & Richards, 1999), varying concentrations of sucrose in the brine could possibly affect the location of the coldspot. Preliminary trials confirmed that the coldspot remained in the same lower location in the container, regardless of the sucrose concentration.

4. Conclusions

This study demonstrated that it is feasible to use mixed models to evaluate and predict the effects of process conditions (jar size, carrots to brine ratio, carrot spear diameter, brine temperature, blanching or not blanching prior to filling, and concentration of sucrose in the brine) on the accumulated lethality values of thermally processed pickled carrots. It is expected that this experimental setup can be applied to further evaluate the impact of variations in processing conditions on the accumulated lethality values reached through thermal processing of similar foods. Food processors and process authorities can use the results obtained from the models to establish processing guidelines and evaluate current processes for production of shelf-stable pickled foods. As demonstrated through validation trials, this can be applied to carrots or other similar products with pH values from < 3.9 to 4.4. It was confirmed that the correct position of the temperature probe for data collection is essential for precise measurements, even in low-temperature processes such as those applied to pickled vegetables.

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CHAPTER 4

EVALUATION OF A HOT-FILL-HOLD OPERATION: EFFECTS OF PROCESS CONDITIONS ON ACCUMULATED LETHALITY AND VACUUM FORMATION FOR pH CONTROLLED FOODS

Abstract

The hot-fill-hold process is commonly used for processing fluid acid and acidified foods with pH values below 4.4. There are scarce processing guidelines or published data indicating temperatures and times required to achieve proper thermal treatment of the processed foods in a given container and adequate vacuum. We evaluated a hot-fill-hold process by assessing the effect of process conditions on vacuum formation and on accumulated lethality values based on the container's coldspot, corresponding to the underside of the lid, and modeled the conditions' effects. Sucrose solutions (10 to 50°Brix) were prepared and heated (79.4 to 96.1°C). Glass canning jars (473 and 946 mL) were used, with two lid widths (6.2 and 7.6 cm). After hot-filling leaving 6 to 15% headspace (depending on the jar's volume and lid's width), the temperature on the underside of the lid was measured for 5 min using a surface thermocouple. After cooling at room temperature, container vacuum was measured. Mixed models (residual unexplained replicate-to-replicate variability was always < 2%) showed that all three- and four-way, and most two-way interactions were not significant in either model (accumulated lethality and vacuum). Overall, lethality values ranged between 0.001 and 0.1 min (T_{ref} 93.3°C, z-value 8.9°C), and vacuum between -20 and -60 kPa. These results contribute to the establishment of processing guidelines ensuring production of

safe and stable products with optimized processing temperatures and times, to enhance quality of products processed using hot-fill-hold operations.

1. Introduction

According to US federal regulations (CFR Title 21, Chapter 1, Subchapter B, Part 114.3(e)), a scheduled process for an acidified food is the process selected by a processor as adequate for use under the conditions of manufacture for a food in achieving and maintaining a food that will not permit the growth of microorganisms having public health significance (GPO, 2013). Since *Clostridium botulinum* will not grow in media that has a pH lower than 4.6 (acid), acid and acidified foods do not require as severe a process as those with a pH above 4.6 (low acid). Two basic processes are usually applied to attain pasteurization of acidified foods: (a) hot-fill-hold, in which fluid products are heated and held at a given temperature for a given time, filled into containers, closed and inverted or rotated to pasteurize the internal surfaces of the container, including the closure, and held hot for a specified amount of time; and (b) water bath, in which products are filled into the container, and then thermally processed, usually using hot water baths, sprays or canals (Larousse & Brown, 1997).

Acidified and acid fluid foods such as applesauce and other fruit purees, as well as tomato-based sauces are processed using the hot-fill-hold method. Although this process is widespread in the food industry, not many studies have addressed the effect of process conditions on the products' quality parameters and microbial targets (Silva, Martins, & Silva, 2003; Silva & Silva, 1997), or on the resulting lethality values

(Sandoval, Barreiro, & Mendoza, 1994). No study has been found in the literature that addresses the effect of process conditions on the efficacy of the hot-fill-hold operation to allow the underside of the lid (which is the area of the container that presents the lowest time / temperature exposure) to achieve proper pasteurization. Although some molds have been found to be able to grow at atmospheric oxygen concentrations as low as 0.5% or 2% (depending on the experimental conditions), molds encountered in food spoilage are usually considered strict aerobes. Consequently, limiting oxygen as a substrate is an effective way to inhibit mold growth (Dagnas & Membre, 2013). Therefore, the process conditions must also ensure that vacuum is achieved after packaging, so anaerobic conditions are attained.

This study will use the measure of accumulated lethality to determine the ability of a hot-fill-hold process to achieve the desired pasteurization levels on the underside of the lid, while assessing the vacuum formed after closure of the container. Results obtained from this project can be used to establish processing guidelines and to evaluate current processing conditions to ensure production of safe, shelf-stable acidified and acid products.

2. Materials and methods

2.1 Experimental setup

Solutions of distilled water and sucrose covering typical concentrations of hot-packed foods (ranging from 10 to 50°Brix, at 10°Brix intervals) were prepared, resulting in water activities (average ± standard deviation, n = 3) of 0.997 ± 0.001, 0.988 ± 0.001, 0.978 ±

0.002, 0.963 ± 0.001, and 0.940 ± 0.003. Concentration of sucrose (ºBrix) was measured using a digital Abbe refractometer (Leica Inc., Buffalo, NY), and water activity was measured using an AquaLab 4TE water activity meter (Decagon Devices Inc., Pullman, WA). Solutions were heated to 79.4, 85.0, 90.6 and 96.1ºC (175, 185, 195 and 205ºF). Mason jars (Jarden Home Brands, Daleville, IN) of sizes 473 and 946 mL (16 and 32 fl oz), commonly used for acid and acidified foods, were used, with two varying lid widths: 6.2 and 7.6 cm. The hot sucrose solutions were placed in the glass jars leaving headspace volumes of 6, 10, 8 and 15% the total volume of the jars, for combinations of jar size/lid width: 946 mL/6.2 cm, 473 mL/6.2 cm, 946 mL/7.6 cm and 473 mL/7.6 cm, respectively. The volume of solutions was kept constant for each jar size/lid width combination, regardless of sucrose concentration. Therefore, the masses of solutions varied according to their density.

After the solutions were poured in the jars, the lids were secured in place, and jars were inverted. The process of filling, securing the lid and inverting the jars took 20 sec. After the jars were held inverted for 5 min, they were returned to the upright position. During the inversion period, the temperature on the underside of the lid (coldest point after hot-packing, determined by preliminary trials) was measured every 10 sec, using a CALPlex temperature logger and CALSoft5 thermal processing software (TechniCAL, Inc., New Orleans, LA) equipped with a 20114 Micro-Foil surface thermocouple (RdF Corporation, Hudson, NH). The surface thermocouple was fixed at the center of the underside of the lid. Preliminary trials to confirm that the underside of the lid corresponds to the coldspot of the containers during the hot-fill-hold operation were conducted using needle and

flexible, type T thermocouples (Ecklund-Harrison Technologies Inc., Fort Myers, FL), in addition to the surface thermocouple. The needle thermocouple was located at the center of the jar, and the flexible thermocouple was used to measure temperature at the bottom of the jar. The hot-fill-hold operation was conducted and measurements were recorded as described.

The same experimental setup described above was repeated without the thermocouples, in order to measure vacuum formation. After filling, lidding, inverting, holding and returning to the upright position, jars were left to cool for at least 16 h at 24 ± 2°C. Vacuum produced inside the jars was measured using a Series 6000 Zahm Model D.T. piercing device (Zahm & Nagel Co., Inc., Holland, NY) equipped with a vacuum gauge, by puncturing each jar's lid and recording the measured vacuum.

