IDENTIFICATION OF CRITICAL PARAMETERS FOR THE MICROBIOLOGICAL SAFETY AND STABILITY OF SELECT ACIDIFIED FOODS

A Thesis

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by

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ABSTRACT

Science-based guidelines are needed to comply with federal safety requirements for acidified foods production. A microbial challenge study of cold-processed pickled eggs (4.4 and 4.0 equilibrated pH) using 2.5% and 5% acetic acid brine (with and without sodium benzoate) was conducted with pertinent pathogens: *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* and *Staphylococcus aureaus*. Pathogens died off after 12-16 days of packing. A shelf-life study was conducted of an 80:20 apple:carrot juice blend acidified with malic (MA) or acetic (AA) acids, to pH 3.3, 3.5 and 3.7, filled at 63, 71 and 77ºC into glass or PET bottles. Stability for an equivalent of 120 days at 25ºC was achieved under the following conditions: AA, PET bottles, any pH or fill temperature; AA, pH 3.3, glass, all fill temperatures; MA, pH 3.3, PET, 71 or 77ºC fill. Other treatments were not stable reflecting initial juice microbial load and container cooling rates.

BIOGRAPHICAL SKETCH

Elizabeth Sullivan grew up in Phelps, New York, a small town east of Rochester. She was awarded her Bachelors of Science in Mathematics and English from Dickinson College in Carlisle, PA in 1998, and her Master of Arts in Rhetoric and Composition from the University of Massachusetts, Amherst in 2004. She spent two years teaching English and History in an international school in Shanghai, China and has traveled to various cities in China, Vietnam, Cambodia, Thailand, Malaysia and Singapore as well as numerous places in Western Europe. Elizabeth has worked in the New York State Food Venture Center for the past five years, two of them while completing her Master of Science in Food Processing. She was awarded the Western New York Institute of Food Technologists award in 2012 for her graduate research, and has done numerous presentations to regulators and to small-scale processors regarding regulation of processed food products. She currently lives in Fairport, NY with her husband and daughter, six parakeets and a cat.

For LHS and BTS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Pickling is a traditional method of food preservation worldwide whereby foods including meats, vegetables and fruits, are preserved through the addition of acid and/or salt. Prior to the introduction of canning and refrigeration, pickling and dehydration were two of the most common methods of food preservation (12). Modern pickled goods are still made at home, but are also widely available from small-scale food processors as well as from large manufacturers. Pickled foods, which are termed "acidified" by the U.S. Food and Drug Administration (FDA), permeate commerce and are subject to state and federal regulations. In addition, acid foods and beverages are subject to increasing scrutiny because of association with acidified foods and with outbreaks of food borne illness.

Safety: Acidified Foods are defined by the FDA as a low acid food, or a food with a significant proportion of low acid ingredients, with a water activity above 0.85 to which acid(s) or acid ingredients are added in order to lower the equilibrium pH to 4.6 or below. When FDA wrote the regulations governing acidified foods, stated in the Code of Federal Regulations, Title 21, Part 114 (21CFR114), the microorganism of primary concern was *Clostridium botulinum* and the regulations are written for its control. The regulations require acidified foods to be produced under a scheduled process written and/or approved by a process authority, someone defined in 21CFR114.83 as someone with 'expert knowledge', acquired through training and experience, to determine an appropriate processing procedure for a product. The regulations also require either a thermal process to achieve lethality for a product, or a microbial challenge study verifying the lethality of the processing procedure. Since the adoption of 21CFR114, pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella* ssp., and *Staphylococcus aureus* have demonstrated a worrying

ability to survive in acid and acidified foods, focusing attention of regulators and process authorities on these microorganisms as well as *C. botulinum*.

The FDA Acidified Foods Draft Guidance, currently being followed by FDA regulators, views any food with at least 10% low acid ingredients as acidified, and also considers the shift in pH relative to the pH of the product's acid components alone in acidified determination (15). Shifts considered significant (Table 1.1) confer the "acidified" classification to a food along with all requisite federal requirements. The FDA Acidified Foods Draft Guidance effectively expands the definition of "significant" with the result that many foods, including tomato sauces and salsa, which were not traditionally considered acidified, now fall into that category. Finally, the Food Safety Modernization Act (FSMA), while much of it is still to be decided in practice, suggests that increased attention will be paid to all foods in the future. As far as fruit and vegetable based products are concerned, scrutiny has been reserved for low acid, acidified and water-activity controlled foods with a water activity between 0.85 and 0.91. The Acidified Foods Draft Guidance and what we know thus far regarding FSMA implementation suggest that most if not all food products will be the subject of increased attention, if not increased regulation.

Table 1.1: Recommendations for Significant Difference in pH for Acidified Foods

If the equilibrium pH of the predominant acid or acid food is:	Then you should consider a shift in pH to be significant when:
> 4.2	Any shift in pH is present
4.2	The shift in pH is > 0.2
≥ 3.8 and < 4.2	The shift in pH is > 0.3
< 3.8	The shift in pH is > 0.4

*From: Guidance for Industry: Acidified Foods – Draft Guidance. FDA. 2010 (15).

The atmosphere of heightened regulatory attention to acidified foods means that many food safety parameters, both for ingredients and for processes, which have long been accepted by process authorities and regulators alike without question, now require scientific proof, in many cases peer-reviewed. For acidified foods, thermal processes are required to achieve lethality for target pathogens, resulting in a commercially sterile product (21CFR114.83). It has long been assumed that acidified foods with an equilibrated pH of 3.3 or below required only a hold time of a few days at room temperature to achieve safety; stability could be assured through the use of preservatives. A "few days" at about 25ºC was the thermal treatment. In the current regulatory climate, scientific studies must validate the safety of such processes at specific temperatures and for specific hold times.

Once product safety has been verified through microbial challenge studies, scientific studies (shelf life studies) or scientific parameters for microbial growth must validate the appropriateness of the process and the final product container to maintain a commercially sterile product. So far, FDA has been concerned primarily with food safety. However, stability is linked to safety; mold growth has been linked to an increase in product pH, for example, making a once safe food unsafe. Thus, increased FDA attention to stability can be assumed. The scheduled process must be developed with attention to the thermal treatment required by regulation, the properties of the particular food in question, and to the expectations and production realities of the food producer including quality considerations, fill line abilities, and container preferences. With the regulations in flux, and with all indications being that regulatory attention to all product classes will increase, the act of balancing regulatory requirements against the needs and realities of the product and the producer becomes more challenging, especially where there is an absence of scientific data to support specific processing parameters.

To begin to address the dearth of published scientific support, Breidt et.al. have published two articles addressing the efficacy of various thermal processes to achieve safety

through the destruction of *E. coli*, *L. monocytogenens* and *Salmonella*. One establishes lethality for these pathogens for acidified foods relying primarily on acetic acid far a pH of 3.3. A 5-log reduction can be achieved at 12.8ºC (6 days) and 25ºC (48 hours) (7). The second article addresses products with a pH of 4.10 processed at temperatures between 60 and 82.8ºC (8). While these articles are helpful, they do not address lethality, either for safety or for sterility, at temperatures between 12.8 and 25ºC nor at pH values between 3.3 and 4.10 and between 4.10 and 4.60. Pflug provides a table of lethality to achieve commercial sterility for pH range of 3.9 to 4.6 at temperatures equivalent to 200ºF which is widely accepted (31), but in general, little attention has been paid to publishing lethality requirements for pH values under 3.9.