2.2 Data analysis

A full factorial design was used to test the effects of the solution's sucrose concentration (10 to 50°Brix), filling temperature (79.4 to 96.1°C), jar size (473 or 946 mL) and lid width (6.2 or 7.6 cm). Two mixed models were constructed with responses: (a) logarithmic transformation of accumulated lethality measured on the underside of the lid and (b) vacuum formed. Based on time and temperature data, accumulated lethality (F) was calculated according to the following equation:

$$F = \int_0^{t_f} 10^{\left(\frac{T-T_{ref}}{z}\right)} dt$$

where T corresponds to the temperature, T_{ref} to the reference temperature (93.3°C), z to the thermal resistance (8.9°C), and t to the time. The fixed effects tested were the

63

process conditions and their interactions. A random effect of replicate was also added.

Every combination of processing conditions (trial) was replicated three times. Significant

terms of the model were selected through backwards elimination. Terms were

considered significant at $P < 0.01$. Analyses were performed using the statistical

software JMP® Pro 11.0.0 (SAS Institute Inc., Cary, NC).

2.3 Validation experiments

Four validation experiments were carried out following the experimental setup described

in section 2.1. Each experiment was replicated three times, and for each replicate,

measurements of accumulated lethality on the underside of the lid and vacuum formed

were recorded. Table 4.1 shows the processing conditions tested, which were randomly

selected.

Table 4.1. Experimental conditions evaluated in the four validation experiments.

Sucrose concentration (ºBrix)	Fill temperature (ºC)	Jar size (mL)	Lid width (cm)
22	87.8	946	6.2
22	87.8	473	6.2
36	93.3	946	7.6
36	93.3	473	7.6

The validation experiments were repeated, but instead of using sucrose solutions, two

products typically processed using a hot-fill-hold process were chosen. Applesauce

(22ºBrix) and barbeque (BBQ) sauce (36ºBrix) (both purchased from a local

supermarket) were used. The measured values of logarithmic transformation of accumulated lethality on the underside of the lid and vacuum formed during the validation experiments correspond to the observed values. The predicted values of the responses at the tested conditions of the validation experiments were obtained from the models built from the experiment described in section 2.2, after the non-significant terms were eliminated. 95% confidence intervals were calculated for both observed and predicted values.

3. Results and discussion

3.1 Coldspot location trials

Preliminary trials were conducted to confirm the location of the coldspot in the containers during the hot-fill-hold operation. Two locations were selected: the underside of the lid and the bottom of the jar. As a reference, temperatures were also recorded on the center of the jar. Figure 4.1 shows representative temperature profiles and calculated accumulated lethalities, which confirm the underside of the lid as the container's coldspot. The process conditions (jar size, lid width, fill temperature and sucrose concentration) that yielded data shown in Figure 4.1 are: 473 mL, 7.6 cm, 79.4ºC and 50ºBrix. These conditions correspond to the trial that produced the lowest accumulated lethality value measured on the underside of the lid. Similar results were obtained with other process conditions.
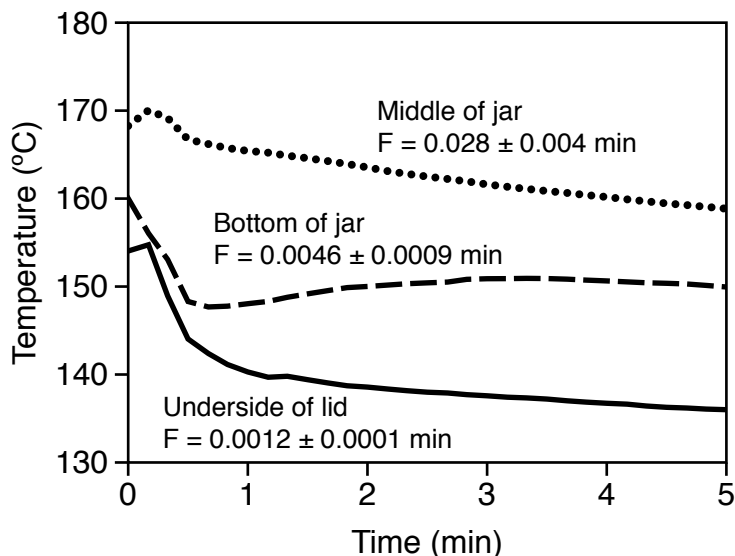
Figure 4.1. Average temperature profiles and accumulated lethalities measured during the preliminary coldspot location trials (n = 3). Process conditions (jar size, lid width, fill temperature, sucrose concentration): 473 mL, 7.6 cm, 79.4°C, 50°Brix.

3.2 Evaluation of effects of process conditions

Table 4.2 shows the results from the model that was used to evaluate the effects of process conditions on the logarithmic transformation of accumulated lethality. The amount of residual variance explained by the model is 98.20%. Jar size had a significant effect on accumulated lethality measured on the underside of the lid, but effect of lid width was substantially less marked. The effects of sucrose concentration and fill temperature on the resulting accumulated lethality follow a logarithmic trend. At the average sucrose concentration, higher fill temperatures produce higher accumulated lethality values. However, the positive trend becomes weaker with an increase in sucrose concentration, as indicated by the interaction term. Inversely, at the average fill temperature, higher sucrose concentrations produce lower accumulated lethality values.

66

The interaction term indicates that this negative trend increases with an increase of the fill temperature.

The effect of the concentration of sucrose on accumulated lethality values is likely caused by changes in the mass of the solution, due to the volume being kept constant for each jar size/lid width combination. Therefore, higher sucrose concentrations produce larger mass of product in the jars. Likewise, varying concentrations of sucrose will affect the product's density, viscosity, thermal conductivity, and specific heat, which can affect the natural convection process of heat transfer (Datta & Teixeira, 1988; Earle, 2004). In turn, these convection forces will affect how the product transfers heat to the underside of the lid. Ghani, Farid, Chen, and Richards (1999) have studied the effect of the natural convection current on the movement of the coldest or zone in a can of liquid food, and found that, predictably, the action of natural convection forced the slowest heating zone to migrate towards the bottom of the can. The results from the preliminary coldspot location trials are also explained by natural convection.

Table 4.2. Results from the model for logarithmic transformation of accumulated lethality, according to a full factorial design. Dummy variables coded 0 and 1 were used for nominal factors. Both sucrose concentration (ºBrix) and fill temperature (ºC) were centered.

Term	Estimate	Standard error	$P > |t|$
Intercept	- 21.7	0.2	<0.0001
Jar size (946 mL = 1)	0.31	0.02	<0.0001
Lid width (6.2 cm = 1)	- 0.02	0.02	0.3408
Sucrose concentration (ºBrix)	- 0.012	0.001	<0.0001
Jar size (946 mL = 1) × (Sucrose concentration (ºBrix) - 30.03)	- 0.005	0.001	0.0027
Fill temperature (ºC)	0.198	0.002	<0.0001
Lid width (6.2 cm = 1) × (Fill temperature (ºC) - 87.99)	- 0.020	0.003	<0.0001
(Sucrose concentration (ºBrix) - 30.03) × (Fill temperature (ºC) - 87.99)	- 0.0003	0.0001	0.0068