While there is room for study regarding conditions to achieve commercial sterility in acidified foods, much work has been done regarding the effectiveness of various organic acids, particularly against pathogens. The impetus for this work is usually cited as the outbreaks of *Salmonella* and *E. coli* O157:H7 in acidic fruit juices. Hsiao and Siebert found lactic, citric and acetic acids to be stronger (lower minimum inhibitory concentrations for certain spoilage microorganisms) than malic acid on lab media at pH 5.25 (18). Their work summarizes much of the available literature. Lactic acid is considered a stronger organic acid, and malic a weak acid, with citric and acetic acids somewhere in between, depending on the study. Although organic acids are universally found more effective against pathogens than inorganic acids like hydrochloric acid, the effect of particular acids on specific pathogens varies and seem to depend largely on the parameters of the study: the microorganisms, acid concentrations, equilibrated pHs, media, and temperature(s). Numerous authors have cited these circumstances to explain or qualify results (10, 19, 21, 22, 27, 28). Pérez-Díaz et.al. found citric acid together with sorbic or benzoic acids sufficient to prevent growth of *L. monocytogenes* in sweet potato puree (30). Acetic acid has been found by some studies to be the most effective organic acid against *E. coli* O157:H7, *L. monocytogenes,* and *S. aureus* (32,

34), although Han and Linton (17) and Buchanan and Edelson (10) both found lactic acid to be most effective in controlling *E. coli* O157:H7. Bjornsdottir et.al. found low concentrations (5 mM) of lactic acid, malic acid or acetic acid to be less effective than gluconic acid against acid-adapted *E. coli* O157:H7 (5).

Many of the studies regarding the usefulness of organic acids have been conducted on a variety of homogenous mixtures (6, 9, 10, 21, 25, 30, 34). Less information is available for commercial products with distinct solid and liquid components. Beuchat et. al. and Tsai and Ingham both conducted studies on commercially-produced products, although these products were, with the exception of the relish studied by Tsai and Ingham, homogenous mixtures such as dairy-based salad dressings, mustard and ketchup (4, 41). While studies on fruit and vegetable purees, and on acidic solutions, are useful in analyzing the effect of organic acids against pathogens in a homogenous mixture, such studies do not provide a complete picture of the acidification process for products with distinct solid and liquid component, like pickles, where surface contamination and brine strength are important considerations.

Moreover, studies generally have focused on systems with a low buffer capacity rather than systems, such as pickled eggs or bean salsa, with a much higher buffer capacity. Bjornsdottir et.al. and Breidt et. al. both used gluconic acid as a noninhibitory buffer to look at the effects of acetic acid on *E. coli*, and both found the effect of acetic acid to be lower than that of pH alone (5, 6). Richard and Cutter (33) published a study validating a process for a product (pickled eggs) with both distinct solid and liquid components and a high buffer capacity, but the brine used in the study had a high concentration of salt and can therefore only be referenced in similar salt/acid brined products. The acidification rates for high buffer-capacity systems are of interest since, by federal regulation, products that do not reach pH 4.6 or below within 24 hours must be refrigerated until the product reaches pH 4.6 or below in order to prevent the growth of *Clostridium botulinum* (9CFR318.300(b)). If lethality parameters for pertinent pathogens are determined in the absence of refrigeration when

refrigeration is required as part of the processing conditions, those lethality factors are no longer applicable.

To address the foodborne illness outbreaks linked to apple and citrus juices, FDA has developed regulations stated in 21CFR120 requiring treatment resulting in a 5-log reduction in the pertinent pathogen. The FDA recommends a series of time/temperature combinations as sufficient for pasteurizing fruit juice, the relevant pathogen being *Cryptosporidum parvum.* The FDA notes a lack of research into heat resistance of *C. parvum*, and allows for the use of pasteurization regimes validated for *E. coli* instead (14). Mak et.al. have validated 68.1ºC for 14 seconds for *E. coli, L. monocytogenes* and *Salmonella* (23) and this is used as the recommended treatment for cider in Wisconsin. However, New York State allows pasteurization at 71ºC for 6 seconds to ensure a 5-log reduction in *E. coli* (14), although a pasteurization step of 60ºC held for at least 5 minutes is also recognized by the FDA as part of the Juice HACCP (Hazard Analysis and Critical Control Point) validated procedures. Both pasteurization treatments are based in work by Mazzotta which studied fruit juice inoculated with *E coli*, *L. monocytogenes* and *Salmonella* (24).

Stability: Since refrigeration will control the growth of *C. botulinum*, the federal regulation of acidified foods only applies to shelf-stable products. Lethality for pasteurization, regarded as the time/temperature combination needed to ensure pathogen destruction, is only a part; commercial sterility requires control of spoilage microorganisms as well as pathogens. Since many spoilage microorganisms are hardier than the pathogens relevant to acidified foods, commercial sterility requires greater applications of heat over time than pasteurization, and may require the use of preservatives such as sodium benzoate and potassium sorbate. The greater demands of commercial sterility can result in quality problems such as color degradation, flavor changes, and changes in product texture. In addition, required processing temperatures limit the types and sizes of appropriate containers. Processing requirements also

have implications for utility costs, storage and distribution costs, and time constraints.

While use of various organic acids can offer varying degrees of antimicrobial activity, additional processing is required in most cases to yield a shelf-stable product or prolong refrigerated shelf life. Traditionally, processing has involved thermal treatment of the product which can negatively impact nutrient content as well as flavor, texture and color of food products. Attention has been widely focused on high hydrostatic pressure (HHP) processing and on ultrahigh pressure processing (UHP) since these two processing methods show promise in simultaneously destroying pathogenic bacteria and in prolonging shelf life while maintaining nutritional and organoleptic quality. A literature search for articles only published in the last few years yields a wealth of research regarding safety and stability for products from acidic juices like blueberry, pomegranate and apple (2, 13, 16, 42) to vegetable juice (3, 29) to milk and milk/fruit juice blends (1, 40). Researchers have examined vitamin retention and the effect of HHP and UHP on anthocyanin and polyphenol content (12, 13, 36, 43) as well as spoilage microorganisms like *Alicyclobacillus acidoterrestris* (29, 36).

With regard to spoilage microorganisms and shelf stability of acidified products in general and juices in particular, Sperber states that, for acidified food products that rely on acetic acid for their pH, spore forming bacteria are not a concern (38). The concerns come mainly from lactic acid bacteria and yeasts, with few molds tolerating acetic acid (38). For acid fruit juices, considerable attention has been paid to *Alicyclobacillus acidoterrestris*, a spoilage microorganism isolated as spore-forming bacilli by Splittstoesser et.al. which has shown ability to withstand both low pH and processing temperatures which were long considered adequate for stability (39). Silva et.al. tested various processing temperatures, ºBrix levels and pH levels to determine adequate pasteurization regimes for *Alicyclobacillus acidoterrestris,* finding that 85ºC and 91ºC were sufficient for juice with a pH of 3.5 or 3.7 and a ºBrix of about 11 (37). In 2012, Silva et.al. studied high pressure processing combined with heat to inactivate *Alicyclobacillus acidoterrestris* and concluded that pressure could

lower processing temperatures (36). Work has been done recently by Irkin and Korkuluoglu to demonstrate the usefulness of spices and essential oils as antimicrobial agents against *E. coli*, *L. monocytogenes*, *Salmonella*, *B. cereus* and several yeasts in fruit juices as a way to address a desire for "natural" preservatives on the part of consumers (20).

There is less literature regarding the effectiveness of various organic acids against spoilage microorganisms than against pathogens. However Hsiao and Siebert concluded that lactic, acetic and citric acids are stronger (lower minimum inhibitory concentrations) against several spoilage microorganisms than, for instance, malic acid (18). Savard et.al. reported a combination of lactic, acetic and propionic acids most effective against two spoilage yeasts applied to fermented (lactic acid bacteria) vegetable juices at pH 3.74 (35). Calder et.al. showed that application of sodium acid sulfate had a similar antimicrobial action against aerobic bacteria to citric acid when used as an anti-browning agent on French-fry cut potatoes (11). Mosneaguta et.al. extended these conclusions to malic acid (26).

In product development, processors have to balance product quality, equipment realities and container desires with thermal requirements yielding shelf stability. The established processing parameters for shelf stability often have negative implications for product quality – loss of nutrients, flavor change, color loss and browning. Juice processors and processors of value-added or gourmet products often rely on product nutrients, flavor and color to give their products the differentiation that will convince consumers to pay higher prices. To limit the costs of food processing, storage and shipping, and to preserve product quality, alternative production parameters must be determined for acidified foods and validated not only for their efficacy against pathogens, but also against spoilage microorganisms. Large businesses can afford the microbial challenge studies required to validate processing procedures. They can also afford shelf life studies to verify stability (commercial sterility). However, small and micro-scale food processing establishments cannot typically afford challenge studies, and may not be able to afford shelf life studies either.

Therefore, it is important to investigate alternative production procedures for acidified foods and publish the results of challenge and shelf life studies in order to give both process authorities and food processors options which help preserve product quality and mitigate production, storage and distribution costs.

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CHAPTER 2

PICKLED EGG PRODUCTION: INACTIVATION RATE OF SALMONELLA, ESCHERICHIA COLI O157:H7, LISTERIA MONOCYTOGENES AND STAPHYLOCOCCUS AUREUS DURING ACIDIFICATION STEP

Abstract. Based on current FDA acidified foods guidelines, regulatory approval of commercial pickled egg production without a final heat treatment requires challenge studies. We conducted challenge studies to verify common pickled egg processing parameters. Hard-boiled eggs were acidified in ambient temperature brine at 60/40 egg/brine ratio. Four brine treatments were studied in triplicate: 5% acetic acid (AA) and 2.5% AA, with and without 0.05% sodium benzoate. Samples were stored at 7ºC until pH at the yolk center ≤ 4.6; subsequently, samples were held at ambient temperature. Egg pH was measured at 24-48 hour intervals until equilibrium pH was reached (4.0 and 4.4). Eggs and lids were challenged with separate pathogen cocktails (6 strains/serovars): *Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*. It took 5 and 9 days respectively for pH to fall below 4.6 for 5% and 2.5% AA-brined eggs. Sodium benzoate did not affect acidification rate for these brine treatments ($p \geq 0.05$), nor was sodium benzoate observed to affect pathogen die-off. *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* were not detected by enrichment (< 1 CFU/g), for pickled eggs in 5% AA brine at 72 hours; *S. aureus* was not detected after 7 days. In 2.5% brine, *Salmonella* was not detected after 10 days; no pathogens were detected by 14 days. No pathogens were detectable on lids within 72 hours for 5% AA brines. Only *S. aureus* was detected on lids after 72 hours in 2.5% AA brine

and died off rapidly at ambient temperature. Although pathogens began die-off under refrigeration, heat treatment (ambient temperature storage) was required to reach undetectable levels; minimal inversion was adequate treatment for lids. Pickled eggs should be held under refrigeration for the length of time needed to acidify them to ≤ 4.6, and then held at ambient temperatures to ensure pathogen inactivation.

Introduction. Pickling is a common method of preserving vegetables, fruit and meat. Hard-cooked eggs pickled in an acidic brine are also common, but the egg, as opposed to vegetables or fruit, poses unique challenges to proper preservation. Inadequate home preservation of pickled eggs has been linked to at least one case of botulism (12). Because of safety concerns, instructions for home pickling of eggs stipulate that the eggs be held under refrigeration and removed only for consumption in order to mitigate pathogen growth (22, 27). Production procedures for use by home and small-scale producers typically do not include a pasteurization step for the final product thus offering a potential environment for typical food borne pathogens such as *Samonella* spp. and *Listeria monocytogenes*. Moreover, outbreaks of *Escherichia coli* O157:H7 in acid conditions (fruit juices) have raised concern over in acidic food products, including acidified products such as pickles. Further, pickled eggs and similar pickled products are subject to staphylococcal contamination since hard-cooked eggs must be peeled, often by hand, prior to acidification.

Federal regulations for acidified foods are stated in the Code of Federal Regulations, Title 21 Part 114 (21CFR114). The regulations specify basic criteria with regard to pH measurement, record keeping, and the need for a production process resulting in a product

free from microorganisms of public health concern. Regulation also stipulates thermal treatment of acidified foods with an equilibrated pH greater than 3.3, unless microbial challenge studies verify the efficacy of the process against microorganisms of public health concern. The regulations leave the particulars of processing up to a recognized process authority, someone with the expertise to determine an appropriate processing procedure. The procedure must be developed with an eye both to the thermal treatment requirement, and to the requirement that pickled products reach a pH at or below 4.6 within 24 consecutive hours if held at room temperature, which is based on the USDA acidified meats definition found in 9CFR318.300(b) and repeated in 9CFR381.300(b). In practice, refrigeration is required as deterrent to microbial growth for products that take longer than 24 hours to acidify to pH ≤ 4.6.

Studies regarding the development of acid and cold tolerance by pathogens could complicate the refrigeration requirement for products that take longer than 24 hours to acidify to pH ≤ 4.6. Relevant studies explore the effects of acid shock on a variety of pathogens, typically shocking pathogens in media at pH values of 4.5 to 5.5 prior to exposing these pathogens to acid, heat and/or salt (3, 6, 7, 10, 13, 17, 19, 21, 24, 26). Once pathogens develop acid tolerance, the strength of acid and/or time of exposure required for pathogen lethality increases. The heat required to kill acid-shocked pathogens may also increase, although some studies have found that acid-stressed cells die off more quickly under cold conditions than non-stressed cells (2, 4, 9, 11, 14, 25). Since refrigerated storage delays pathogen destruction, such storage has the potential to create an environment where pathogens could develop tolerance of acidic conditions if the brine is not acidic enough to kill or

inactivate pathogens. Extended refrigeration also leads to some concern that pathogens may develop cold tolerance. Han and Linton have suggested that cold tolerance in *L. monocytogenes* may lead to development of acid tolerance as well (15).

Since acidified pickled eggs (as opposed to salt-preserved eggs) are usually pickled in vinegar, the efficacy of acetic acid in controlling pathogens and the time required for the eggs to acidify is paramount in creating and evaluating the safety of processes for production. Commercially, pickled eggs are often made with vinegar (acetic acid) brines, sometimes with the addition of citric and/or phosphoric acid to enhance acidification. Although weak organic acids are universally found more effective against pathogens than inorganic acids like hydrochloric acid, the effect of particular acids on specific pathogens in particular systems varies, and seems to be related to the pH and the composition of the media used for the study and the temperature at which the study was conducted. Acetic acid has been found by some studies to be the most effective organic acid against *E. coli* O157:H7*, L. monocytogenes, and Staphylococcus aureus* (16, 21, 24), although Han and Linton (15) and Buchanan and Edelson (10) both found lactic acid to be the most effective in controlling *E. coli* O157:H7. Bjornsdottir et.al. found low concentrations of lactic acid, malic acid or acetic acid to be less effective than gluconic acid against acid-adapted *E. coli* O157:H7 (7).

While there is a wealth of information regarding the efficacy of various organic acids and acid and cold tolerance in pathogens in a variety of homogenous mixtures, less information is available for commercial products with distinct solid and liquid components (8-10, 16, 18, 20, 24). Beuchat et. al. and Tsai and Ingham both conducted studies on commercially-produced products, although these products were, with the exception of the

relish studied by Tsai and Ingham, homogenous mixtures such as dairy-based salad dressings, mustard and ketchup (5, 26). While studies on fruit and vegetable purees, and on acidic solutions, are useful in analyzing the effect of organic acids against pathogens, such studies do not provide a complete picture of the acidification process for heterogeneous products like pickles. Moreover, a focus on fruit and vegetable products does not easily translate to a high protein product such as pickled eggs which has a much higher buffer capacity. Richard and Cutter have recently published a study looking at the efficacy of one industrial egg pickling process involving very low equilibrated pH and high salt content (23). Our purposes in this study were to determine the acidification rate in pickled eggs in vinegar brine under four brine treatments resulting in equilibrated pHs of 4.0 and 4.4, and to determine the effectiveness of acetic acid against *E. coli* O157:H7, *L. monocytogenes*, *S. enterica* and *S. aureus* in such pickled egg systems.