Figure 4.2 shows contour plots with accumulated lethality as the response, as a function of the process conditions, based on the constructed model.
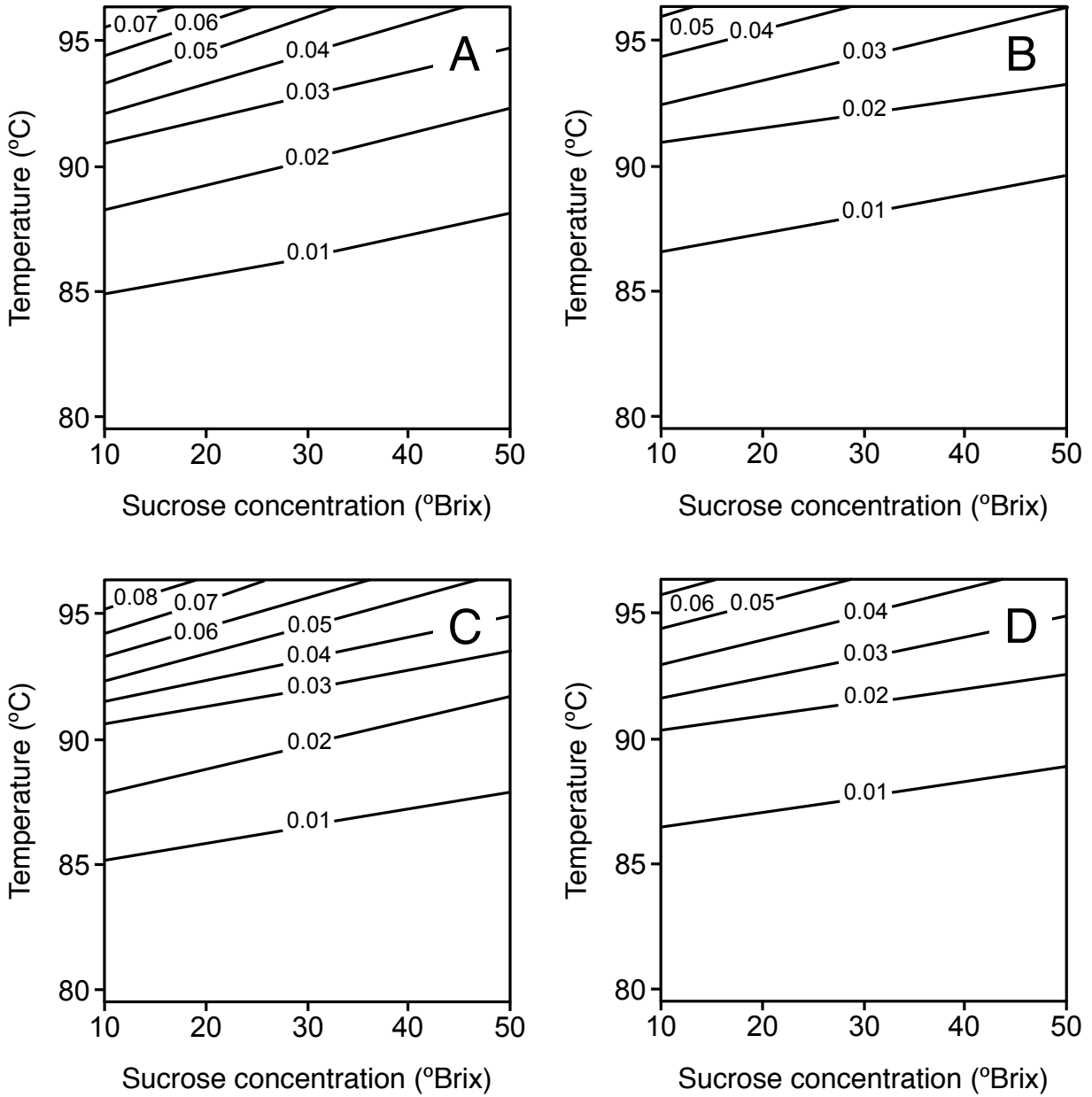
Figure 4.2. Contour plots from the constructed model with accumulated lethality (min) as the response, as a function of fill temperature and sucrose concentration. Other process conditions (jar size, lid width) and resulting headspace: (A) 946 mL, 6.2 cm, 6%; (B) 473 mL, 6.2 cm, 10%; (C) 946 mL, 7.6 cm, 8%; (D) 473 mL, 7.6 cm, 15%.

Table 4.3 shows the results from the model that was used to evaluate the effects of process conditions on the vacuum formation. The amount of residual variance explained by the model is 98.05%. Jar size had a significant (and sizeable) effect on vacuum formation, while the effect of lid width was also significant, but had less impact on the response. Larger jars produced more vacuum in the headspaces, while larger lid diameters had the inverse effect on the response. As the significant interaction term between these two nominal factors denotes, the effect of jar size on vacuum formation was affected by lid diameter, and vice versa. Overall, the magnitudes of vacuum achieved don't correspond with the jar's absolute headspace volume, but with the ratio of headspace volume to total jar volume.

The effects of sucrose concentration and fill temperature on the resulting vacuum formation follow a linear trend. Similar to the effect on accumulated lethality, at the average sucrose concentration, higher fill temperatures produce higher vacuum values, and the trend becomes weaker with an increase in sucrose concentration. At the average fill temperature, higher sucrose concentrations produce lower vacuum values, and the trend increases with an increase in the fill temperature. The effect of temperature on vacuum production is caused by the increase of the vapor pressure of water in the headspace of the jars. Related to this phenomenon, the effect of the concentration of sucrose on the vacuum is due to a decrease of the vapor pressure of water in the product as the concentration of sucrose increases.

Table 4.3. Results from the model for vacuum formation, according to a full factorial design. Dummy variables coded 0 and 1 were used for nominal factors. Both sucrose concentration (ºBrix) and fill temperature (ºC) were centered.

Term	Estimate	Standard error	*P* > |t|
Intercept	47	2	<0.0001
Jar size (946 mL = 1)	- 13.0	0.3	<0.0001
Lid width (6.2 cm = 1)	- 5.2	0.3	<0.0001
Jar size (946 mL = 1) × Lid width (6.2 cm = 1)	- 4.3	0.4	<0.0001
Sucrose concentration (ºBrix)	0.10	0.01	<0.0001
Lid width (6.2 cm = 1) × (Sucrose concentration (ºBrix) - 30.35)	- 0.04	0.01	0.0030
Fill temperature (ºC)	- 0.90	0.02	<0.0001
Jar size (946 mL = 1) × (Fill temperature (ºC) - 87.92)	- 0.18	0.03	<0.0001
(Sucrose concentration (ºBrix) - 30.35) × (Fill temperature (ºC) - 87.92)	0.006	0.001	<0.0001

Figure 4.3 shows contour plots with formed vacuum as the response, as a function of the process conditions, based on the constructed model.
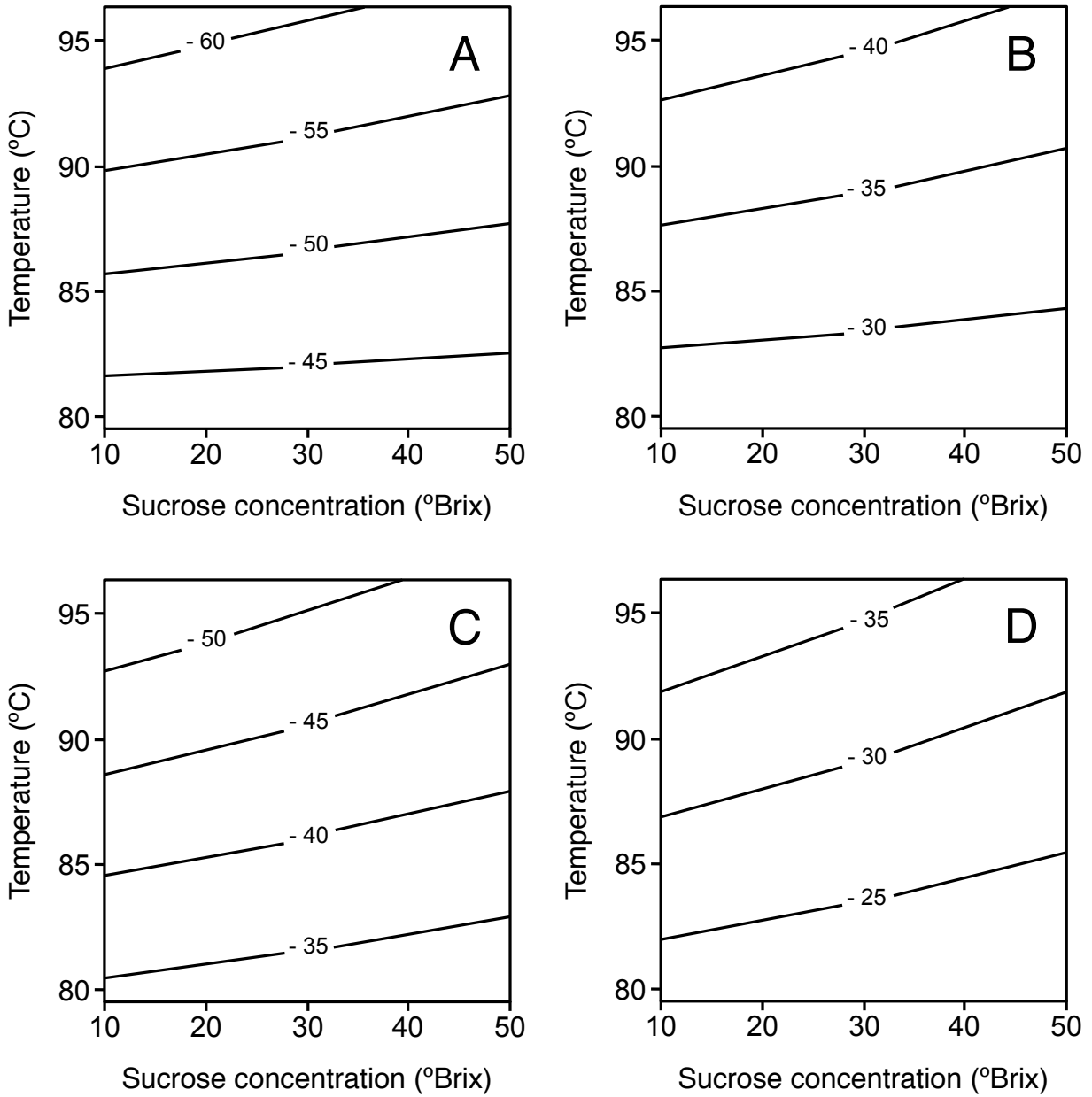
Figure 4.3. Contour plots from the constructed model with formed vacuum (kPa) as the response, as a function of fill temperature and sucrose concentration. Other process conditions (jar size, lid width) and resulting headspace: (A) 946 mL, 6.2 cm, 6%; (B) 473 mL, 6.2 cm, 10%; (C) 946 mL, 7.6 cm, 8%; (D) 473 mL, 7.6 cm, 15%.