Materials and Methods

Brine Preparation. White distilled vinegar (5% acetic acid) was obtained from a local grocery store. Sodium benzoate (T. J. Baker, Phillipsburg, NJ) was added as a percentage by weight of the brine. Four brine solutions were prepared: 5% acetic acid brine (5AA); 5% acetic acid brine with 0.05% sodium benzoate (5AASB); 2.5% acetic acid brine (2.5AA); 2.5% acetic acid brine with 0.05% sodium benzoate (2.5AASB). Acetic acid concentrations in brine were based on previous studies that indicated that 5% brine strength at a 60/40 egg to brine ratio would result in an equilibrium pH of approximately 4.0; acetic acid concentration of 2.5% would result in an equilibrium pH of approximately 4.4. Sodium benzoate was added

at 0.05% to determine the effect, if any, of the preservative on the rate of acid penetration into the eggs. Sodium benzoate is commonly used in industry to prevent the growth of spoilage microorganisms in pickling brines.

Sample Preparation for pH measurement. Medium grade, hard-cooked eggs were obtained from Lehman's Egg Service (Chambersburg, PA). Eggs were placed, nine to a jar, in 32 oz glass canning jars. Based on average egg weight (43g), the weight of brine necessary to yield a 60/40 egg to brine ratio was determined. Weight of the brine was converted to volume (290 mL). Brine was poured over the eggs in each sample jar at ambient temperature. Jars were then capped with a two-piece lid and inverted once to simulate minimal brine/lid contact during distribution. Samples were made in triplicate for each of the four brine treatments; 5AA, 5AASB, 2.5AA and 2.5AASB.

pH measurement. All pH measurement was done using an Orion PerpHecT ROSS Combination pH Micro Electrode (Thermo Scientific, Hanover Park, IL) connected to an accumet Basic AB15 pH meter (Fisher Scientific, Pittsburgh, PA) with temperature compensation. The electrode tip has a 1.3mm diameter and requires immersion of 1 mm into testing media. Brine pH was measured by immersing the probe in the brine in the sample jar. Egg pH was measured by first cutting the egg off-center transversally, resulting in two unequal portions. The smaller portion was reserved. The larger portion was used for point pH determination as shown in Figure 2.1.

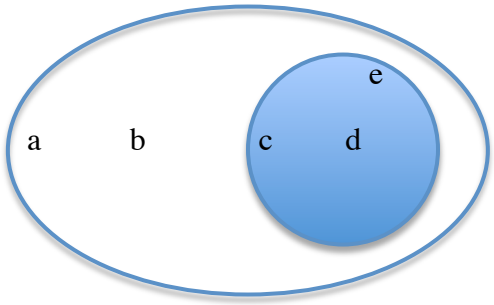

Figure 2.1: Sampling diagram showing transversal cut of egg with sampling points for pH.

Initially, four points were identified for pH measurement to determine the progression of acid through the egg. Point E was added 48 hours after egg brining began to provide a more complete picture of egg yolk acidification. Once point measurements of pH for a sample were complete, the whole egg yolk, both that part used for point measurement and that part reserved, was finely chopped and tested for pH using the same probe and no more than 20% distilled water (added to facilitate pH measurement). The total egg pH was determined by combining both portions of egg white with the chopped up egg yolk, grinding to a paste in an electric coffee grinder (obtained from a local retailer), and inserting the pH probe into the mixture.

Pathogens. Pathogens used for this study consisted of 6 strains/serovars of each of four pathogens: *Salmonella enterica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus* as shown in Table 2.1.

Table 2.1. Pathogen Strains/Serovars used.

S. enterica	*E. coli* O157:H7	*L. monocytogenes*	*S. aureus*
Gaminara	ATCC 43894	strain 2289	ATCC 8095
Rubislaw	ATCC 43895	strain L-99	ATCC 9144
Hartford	ATCC 43890	strain H0222	ATCC 25923
Montevideo	ATCC 43889	strain F2586-VI	strain T-50-32
Michigan	35150	strain 104025	strain 50-1-32
Anatum	933	strain 19112	strain 3-C5

Egg inoculation. The entire contents of two 5 gallon shipping containers of eggs were aseptically removed from containers and allowed to partially dry in a BL II biosafety cabinet. The five overnight cultures (5 ml) grown in Tryptic Soy Broth (TSB) at 37°C with 250 rpm shaking, were mixed together in equal volumes to comprise the resulting cocktail for each of the four pathogens (25 ml total volume). The cocktail was then diluted in 2000 ml of sterile peptone water and the eggs were allowed to sit at room temperature in the inoculum for 10 minutes. Eggs were then removed from the inoculum and allowed to air dry on sterile cheese cloth in the BL III cabinet.

Inoculated eggs were placed in 32 oz glass canning jars, nine eggs to a jar. Brine was added to the jars at 290 mL per jar yielding a 60/40 egg/brine ratio by weight. Inoculated lids (explanation follows) were put on jars and all samples were held at 7ºC until pH analysis indicated the center point of the yolk (point D) had acidified to pH ≤ 4.6; prior studies indicated that none of the brine treatments resulted in a pH at point D below 4.6 after 24 hours

at room temperature. At this point, challenge study samples corresponding to the brine treatment in question were removed from refrigeration and held at room temperature. Samples were made in triplicate for each of the four brine treatments (5AA, 5AASB, 2.5AA and 2.5AASB) and for each of the four pathogens tested.

Lid inoculation. Lids were labeled with seven sampling points corresponding to the seven sampling times determined (0, 8, 24, 48 and 72 hours and 7, and 10 days). Each pathogen strain/serovar was grown in Tryptic Soy Broth at 35ºC for 24 hours at 225 rpm shaking. Separate strains/serovars for each species were pooled and 20 μl of each cocktail was added to each of the seven time point locations on the appropriate lid. Lids were then placed in a laminar flow hood until the spots had evaporated. Initial time sampling was taken. Lids were immediately placed onto corresponding jars filled with inoculated eggs and brine, secured with a canning ring, and the jar inverted once to ensure contact between lid and brine under worst-case distribution conditions.

Microbial analysis. Eggs were sampled at times 0, 8, 24, 48 and 72 hours and 7, 10, and 14 days. Eggs were removed from refrigeration for testing and stored under refrigeration (7ºC) until the pH at the center of the yolk (point D) was below 4.6 in strict compliance with regulations. A representative whole egg was sampled from each container for each sampling time, weighed and diluted with 10 times the volume of sterile phosphate buffer. The sample was then homogenized for 2 minutes. Serial dilutions were made in sterile peptone water and 1mL samples of the various dilutions were pour plated in duplicate with tempered TSA. The TSA plates were incubated for 24-48 hours at 37°C and then the plates of the appropriate dilutions (30-300 colonies) were enumerated. Each of the samples was performed in

triplicate.

Data analysis. A multiple linear regression analysis was carried out in order to evaluate the effect of sodium benzoate on the acidification rate of the center of the yolk, for hard-cooked eggs pickled in 2.5 and 5 % acetic acid brines. Effects were considered significant when $p<0.05$.

Results

Acidification. Egg acidification as shown by pH measurements for 5AA and 5AASB, and for 2.5AA and 2.5AASB were not significantly different ($p \leq 0.05$) (data not shown). Lack of difference in pH data between brines with sodium benzoate and brines without sodium benzoate is most likely a reflection of the minimal amount of the preservative in the pickled egg system as a whole (0.02% by weight). When sodium benzoate is used in industrial egg pickling, it is often added at about 250 ppm in the pickled egg system as a whole. Greater concentrations of sodium benzoate result in off flavors. In this study, sodium benzoate was added to the brine, as it is in industry, to control surface growth of spoilage microorganisms since packaging was new and eggs were hard-boiled, both resulting in minimal microbial load.