3.3 Validation experiments

Figure 4.4 presents results from the conducted validation experiments, including the predicted and observed log transformed accumulated lethality values (averages from three trials) as well as their respective 95% confidence intervals. Overall, observed values for sucrose solutions are in agreement with their respective predicted values (except for one of the trials, for which the model seems to under predict the log transformed accumulated lethality values). However, the model fails to predict results from the validation trials carried out with applesauce and barbeque sauce, over predicting (Figure 4.4A and 4.4B) and under predicting (Figure 4.4C and 4.4D) the log transformed accumulated lethality values, respectively. Additionally, these trials show larger confidence intervals, which indicate a lower reliability of the means estimates.
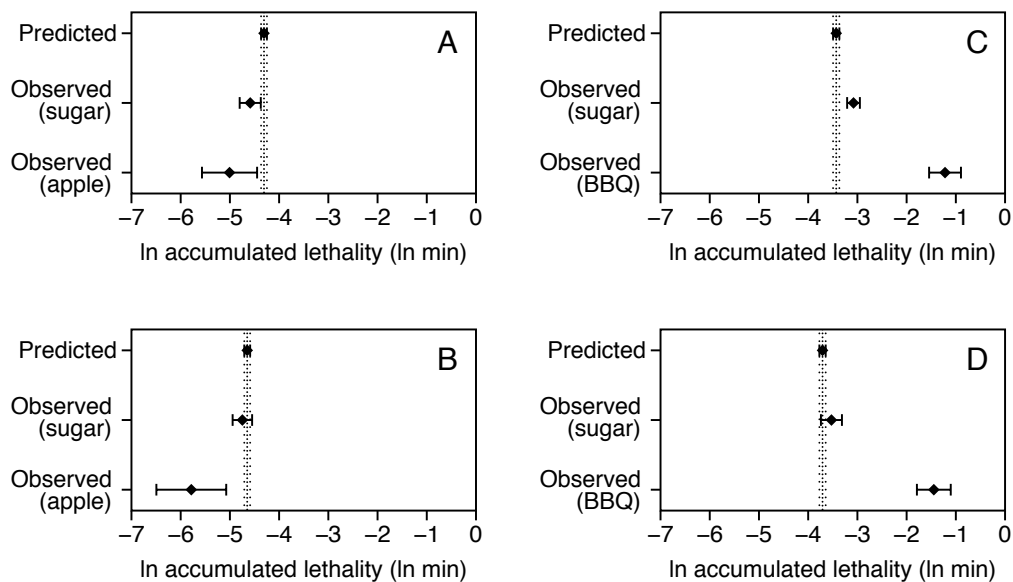

Figure 4.4. Average log transformed accumulated lethality values estimated from the validation experiments. Error bars (and vertical lines for predicted values) represent 95% confidence intervals (n = 3). Process conditions (jar size, lid width): (A) 946 mL, 6.2 cm; (B) 473 mL, 6.2 cm; (C) 946 mL, 7.6 cm; (D) 473 mL, 7.6 cm.

Figure 4.5 shows the average temperature profiles recorded during the validation experiments. The trends show that applesauce and barbeque sauce behave differently when compared to sucrose solutions of the same total soluble solids concentration. Differences in the products' compositions (such as contents of insoluble polysaccharides, lipids and proteins), likely affect viscosity, thermal conductivity, and specific heat. These differences in physical and thermal properties in turn affect the way heat transfers from the fluid to the lid, which alter the temperature profile, and therefore the measured accumulated lethality.
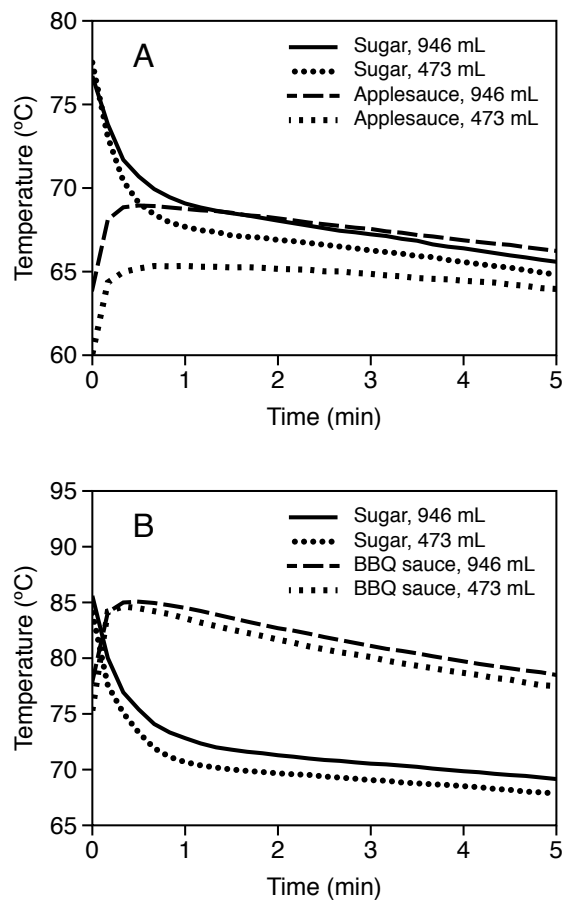

Figure 4.5. Average temperature profiles measured during the validation experiments (n = 3). Lid width: (A) 6.2 cm, (B) 7.6 cm.