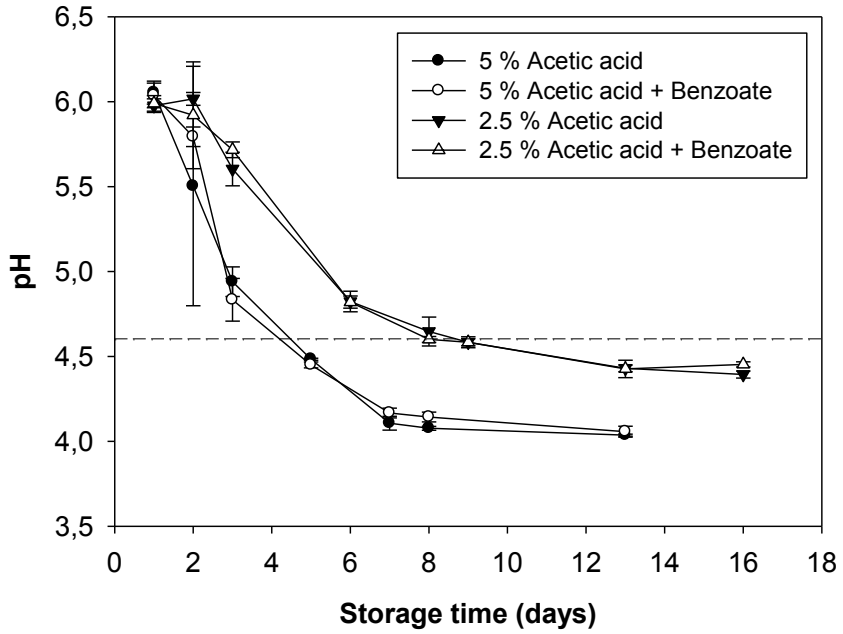

Figure 2.2. Variation of pH (measured at the center of the yolk: point D) with storage time. Error bars represent standard deviation for n = 3. Dashed line represents critical pH of 4.6.

Acid penetration through the egg white was fairly rapid regardless of brine strength. Acid penetration through the egg yolk, however, was affected by brine strength. Acid penetrated sufficiently to lower the pH at point D to below 4.6 within 5 days for 5% acetic acid brine treatments, and within 9 days for 2.5% acetic acid brine treatments (Figure 2.2). Equilibrium was reached by day 13 for 5% acetic acid brine treatments, and by day 16 for 2.5% acetic acid brine treatments (equilibration determined by pH values ± 0.01 at all sampling points, Total Yolk and Total Egg). The multiple linear regression analysis (R^2 = 0.91) showed no significant effect ($p>0.05$) of sodium benzoate on the acidification rate of the center of the yolks.

Challenge Study. Results from the challenge study indicate that all pathogens in all brine treatments were at undetectable levels on both pickled eggs and jar lids within 14 days. There was no difference observed in the rates of pathogen die-off between brines containing 0.05% sodium benzoate and those without the preservative. In treatments 5AA and 5AASB, *E. coli* O157:H7, *S. enterica* and *L. monocytogenes* died off on pickled eggs within 72 hours with *S. aureus* at undetectable levels by day 7 (Figure 2.3). For 2.5AA and 2.5AASB, *S. enterica* died off on pickled eggs within 10 days; *E. coli* O157:H7, *L. monocytogenes* and *S. aureus* were not detected by day 14 (Figure 2.4).

Although there was an initial die-off of all pathogens on pickled eggs, *S. aureus* was detectable at fairly constant levels until samples were removed from refrigeration. In samples treated with 2.5% acetic acid brine, complete die off of both *S. aureus* and *L. monocytogenes* on pickled eggs was not observed until after samples were removed from refrigeration (Figure 2.4). Although these pathogens did not grow under refrigerated storage, pathogen destruction was limited.

Jar lids demonstrated die off within 16 hours for 5AA and 5AASB for all pathogens except *S. aureus*. By the 72 hour sampling, no pathogens were detected in the 5AA or 5AASB treatments, and no pathogens except *S. aureus* were detected in 2.5AA and 2.5AASB treatments. *S. aureus* lingered on the lids of 2.5AA and 2.5AASB until samples were removed from refrigeration; *S. aureus* was not detected on jar lids by the 10 day sampling point (Table 2.2).

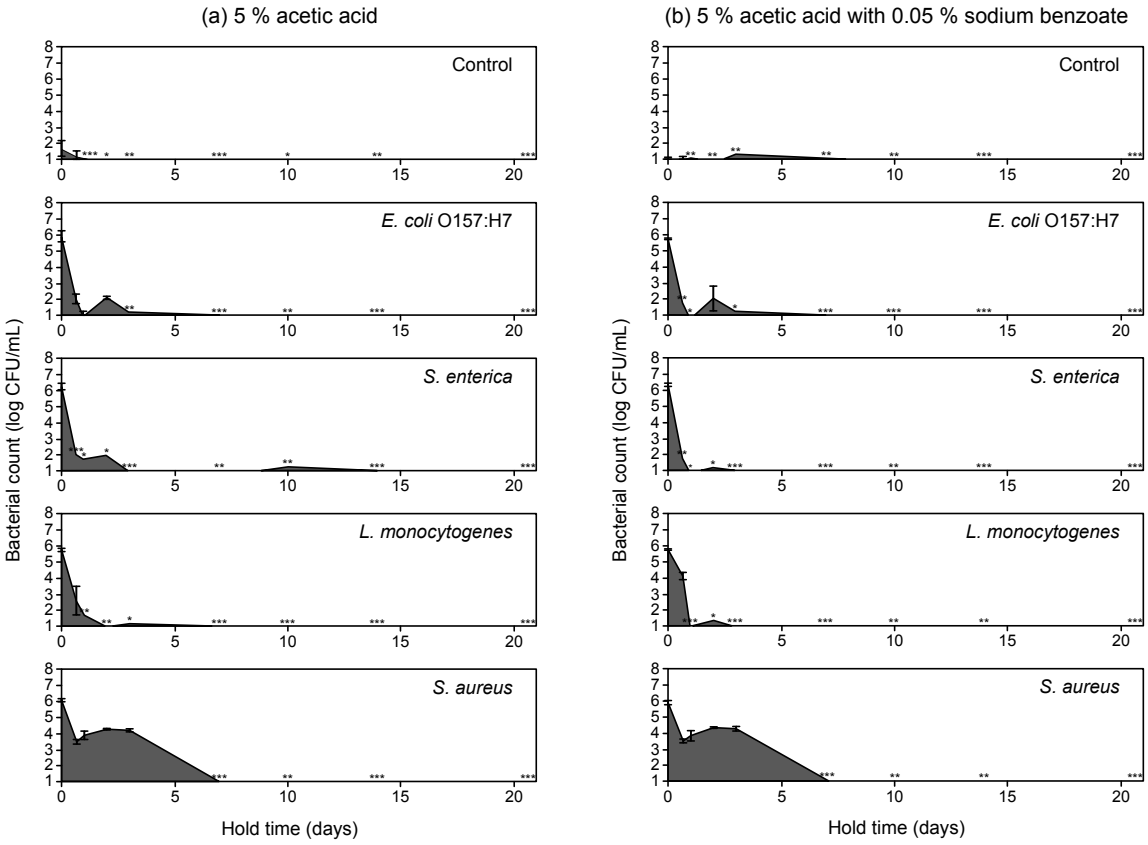

Figure 2.3. Variation of bacterial counts on pickled eggs with storage time for (a) 5 % acetic acid, (b) 5 % acetic acid with sodium benzoate. Error bars represent standard deviation for n = 3, * indicates one of the replicates resulted in a zero plate count (the average is assumed to be less than the indicated point), ** indicates two of the replicates resulted in a zero plate count (the average is assumed to be less than the indicated point), *** indicates the three replicates resulted in a zero plate count (the average is assumed to be less than the indicated point, which is the detection limit of 1.0 log value).