Figure 4.6 presents results from the validation experiments concerning production of vacuum. Predicted and observed values are shown, as well as their respective 95% confidence intervals (averages from three trials). Once again, observed values for sucrose solutions are in agreement with their respective predicted values (except for one of the trials, for which the model seems to under predict the produced vacuum). The model fails to predict results from the validation trials carried out with larger jars (946 mL), under predicting the produced vacuum: 84% in the case of applesauce and 91% for barbeque sauce. These trials show larger confidence intervals, which indicate a lower reliability of the means estimates.
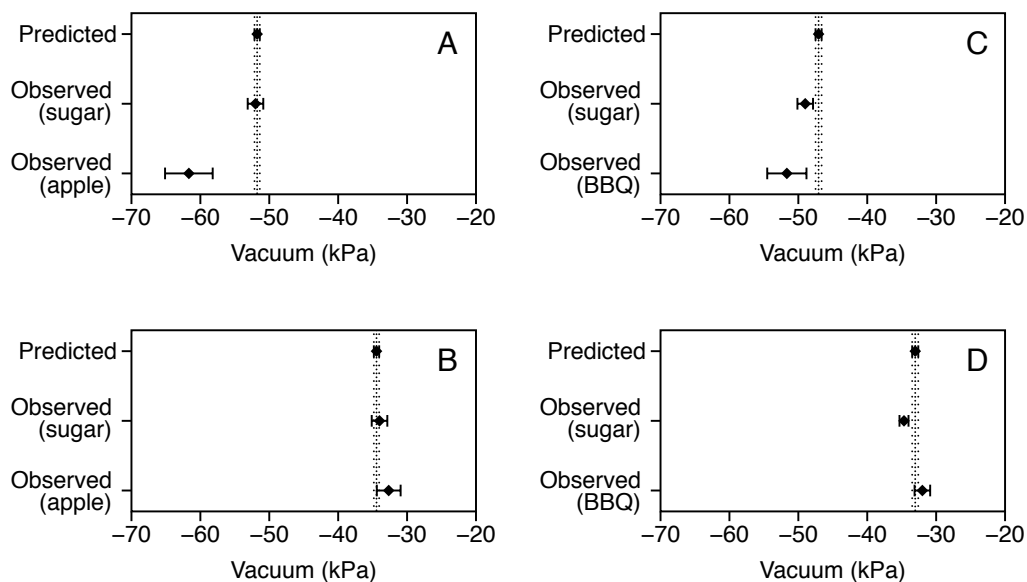

Figure 4.6. Average vacuum values estimated from the validation experiments. Error bars (and vertical lines for predicted values) represent 95% confidence intervals (n = 3). Process conditions (jar size, lid width): (A) 946 mL, 6.2 cm; (B) 473, 6.2 cm; (C) 946 mL, 7.6 cm; (D) 473 mL, 7.6 cm.

4. Conclusions

Results from this study show that current hot-fill-hold process recommendations for temperature and time should also consider type of fluid and characteristics of the containers and closures. A better understanding of the operation's conditions might improve control of spoilage microorganisms, or allow modifying inversion times and filling temperatures of established or future processes. Although the hot-fill-hold process is typically used for pH controlled foods (finished equilibrium pH of 4.4 or below, and water activity above 0.85), these results can also be useful for processors of water activity controlled foods, such as simple syrups and dessert sauces. The observed low values of accumulated lethality measured on the underside of the lids also emphasize the importance of proper sanitization of packaging materials. The relatively simple methodology of this experiment as well as the low amount of residual variance unexplained by the models demonstrate that it is feasible to use mixed models to evaluate and predict the effects of process conditions on the accumulated lethality values measured on the underside of the lid and vacuum formation. This experimental setup can be applied to further evaluate the impact of variations in processing conditions on desired pasteurization levels on packaging materials.

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CHAPTER 5

EFFECT OF WATER ACTIVITY (0.85, 0.90 AND 0.93) ON THE THERMAL TOLERANCE AND SURVIVAL OF *SALMONELLA ENTERICA* TENNESSEE AND SENFTENBERG IN MILK CARAMEL

Abstract

Outbreaks involving low water activity (a_w) foods, such as peanut butter, contaminated with *Salmonella* have raised concerns about the safety of these products. Nonetheless, ensuring the safety of a_w controlled products becomes a challenge due to the enhanced thermal tolerance that *S. enterica* has shown in low a_w environments. Only a few studies regarding heat tolerance and survival responses of *S. enterica* in foods with a_w ranging between 0.85 and 0.93 have been published. Therefore, this study aims to evaluate the effect of a_w on the thermal tolerance and survival of *S. enterica* serovars Tennessee and Senftenberg. The decimal reduction time (*D*-value) at 76, 78 and 80ºC, change in temperature necessary to produce a 10-fold change in *D*-value (*z*-value), and survival at 20 ± 0.5ºC for 10 weeks for the two *S. enterica* serovars were determined in goat milk caramel samples at three a_w values (0.85, 0.90 and 0.93). Experiments were performed in triplicate. For thermal inactivation, a significant triple interaction between serovar, temperature and a_w was found ($P < 0.0001$). Highest tolerance was observed at a_w 0.85 for *S. enterica* Senftenberg ($D_{76ºC}$ of 2.9 ± 0.3 min), and the lowest at a_w 0.93 for the Tennessee serovar ($D_{80ºC}$ 0.129 ± 0.007 min). After a natural log transformation of the *z*-values, a significant interaction between serovar and a_w was found ($P < 0.0001$). The same double interaction was noted during survival experiments ($P < 0.02$). A greater

than 4-log reduction of both serovars (regardless of a_w), was observed after 10 weeks of storage, but positive results were still found at that sampling point. Our findings may assist the food industry on the establishment of critical limits for the safe thermal treatment of a_w controlled products. Likewise, our results stress the relevance of maintaining good manufacturing and sanitization practices as a preventative action during processing of a_w controlled products.

1. Introduction

Low water activity (a_w) is a barrier to growth for many vegetative pathogens (Podolak, Enache, Stone, Black, & Elliott, 2010). However, salmonellosis outbreaks linked to contaminated low a_w food products have occurred and brought attention towards the microbial safety of these products (He, et al., 2013). These incidents raise more concerns due to the abundant scientific evidence available about the persistence of foodborne pathogens in low a_w foods (Baylis, et al., 2004; Clavero, Brackett, Beuchat, & Doyle, 2000; Kenney & Beuchat, 2004; Nummer, Shrestha, & Smith, 2012).

Thermal processing is still the preferred technological approach used and approved by regulatory agencies to inactivate pathogenic bacteria (Bermudez-Aguirre & Corradini, 2012) and to increase the shelf-life of foods. To achieve these goals, the required time and temperature combinations are established, usually based on challenge tests, legislation and product safety history. In order to assess the adequacy of a heating step, one option is to estimate log reductions of bacteria, based on the *D*- and *z*-values concept (Van Asselt & Zwietering, 2006). Heat pasteurization of low a_w products poses

particular challenges due to the enhanced heat tolerance that some pathogens such as

Salmonella have shown in low a_w environments (Ma, et al., 2009; Silva & Gibbs, 2012).

Regarding this phenomenon, He, et al. (2013) suggested that the environmental stress

that *S. enterica* encounters in low a_w foods causes a reduction in the pathogen's cellular

size, change that may constitute an adaptation strategy to the low a_w stress. This stress

adaptation may subsequently cross-protect the bacteria from other environmental

challenges, such as heat, making the desiccation-stressed *S. enterica* more tolerant to

heat. Podolak, et al. (2010) also pointed out that *Salmonella* cells can subsist in a

dormant state and return to active cell growth when the environmental conditions are

again favorable for growth.

Until now, scarce information regarding the *D*- and *z*-values and survival of *S. enterica*

in low a_w foods is available. Likewise, little is known about the influence of a_w on the

inactivation and survival of Salmonellae when present in products with a_w of 0.85 or

above. Thus, the establishment of the adequate processing conditions for a mild heat

treatment and storage of low a_w foods is a challenge for process authorities, and

therefore the food industry. Hence, the present study aims to evaluate the effect of

different a_w levels on the thermal tolerance parameters and survival of two *S. enterica*

serovars. Milk caramel was selected as a model food product due to its highly nutritious

profile (high fat, protein and simple carbohydrate content), characteristic that is

beneficial for pathogen growth and survival because these nutrients may represent

protective barriers for Salmonellae during the application of heat (Van Asselt &

Zwietering, 2006). Dega, Goepfert, and Amundson (1972) found that increasing the

concentration of solids in milk from 10 to 42% caused an increment in the $D_{55°C}$-value from 4.7 to 18.3 min. Since significant differences on the thermal tolerance among *S. enterica* serovars have been reported (Geopfert & Biggie, 1968), we considered pertinent to study at least two serovars, including *S. enterica* Senftenberg, one of the most heat resistant serovars (Bermudez-Aguirre & Corradini, 2012; Doyle & Mazzotta, 2000) and *S. enterica* Tennessee, which although is rarely implicated with foodborne infections, caused the multistate outbreak in 2006 and 2007 with peanut butter as a new vehicle of transmission for *Salmonella* (Sheth, et al., 2011). The overall goal of investigations in low a_w food products should be to advance knowledge of the behavior of foodborne pathogens in these products, with the ultimate aim of developing and implementing interventions that will reduce foodborne illness associated with this food category (Larry R. Beuchat, et al., 2013). Thus, we believe that the elucidation of the thermal tolerance parameters and survival for the two *S. enterica* serovars in a product with a_w between 0.85 and 0.93 (range were the scientific information is even more limited) will be useful for the establishment of the critical limits for the safe thermal processing of milk caramel and fluid products with a similar a_w, such as some oil-based sauces, dessert sauces and syrups.