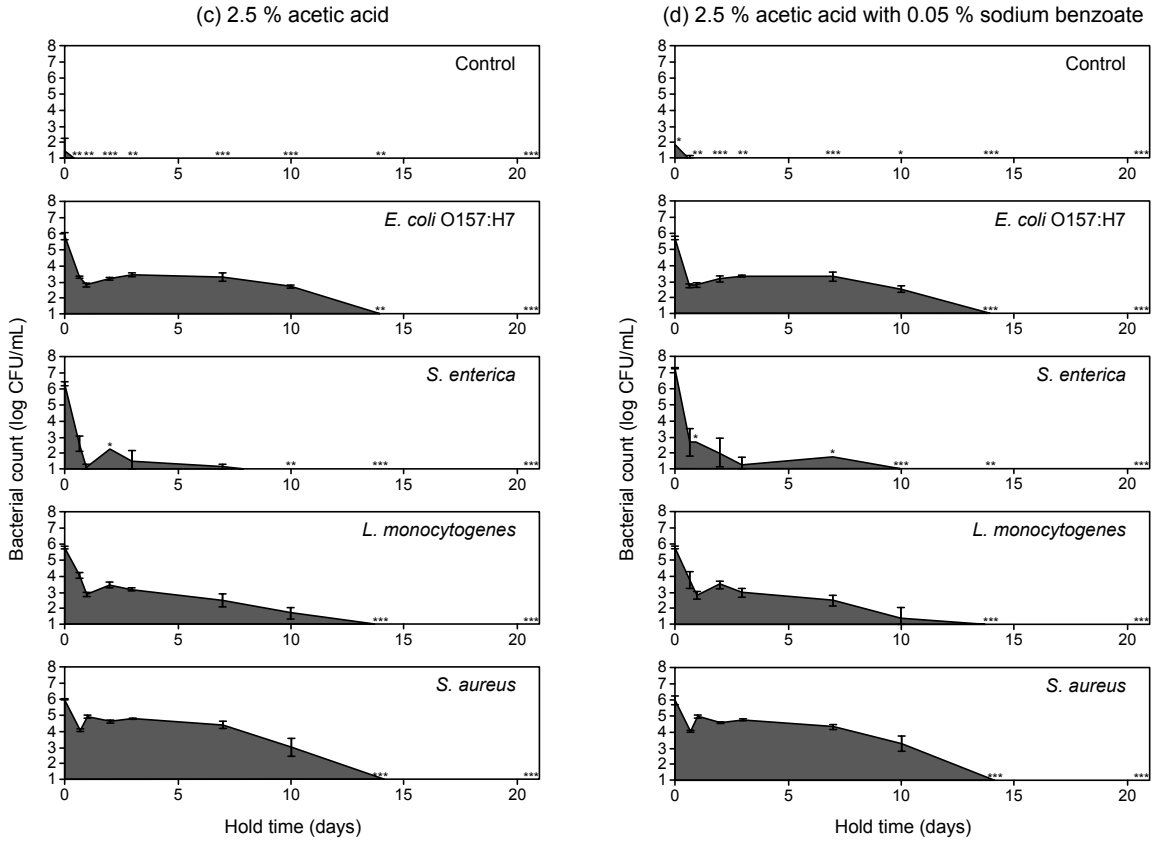

Figure 2.4. Variation of bacterial counts on pickled eggs with storage time for (a) 2.5 % acetic acid, (b) 2.5 % acetic acid with sodium benzoate. Error bars represent standard deviation for n = 3, * indicates one of the replicates resulted in a zero plate count (the average is assumed to be less than the indicated point), ** indicates two of the replicates resulted in a zero plate count (the average is assumed to be less than the indicated point), *** indicates the three replicates resulted in a zero plate count (the average is assumed to be less than the indicated point, which is the detection limit of 1.0 log value).

Discussion

Total Egg pH versus Total Yolk pH. When pickled eggs are tested for pH in industrial settings, all of the egg, both white and yolk, is generally ground up for testing. As demonstrated by Acosta (1) and extended to lower acetic acid concentrations by this study, total egg pH is not a good indicator of egg acidification because the egg white acidifies rapidly and skews pH data for the egg as a whole.

Table 2.2. Variation of bacteria counts on lids over storage time for 5 % acetic acid (5AA), 5 % acetic acid with sodium benzoate (5AASB), 2.5 % acetic acid (2.5AA), 2.5 % acetic acid with sodium benzoate (2.5AASB).

Pathogen	Treatment	0 Hour	16 Hour	24 Hour	48 Hour	72 Hour	7 Day	10 Day
Control	5AA	0.39 ± 0.616	< -0.30[b]	< -0.30[b]	0.00 ± 0.426[a]	< -0.30[b]	nd[c]	< -0.15 ± 0.213[a]
	5AASB	< -0.15 ± 0.213[a]	0.09 ± 0.357	nd[c]	nd[c]	0.00[b]	nd[c]	< -0.30[b]
	2.5 AA	< 0.18[b]	< -0.15 ± 0.213[a]	0.02 ± 0.551	<-0.30[a]	< -0.30[b]	0.00[b]	nd[c]
	2.5 AASB	< 0.18[b]	0.00 ± 0.426a	< -0.30[b]	< -0.30[a]	< -0.30[a]	nd[c]	< -0.30[b]
Escherichia coli 0157:H7	5AA	5.52 ± 0.465	< 1.18[b]	< 0.62 ± 0.879[a]	< -0.30[b]	nd[c]	nd[c]	< -0.30[b]
	5AASB	5.94 ± 0.062	<1.18[b]	< 0.51 ± 0.473[a]	nd[c]	nd[c]	< -0.30[b]	nd[c]
	2.5AA	5.84 ± 0.129	2.02 ± 0.185	1.10 ± 0.913	0.66 ± 0.870	<1.18[b]	< -0.30[b]	nd[c]
	2.5AASB	5.79 ± 0.205	1.31 ± 0.751	1.47 ± 0.671	1.72 ± 0.667	<1.03 ± 0.469[a]	0.17 ± 0.576	nd[c]
Salmonella enterica	5AA	6.07 ± 0.059	< 1.0[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
	5AASB	5.91 ± 0.021	< 1.0[c]	<0.40[b]	nd[c]	nd[c]	nd[c]	nd[c]
	2.5AA	5.75 ± 0.109	<1.09 ± 0.550a	nd[c]	< -0.30[b]	nd[c]	< -0.30[b]	nd[c]
	2.5AASB	5.70 ± 0.077	<1.0[b]	<0.24 ± 0.337[a]	< -0.30[b]	nd[c]	< nd[c]	< -0.30[b]
Listeria monocytogenes	5AA	4.82 ± 0.204	< 0.70[a]	< -0.30[b]	nd[c]	nd[c]	nd[c]	< -0.30[a]
	5AASB	4.83 ± 0.733	< 1.0[c]	nd[c]	< -0.30[b]	nd[c]	< 0.18[b]	< -0.30[b]
	2.5AA	4.85 ± 0.269	1.00 ± 0.301	nd[c]	< -0.15 ± 0.213[a]	nd[c]	nd[c]	< -0.30[b]
	2.5AASB	4.88 ± 0.158	< 1.18[b]	nd[c]	< 1.06[b]	< 0.18[b]	nd[c]	< -0.30[b]
Staphylococcus aureus	5AA	6.09 ± 0.042	3.07 ± 0.358	0.65 ± 0.772	0.00[b]	0.00[b]	nd[c]	0.00[a]
	5AASB	6.12 ± 0.136	4.28 ± 0.184	2.30 ± 0.501	1.54 ± 0.538	0.00[b]	nd[c]	< -0.30[b]
	2.5AA	6.09 ± 0.084	4.65 ± 0.251	3.27 ± 0.311	4.56 ± 0.094	nd[c]	1.12 ± 0.558	nd[c]
	2.5AASB	6.12 ± 0.108	4.95 ± 0.096	3.57 ± 0.346	2.36 ± 0.639	<2.40 ± 0.019[a]	1.00 ± 0.533	nd[c]

a: One of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. Both the average and standard deviations are calculated using two values. The average is assumed to be less than the calculated average based on the plate count of the clean third plate.

b: Two of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is simply the remaining log value. A standard deviation is not given. The average is assumed to be less than the given average based on the plate counts for the two clean plates.

c: Three of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is given as being less than our detection limits (1.0 log value for the lid samples).

While industry cannot be expected to purchase the expensive pH probes that would allow more precise point analysis of acidification, pH of the whole yolk can be taken with current, standard pH probes and is a more accurate indicator of egg acidification than total egg pH. In fact, such protocols are suggested by regulation (9CFR318.300(b)). Further, this study suggests that eggs should not be removed from refrigeration immediately after the total yolk pH measurement drops below 4.6. Rather, eggs may be removed from refrigeration 4-7 days after the total yolk pH drops below 4.6, depending on brine strength, to account for differences between total yolk pH and the pH at the center of the yolk (point D).

Challenge Study. Given that pathogens were not detected on sample lids within 72 hours for 5% acetic acid brine concentrations, and that no pathogens except *S. aureus* were detected on lids within 72 hours for 2.5% acetic acid brine concentrations, minimal contact between the lid and brine seems necessary and adequate at equilibrated pickled egg pHs of 4.0 and 4.4 to ensure pathogen destruction on the lids over the time needed to ensure destruction in the system as a whole (Table 2.2). Procedures stipulating tilting or inversion of sealed containers to ensure contact between the lid and the brine should be adequate for pathogen destruction on lids.

At the levels at which sodium benzoate was added in this study, there was no observable impact of the preservative on pathogen survival, either enhancing or inhibiting pathogen survival. Results of studies of *L. monocytogenes* and cold shock indicate that sodium benzoate at 0.06% can provide an inhibitory effect to the pathogen at 4ºC (20). Since sodium benzoate was added at 0.05% by weight of the brine amounting to a concentration of

0.02% in the overall pickled egg system in this study, it is probable that the concentration was too low to impart any additional *L. monocytogenes*-inhibitory effect to the system.

Although the 5% acetic acid brine treatments reached equilibrium pH within 13 days, and the 2.5% acetic acid brine treatments within 16 days, the challenge study data shows the importance of removing eggs from refrigeration as quickly as possible, as soon as the pH of the solids drops below 4.6 to mitigate the growth potential for *Clostridium botulinum*, rather than waiting until for the pH to equilibrate. Processes for pickled egg production must take this into account. To be effective against pathogens, the process must provide a heat treatment in the form of storage at ambient temperature subsequent to the refrigerated hold time required to acidify the eggs to pH ≤ 4.6. This study found *S. aureus* particularly resistant to die-off under refrigeration (Figures 2.3 and 2.4).

Based on results from the challenge study, it appears that *L. monocytogenes* and *S. aureus* are both able to survive refrigerated storage in brines at 2.5% acetic acid and *S. aureus* in brines at 5% acetic acid, in a protein-rich environment like pickled eggs. At room temperature, pathogen die-off was as expected for inoculated media. Several studies have found links between cold storage and reduced acid tolerance, particularly for *E. coli* and *L. monocytogenes*, the two pathogens about which there is the most literature (13,17, 25, 26). Falerio et.al. reported that lower temperatures impacted growth of *L. monocytogenes* by reducing the pH range the pathogen could tolerate (13). Koutsoumanis et.al. found that tolerance developed through acid shock of *L. monocytogenes* decreased when the temperature at which acid shock occurred decreased, suggesting that refrigerated storage of pickled eggs may not result in the development of an appreciable acid tolerance, at least for *L.*

monocytogenes (17). Results from this study certainly support that hypothesis. Pathogens may not die as quickly under refrigeration, but acid concentrations of 2.5-5% acetic acid brines may be strong enough to counter any potential to develop acid tolerance during refrigerated storage. Since both *S. aureus* and *L. monocytogenes* showed capacity to survive under refrigeration, it is tempting to suggest that removal from refrigeration should be based on total egg pH rather than total yolk pH, since the latter would result in a longer refrigerated hold. However, the refrigeration requirement eliminates any growth potential for *C. botulinum*, the pathogens in this study did not grow under refrigeration, and once removed from refrigeration, pathogen die-off was rapid, compensating for any delay in die-off occurring during refrigerated hold time for acidification. It is possible that the time under refrigeration may shorten the time needed to achieve undetectable pathogen levels once samples are switched to room temperature storage, although this hypothesis was not within the scope of this study.

Extended refrigeration time, and the additional time required for samples to reach equilibrium pH has implications for industry more of a practical in addition to a food safety nature. The recommendations for refrigerated hold time are for control of *C. botulinum*. However, the ambient hold time is for destruction of *E. coli*, *L. monocytogenes*, *S. enterica* and *S. aureus*. The initial death to all four pathogens studied, even under refrigeration, reflects an initial decontamination of the egg surface. If the pickled eggs were held at room temperature long enough to start decontamination, but were moved to refrigeration within a time frame that ensured product under refrigeration at 24 hours, it is possible that the pathogen destruction would be faster and that we would not see a slight rise in pathogen

numbers over time under refrigeration indicating some recovery and, potentially, acid adaptation at refrigerated temperatures.

Conclusion. At equilibrated pH 4.0, pickled eggs should be held at least 5 days to allow for acidification below 4.6; product should be subsequently held at room temperature for at least 7 days to ensure pathogen die-off. At equilibrated pH 4.4, pickled eggs should be held at least 9 days under refrigeration to allow for acidification below 4.6; product should be subsequently held at room temperature for at least 7 days to ensure pathogen die-off.

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CHAPTER 3

STABILITY OF HOT-FILLED ACIDIFIED APPLE/CARROT JUICE: EFFECT OF FILL TEMPERATURE, ACID TYPE, pH AND CONTAINER TYPE

Abstract. Small-scale food processors have traditionally had limited thermal processing options for production of acidic shelf stable products. In an effort to develop appropriate thermal processing parameters for products with pH below 3.8, a model system of an 80:20 blend of fresh-pressed apple:carrot juice with high microbial load and high insoluble solids was acidified to pH values of 3.3, 3.5 and 3.7 with malic acid or acetic acid. Juice was filled at 63, 71 or 77ºC into either 10 oz glass or 8 oz PET containers (containers at 25ºC), sealed, tilted for cap treatment and held 2 min, and then force cooled in chlorinated water. Samples were stored at 30ºC and analyzed for Total Plate Count and Yeast and Mold at 0, 24 hr, 30 days and 60 days. Samples acidified with acetic acid and filled into PET remained stable throughout the 60 day study as did samples acidified with acetic acid to pH 3.3 and filled into glass and samples acidified with malic acid to pH 3.3 and filled into PET. Data for remaining samples was inconclusive, suggesting higher fill temperatures and/or longer hot hold times are needed to achieve sufficient lethality to guarantee shelf stability for pH above 3.3 and for different acid types.

Introduction. Much work has been done regarding the safety of acid and acidified food products, establishing lethality for such products at various temperatures and hold times, and for various pathogens, mainly *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and *Bacillus cereus*. The United States Food and Drug Administration (FDA) recognizes a series of time/temperature combinations outlined below as sufficient for pasteurizing fruit juice, the relevant pathogen being *Cryptosporidum parvum*

(Table 3.1). The FDA notes a lack of research into heat resistance of *C. parvum*, and allows for the use of pasteurization regimes validated for *E. coli* instead (8). Mak et.al. have validated 68.1ºC for 14 seconds for *E. coli, L. monocytogenes* and *Salmonella* (16) and this is used as the recommended treatment for cider in Wisconsin. However, New York State requires pasteurization at 71.1ºC for 6 seconds to ensure a 5-log reduction in *E. coli* (8), although a pasteurization step of 60ºC held for at least 5 minutes is also recognized by the FDA as part of the Juice HACCP (Hazard Analysis and Critical Control Point) validated procedures. Both treatments are based on work by Mazzotta, who established that a treatment at 71.1ºC for 3 seconds (z-value = 5.3ºC) was sufficient to achieve a 5-log reduction in *E coli*, *L. monocytogenes* and *Salmonella* (17).