2. Materials and methods

2.1 Milk caramel

Commercially available, shelf-stable and preservative-free goat milk caramel (ingredients: goat's milk, organic cane sugar, organic cornstarch and baking soda) was used. In order to determine the effects of three levels of a_w (0.85, 0.90 and 0.93) on the

thermal tolerance parameters (*D*- and *z*-values) and survival of two *S. enterica* serovars (Tennessee and Senftenberg), samples of milk caramel were diluted with sterile deionized water to obtain a_w values of 0.90 and 0.93 (undiluted milk caramel had a_w of 0.85). Samples were kept refrigerated in sterile polypropylene containers at 4ºC until used. To confirm the absence of interfering microorganisms, nine independent samples of milk caramel were pour plated with Trypticase soy agar (TSA) (Difco, BD, Sparks, MD) and incubated for 22 ± 2 h at 37 ± 1ºC.

2.2 Physicochemical measurements

The milk caramel's pH was measured with an Accumet Basic AB15 pH meter (Fischer Scientific, Pittsburgh, PA), its a_w using an AquaLab 4TE water activity meter (Decagon Devices Inc., Pullman, WA), and total soluble solids (expressed as ºBrix) with a digital Abbe refractometer (Leica Inc., Buffalo, NY). All physicochemical analyses were performed in triplicate.

2.3 Bacterial strains and culture condition

A single isolated colony of two serovars of *Salmonella enterica*, Senftenberg ATCC 43845, obtained from the Food Microbiology Laboratory at the New York State Agricultural Experiment Station (Geneva, NY) and Tennessee strain K6443, a clinical isolate from a peanut butter associated outbreak obtained from Larry Beuchat's laboratory at the University of Georgia (Griffin, GA), was transferred into 5 ml of Trypticase soy broth (TSB) (Difco, BD, Sparks, MD), and incubated for 22 ± 2 h at 37 ± 1ºC (to stationary-phase) at 250 rpm. One swab of the stationary-phase inoculum was

streak plated on TSA and incubated for 22 ± 2 h at 37 ± 2ºC. After incubation, the cultures were scraped from the Petri dish and collected in a sterile centrifuge tube using 4 ml of sterile water.

2.4 Thermal tolerance determination

The decimal reduction times (D-values) and the change in temperature required for the thermal destruction curve to traverse 1 log cycle (z-value) were determined following the methodology described by Usaga, Worobo, and Padilla-Zakour (2014). To reduce the product's viscosity and therefore facilitate its inoculation and injection in capillary tubes, the milk caramel was heated and kept at 37ºC. For samples with a_w of 0.85, 10 g of milk caramel were inoculated with 0.1 ml of the *S. enterica* culture and homogenized. Samples with a_w of 0.90 were prepared combining 9 g of milk caramel with 1 g of sterile deionized water and 0.1 ml of the *S. enterica* culture. For samples with a_w of 0.93, 8 g of caramel were mixed with 2 g of sterile deionized water and 0.1 ml of the *S. enterica* culture. The initial population of each serovar of *S. enterica* in all caramel samples ranged from 10^8 to 10^9 CFU·ml^{-1}. A volume of 20 µl of inoculated milk caramel was injected into three glass melting point capillary tubes (1.5 to 1.8 by 100 mm; Kimble Chase, Vineland, NJ) using a 1 ml syringe. Capillary tubes were flame sealed and immediately heat treated in water test tubes contained in a stirred water bath set at a constant temperature (76, 78 and 80ºC). Samples were taken from the water bath at selected sampling times that differed depending on the serovar inoculated and the a_w of the milk caramel. The thermal death time curves had at least 5 sampling points, 4-log reductions, and a coefficient of determination (r^2) greater than 0.90 as suggested by

Usaga, et al. (2014). Inoculated caramel samples, analyzed to determine the microbial population at time zero, were considered non-heated controls. After the application of the heat treatment, the exterior of the capillary tubes was decontaminated by placing the capillaries in test tubes containing 70% cold ethanol and contained in an ice water bath. The excess of ethanol was removed by blotting the capillaries using a sterile filter paper. The three capillary tubes were crushed with a sterile glass rod in a milk dilution bottle containing 20 ml of 0.1% sterile peptone water. A minimum detection limit of 10^2 CFU·ml^{-1} was obtained. Appropriate serial dilutions in sterile 0.1% peptone water were aseptically pour-plated by duplicate in Petri dishes with about 20 ml of a non-selective nutrient medium (TSA). Petri dishes were incubated for 20 ± 2 h at 37 ± 2ºC before colonies were counted. The D-values where calculated as the reciprocal negative value of the slope obtained from plotting the log number of $S.$ $enterica$ survivors against sampling times. The z-values were calculated as the reciprocal negative of the slope of the linear relationship between the $\log_{10}$ of the calculated D-values versus the corresponding temperature for the D-value. A total of three independent replicates were prepared for each treatment (a_w × serovar × temperature).

2.5 Survival of $S.$ $enterica$ Senftenberg and Tennessee in milk caramel

We evaluated the effect of three a_w levels (0.85, 0.90 and 0.93) on the survival of $S.$ $enterica$ Tennessee and Senftenberg in milk caramel stored at 20 ± 0.5ºC. A volume of 30 g of straight (a_w 0.85) or diluted milk caramel (24 g with 6 g of sterile deionized water for a_w 0.93, and 27 g with 3 g of sterile deionized water for a_w 0.90) was inoculated with 0.3 ml of the $S.$ $enterica$ culture, homogenized and aseptically portioned (1 ± 0.1 g) into

sterile centrifuge tubes. Initial counts were determined at day 0 using the pour-plate technique with TSA. Samples were analyzed every two weeks during a 10-week storage period. Petri dishes were incubated for 20 ± 2 h at 37 ± 2°C before enumeration. A total of three independent replicates were prepared for each treatment (a_w × serovar).

2.6 Statistical analyses

Three- and two-way analyses of variance (ANOVA), and Tukey's honestly significant difference (HSD) for multiple means comparisons were performed using JMP® Pro version 11.0.0 (SAS Institute Inc., Cary, NC). Differences were considered significant at a probability (P) value of 0.05.

3. Results and discussion

The milk caramel was characterized by a_w, total soluble solids and pH values shown in Table 5.1. The absence of background microbiota in the product was confirmed by negative results on samples plated on TSA, incubated for 22 ± 2 h at 37 ± 2°C.

Table 5.1. Measured a_w, total soluble solids and pH values of milk caramel used to determine the *D*- and *z*-values of *S. enterica* Senftenberg and Tennessee [a]

Nominal a_w	a_w	Total soluble solids (°Brix)	pH
0.85	0.853 ± 0.006	70.0 ± 0.7	5.6 ± 0.2
0.90	0.897 ± 0.001	64.8 ± 0.7	5.5 ± 0.1
0.93	0.929 ± 0.003	57.2 ± 0.4	5.7 ± 0.1

[a] Values are the average ± standard deviation (n = 3).

3.1 Influence of varying a_w on thermal tolerance

The *D-* and *z*-values of *S. enterica* Senftenberg and Tennessee in milk caramel at the different a_w values tested are presented in Table 5.2. Figure 5.1 shows representative thermal death time curves for *S. enterica* Senftenberg in milk caramel at a_w 0.85. Figure 5.1 illustrates the absence of deviations from the linear decline in the logarithmic number of *S. enterica* survivors over time. Similar curves were obtained for all experimental conditions and were used to calculate the *D*-values.
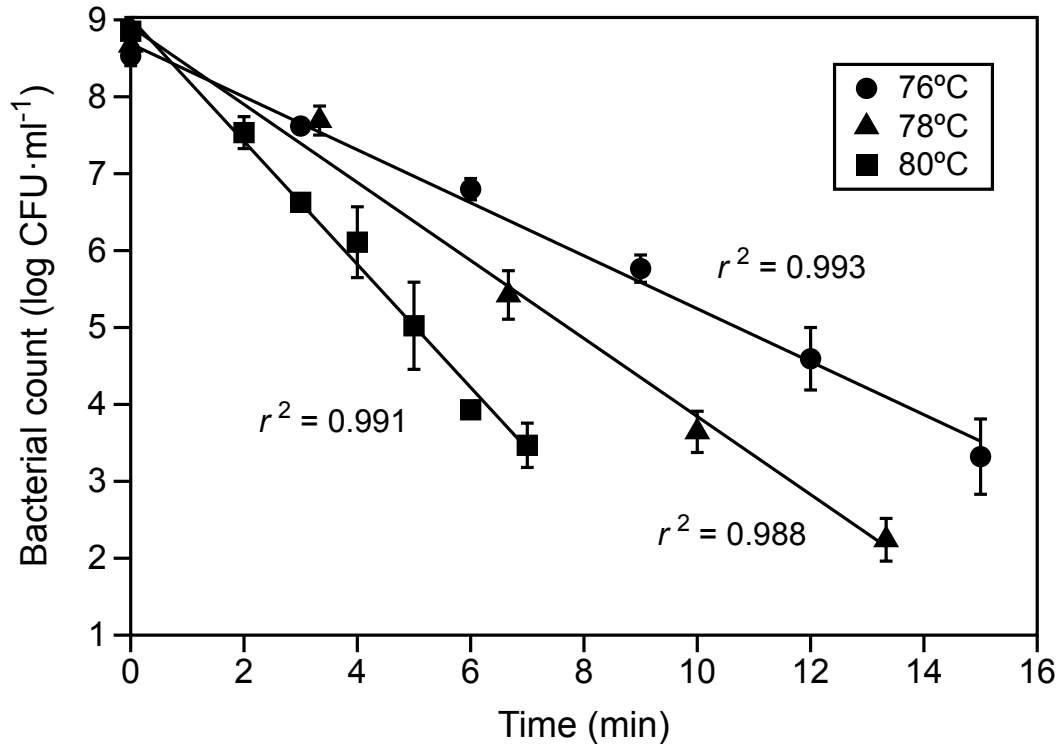

Figure 5.1. Representative thermal death time curves for *S. enterica* Senftenberg in milk caramel at a_w 0.85. Error bars represent standard deviations (n = 3).

After a natural log transformation of the D-values, a three-way ANOVA (r^2 = 0.9969) showed a significant triple interaction between nominal factors serovar, temperature and a_w (P < 0.0001). Results were further analyzed by performing two independent two-way ANOVAs (one for each serovar), with two factors: temperature and a_w. The two resulting models showed r^2 values of 0.9969 and 0.9967 for *S. enterica* Senftenberg and Tennessee, respectively. In both cases, a significant interaction between temperature and a_w was found (P < 0.0001).

For milk caramel samples with the same endpoint a_w, and regardless of the tested serovar, *S. enterica* was found less tolerant at higher temperatures (Table 5.2), which is explained by an accelerated alteration of the microbial cell structure (Farakos, Frank, & Schaffner, 2013), ribosomal degradation (Aljarallah & Adams, 2007) and denaturation of metabolic enzymes due to the heat exposure. These changes adversely affect several biological processes in *Salmonella* and other microorganisms and are responsible for the cell death (Gabriel, 2012). Aljarallah and Adams (2007) previously reported that, as expected, the D-value of *S. enterica* Typhimurium in a heating medium with a_w 0.94 dramatically decreased (heat sensitivity increased) as the temperature augmented, showing a $D_{40°C}$ of 46.1 ± 4.3 min in comparison with a $D_{60°C}$ of 1.6 ± 0.4 min.

Table 5.2. *D*- and *z*-values of *S. enterica* Senftenberg and Tennessee in milk caramel at three a_w values [a]

a_w	*D*-value (min) of *S. enterica* Senftenberg			*D*-value (min) of *S. enterica* Tennessee			*z*-value (ºC)	
	76ºC	78ºC	80ºC	76ºC	78ºC	80ºC	*S. enterica* Senftenberg	*S. enterica* Tennessee
0.85	2.9 ± 0.3 [A]	1.97 ± 0.05 [B]	1.25 ± 0.08 [C]	2.2 ± 0.2 [A]	1.91 ± 0.08 [AB]	0.90 ± 0.08 [C]	10.9 ± 0.8 [X]	10.11 ± 0.05 [X]
0.90	2.3 ± 0.1 [B]	1.17 ± 0.12 [C]	0.39 ± 0.01 [E]	1.59 ± 0.09 [B]	0.62 ± 0.04 [D]	0.30 ± 0.03 [E]	5.3 ± 0.2 [Z]	5.5 ± 0.2 [Z]
0.93	0.58 ± 0.03 [D]	0.29 ± 0.02 [F]	0.153 ± 0.004 [G]	0.31 ± 0.02 [E]	0.22 ± 0.01 [F]	0.129 ± 0.007 [G]	7.0 ± 0.4 [Y]	10.7 ± 0.5 [X]

[a] Values are the average ± standard deviation (n = 3). Values in the same serovar or *z*-value quadrant not sharing a common letter represent significantly different values (*P* < 0.05) based on *post hoc* multiple comparisons with a Tukey correction following a two-way ANOVA run on a log-transformed response.

Regarding the effect of a_w within the same heating temperature, longer exposure times were needed for the inactivation of the test strain as the a_w decreased (Table 5.2). In agreement with our results, several studies have shown the protective effect of a reduced a_w environment against the inactivation of *Salmonella* in low-moisture foods (Archer, Jervis, Bird, & Gaze, 1998; L. R. Beuchat & Scouten, 2002; Doyle & Mazzotta, 2000). For example, survival data at 50ºC of *S. enterica* serovars previously involved in outbreaks in dry foods (Typhimurium, Tennessee, Agona and Montevideo) showed an increased in heat tolerance when decreasing a_w from 0.58 to 0.22 (Farakos, et al., 2013).

Concerning the *z*-values, after a natural log transformation, a two-way ANOVA (r^2 = 0.9834) showed a significant interaction between nominal factors serovar and a_w (P < 0.0001). However, regardless of the tested serovar, no consistent trends were observed for the *z*-values at the three evaluated a_w levels. These results agree with data published by Van Asselt and Zwietering (2006), who reported that the heat resistance of *S. enterica* Senftenberg (in products with a range of a_w values) appeared to be irrelevant compared to the variability in *D*-values reported for all *Salmonella* spp. However, even though no significant differences were found (P > 0.05) among the *z*-values of *S. enterica* Tennessee and Senftenberg at a_w 0.85 and 0.90, at a_w 0.93 the observed significant differences in *z*-values between the two serovars suggest that *S. enterica* Senftenberg has a greater heat tolerance when compared to *S. enterica* Tennessee. These results contrast with those found by He, et al. (2013) who reported

that increased a_w tends to diminish the difference in thermal resistance among different serovars.

Considering the *D*- and *z*-values determined for *S. enterica* Senftenberg at the experimental conditions that triggered the greatest thermal tolerance (a_w 0.85, 78ºC), a minimum treatment of only 6 sec at 100ºC would be required to achieve a 5-log reduction (5*D* process). Considering that products such as caramel sauces, maple and other simple syrups require a concentration step at temperatures higher than 100ºC (which could extend for several minutes), these processes are deemed safe when considering a required 5-log reduction of *S. enterica* Senftenberg. Nonetheless, attention must be given to post-thermal process hygiene practices and conditions of the packaging materials.

3.2 Survival of *S. enterica* Senftenberg and Tennessee in milk caramel

The survival curves for the two *S. enterica* serovars in the milk caramel are depicted in Figure 5.2. A full factorial design was used to evaluate the effect of serovar (two levels) and a_w (three levels) on the survival of these bacteria in the milk caramel. The survival response corresponds to the bacterial count (log CFU·g^{-1}) at week 14. The two-way ANOVA showed a significant interaction between serovar and a_w (*P* = 0.0460). The model showed a coefficient of determination (r^2) of 0.8988. Two separate ANOVAs were conducted (one for each serovar), in order to evaluate the effect of a_w on the survival of the bacteria. For both *S. enterica* serovars the effect of a_w was significant: *P* = 0.0005, r^2 = 0.9202 for *S. enterica* Senftenberg and *P* = 0.0027, r^2 = 0.8606 for *S. enterica*

Tennessee. Figure 5.2 shows results from *post hoc* multiple means comparisons with a Tukey correction, for each *S. enterica* serovar.
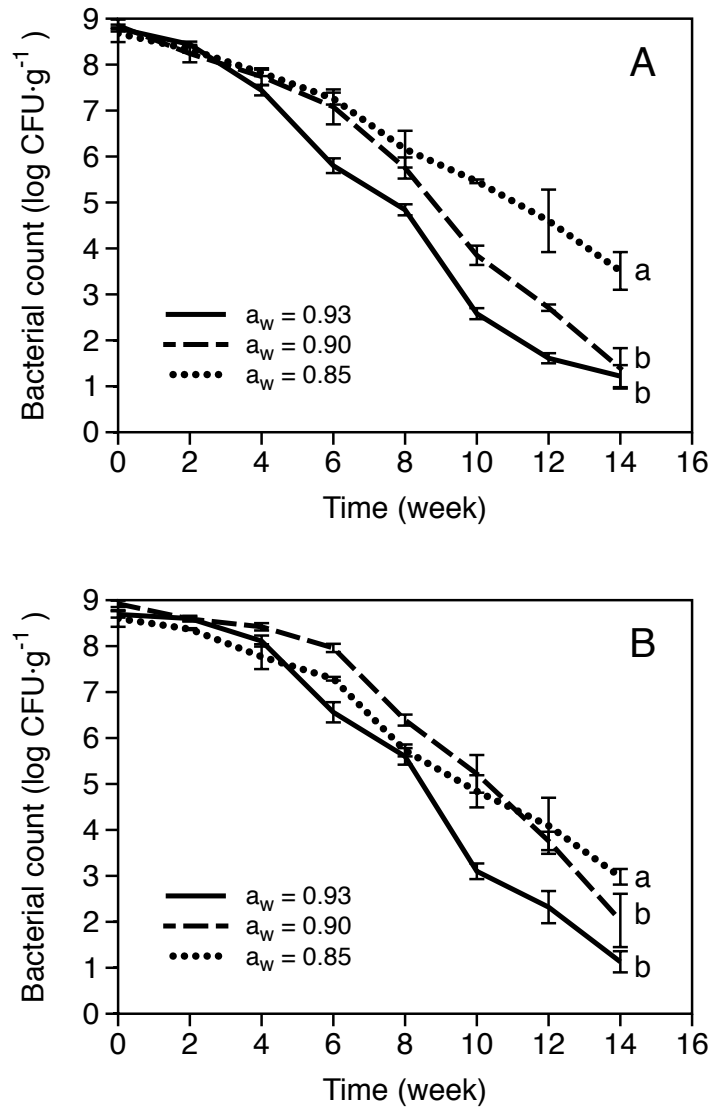

Figure 5.2. Survival curves for two *S. enterica* serovars in caramel sauce at three a_w levels, stored at 20 ± 0.5ºC: (A) *S. enterica* serovar Senftenberg, (B) *S. enterica* serovar Tennessee. Error bars represent standard deviations (n = 3). For each serovar, points at week 14 not sharing a common letter represent significantly different values (*P* < 0.05) based on *post hoc* multiple comparisons with a Tukey correction following an ANOVA.

As pointed out in a previous study (Hajmeer, Basheer, Hew, & Cliver, 2006), in which the survival of *Salmonella* Enteritidis, Gamanara, Newport, Typhimurium and Montevideo was determined in chorizo at 25ºC and with a_w ranging between 0.85 and 0.97, *Salmonella* survival curves are not characterized by a linear correlation. Thus, it is clearly inadequate to use a first-order kinetic model to describe the survival data of this pathogen in a_w controlled foods, such as milk caramel.

The consumption of only a few *Salmonella* cells may be sufficient to cause illness (Beuchat, et al., 2013; Farakos, Frank, & Schaffner, 2013), which increases the risk of extended outbreaks. The safety of milk caramel, when exposed to a post-processing contamination with *Salmonella*, represents a major safety concern due to evidence that even after 10 weeks of storage at 20ºC, positive results were still found with the two tested serovars. A greater than 5-log reduction of *S. enterica* Senftenberg in the milk caramel with a_w 0.93 was observed after 10 weeks of storage. Nonetheless, at the sample sampling time, the log reduction obtained for the same serovar at a_w of 0.90 and 0.85 and for *S. enterica* Tennessee (regardless of the a_w level), were significantly lower than 5-logs. Similarly, Nummer, Shrestha, and Smith (2012) inoculated a peanut butter flavored candy fondant (a_w between 0.65 and 0.69) with two strains of *S. enterica* Typhimurium DT104 (ATCC 700408) and reported a 5-log reduction of the pathogen after 5 weeks of storage at room temperature. A presence of the pathogen was also confirmed in the samples for up to twelve months of room temperature storage.

4. Conclusions

Our findings concerning thermal tolerance of *S. enterica* serovars Tennessee and Senftenberg may assist the food industry on the establishment of critical limits for the safe thermal treatment of a_w controlled products. The survival data presented in this study for the milk caramel stored at room temperature stresses the relevance of implementing and effectively maintain, on a continuous basis, good sanitization, manufacturing and hygiene practices during the production of milk caramel and similar food products. These practices must give special attention to prevent contamination and reduce persistence of *Salmonella*.

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CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The studies carried out as part of this dissertation demonstrate the importance of conducting proper acidification and thermal processing studies. Although foods with low pH or a_w are usually processed with less severe thermal treatments (given the lower public health concerns associated with these products), there's still need to elucidate and optimize the processes. Besides assuring commercial sterility and shelf stability, thermal treatments must be designed to minimize detrimental effects of heat on nutritional and sensory properties of the products, while simultaneously reducing consumption of energy, time and other valuable resources.

The results presented in chapters 2, 3 and 4 illustrate the feasibility and relevance of evaluating and modeling the effects of process conditions on acidification rates and accumulated lethality values. It is expected that these experimental setups can be applied to further evaluate the impact of variations in processing conditions on similar responses. Chapter 5 summarizes results that may assist the food industry on the establishment of critical limits for thermal treatment of a_w controlled products. Also, the survival data stresses the relevance of implementing good sanitization and manufacturing practices during production of these types of food products.

It is envisioned that future projects on the same topics should address other process conditions not included in the experimental designs, as well as their interactions with other effects on the measured acidification rates, accumulated lethality, thermal tolerance and survival of microorganisms. Ideally, results should be analyzed in context with validation and challenge studies that address the persistence of pathogenic and spoilage microorganisms in low pH and a_w products.

The studies described in this dissertation have achieved the goal of providing science-based evidence for food processors and process authorities. These results can be used to establish processing guidelines and evaluate current processes for production of shelf-stable foods. Hopefully, organizations such as the New York State Food Venture Center at Cornell University will utilize these results while continuing to promote economic development through support to food entrepreneurs.