Table 3.1. Pasteurization times and temperatures considered sufficient for juice safety.

Temperature (ºC)	Time (seconds)	Source
60	300	FDA
71.1	6	New York State
71.5	15	Milk Pasteurization
73.9	2.8	FDA
77	1.3	FDA
79.4	0.6	FDA
82.2	0.3	FDA

* Based on FDA. 2004.Guidance for Industry: Juice HACCP Hazards and Controls Guidance First Edition; Final Guidance (8).

With regard to spoilage microorganisms and shelf stability of acidified products in general and juices in particular, Sperber states that, for acidified food products that rely on acetic acid for their pH, spore forming bacteria are not a concern; the concerns come mainly

from lactic acid bacteria and yeasts, with few molds tolerating acetic acid (29). For acid fruit juices, considerable attention has been paid to *Alicyclobacillus acidoterrestris*, a spoilage microorganism isolated as spore-forming bacilli by Splittstoesser et.al. which has been shown withstand both low pH and processing temperatures which were long considered adequate for stability (30). Silva et.al. tested various processing temperatures, ºBrix levels and pH levels to determine adequate pasteurization regimes for *Alicyclobacillus,* finding that 85ºC and 91ºC were sufficient for juice with a pH of 3.5 or 3.7 and a ºBrix of about 11 (28). In 2012, Silva et.al. studied high pressure processing combined with heat to inactivate *Alicyclobacillus acidoterrestris* and concluded that pressure could lower processing temperatures (27). Work has been done recently by Irkin and Korkuluoglu to demonstrate the usefulness of spices and essential oils as antimicrobial agents against *B. cereus, E. coli*, *Salmonella*, *L. monocytogenes*, and several yeasts in fruit juices as a way to address a desire for "natural" preservatives on the part of consumers (14).

When discussing acid or acidified food products, the antimicrobial properties of organic acids become an important part of the discussion given recent outbreaks of *E. coli* in apple juice and *Salmonella* in citrus juices. Many studies have investigated the relative strength of various organic acids against pathogenic microorganisms, mainly in juices and purees. Although weak organic acids are universally found more effective against pathogens than inorganic acids like hydrochloric acid, the inhibitory effect of particular acids varies and seems to be related to microorganisms studied, the concentration of acid, the pH and the composition of the media used for the study and the temperature of treatment and holding. Numerous authors have cited these circumstances to explain or qualify results (5, 12, 15, 19, 21). Acetic acid has been found by some studies to be the most effective organic acid against *E. coli* O157:H7*, L. monocytogenes,* and *S. aureus* (15, 25, 26), although Han and Linton (10) and Buchanan and Edelson (5) both found lactic acid to be the most effective in controlling *E.*

coli O157:H7. Bjornsdottir et.al. found low concentrations (5 mM) of lactic acid, malic acid or acetic acid to be less effective than gluconic acid against acid-adapted *E. coli* O157:H7 (4).

There is less literature regarding the effectiveness of various organic acids against spoilage microorganisms, but Hsiao and Siebert concluded that lactic, acetic and citric acids are stronger (lower minimum inhibitory concentrations) against several pathogens and spoilage microorganisms than, for instance, malic acid (11). Savard et.al. reported a combination of lactic, acetic and propionic acids most effective against two spoilage yeasts applied to fermented (lactic acid bacteria) vegetable juices at pH 3.74 (26). Calder et.al. showed that application of sodium acid sulfate had a similar antimicrobial action against aerobic bacteria to citric acid when used as an anti-browning agent on French-fry cut potatoes (6). Mosneaguta et.al. extended these conclusions to malic acid (18).

While use of various organic acids can offer varying degrees of antimicrobial activity, additional processing is required in most cases to yield a shelf-stable product or prolong refrigerated shelf life. Traditionally, processing has involved thermal treatment of the product which can negatively impact nutrient content as well as flavor, texture and color of food products. Attention in academia has been widely focused on high hydrostatic pressure (HHP) processing and on ultrahigh pressure processing (UHP) since these two processing methods show promise in simultaneously destroying pathogenic bacteria and in prolonging shelf life while maintaining nutritional and organoleptic quality. A literature search for articles published only in the last few years yields a wealth of research regarding safety and stability for products from acidic juices like blueberry, pomegranate and apple (2, 7, 9, 33) to vegetable juice (3, 22) to milk and milk/fruit juice blends (1, 31). Researchers have examined vitamin retention and the effect of HHP and UHP on anthocyanin and polyphenol content (1, 2, 3, 32, 33) as well as spoilage microorganisms like *Alicyclobacillus acidoterrestris* (22, 27).

In product development, processors have to balance product quality, equipment realities and container desires with thermal requirements yielding the longest possible shelf

life - ideally shelf stability. In fact, the most viable products for small-scale processors are shelf stable products because of reduced storage and distribution costs and greater ease of placement in retail outlets. The established processing parameters for shelf stability often have negative implications for product quality – loss of nutrients, flavor change, color loss and browning. Juice processors and processors of value-added or gourmet products often rely on product nutrients, flavor and color to give their products that edge that will convince consumers to pay higher prices. While high pressure processing offers tantalizing possibilities, such processing is currently too expensive and processing facilities too few for the technology to provide a practical option for small-scale processors. Therefore, more traditional thermal processing of acid and acidified foods must be optimized to subject products to the minimal thermal treatment given the product's particular formulation. Our objective was to use an 80:20 apple:carrot juice blend with high microbial load and high insoluble solids, an acidified food, to study the effect of acidifying agent (malic or acetic acid), product pH (3.3, 3.5 or 3.7), fill temperature (63, 71 or 77ºC) and container type (glass or PET) on stability in an effort to test options for producing a minimally processed, shelf-stable acidified product.

Materials & Methods

Juice Processing. An acidified juice blend was produced as follows. Apple juice was pressed using stock apples from Controlled Atmosphere (8-12 months) storage at the New York State Agricultural Experiment Station (Geneva, NY). Due to storage time, visually sound apples were hand-selected from apple exhibiting mold and/or rot and pressed in an accordion press (Goodnature Products Inc., Buffalo, NY). Carrots were purchased from a local supermarket and hand washed in cold, still water to remove visible soil and carrot top remnants. Carrots were processed in a hammer mill (Comminuting Machine Model D, The W.J. Fitzpatrick Co., Chicago, IL) and the resulting pulp was pressed in a small hydraulic rack-and-frame press

(New York State Experiment Station Machine Shop, Geneva, NY). Apple and carrot juices were combined in an 80:20 ratio by weight, the juice blend was divided into six pails, covered, and stored at-2.2ºC until processed. Initially, juice pH was measured at 4.14, ºBrix at 10.91 (pH and ºBrix measured with units described below). Turbidity, measured with a Hach 2100P Turbidimeter (Hach Co, Loveland CO), was measured at 2230 NTU, resembling a high-pulp carrot juice.

Glass bottles (10 oz) and plastisol lined metal lids were obtained from Wixson's Honey, Inc (Dundee, NY). PET bottles (8 oz) and matching lids were obtained from Cherrypharm, Inc. (Geneva, NY). Lids on PET bottles were designed to hold a vacuum. All bottles were stored and held at ambient temperature (about 25ºC). Immediately prior to processing, juice was warmed to about 25ºC. Juice was then acidified with either malic acid (Presque Isle Wine Cellars, Northeast, PA) or glacial acetic acid (Fisher Scientific, Pittsburgh, PA) in order to attain pH values of 3.3, 3.5 or 3.7. Juice was immediately passed through a UHT/HTST Lab-25 HV heat exchanger (Microthermics Inc., Raleigh, NC) and filled at 63, 71 or 77ºC into either glass or PET bottles. See Figure 3.1 for a pictorial explanation.

After filling, bottles were immediately capped and tilted. Bottles were held, tilted, for 2 minutes, righted and immediately force cooled as follows. PET bottles were placed up to their caps in chlorinated (200 ppm) ice water. Glass bottles were submerged in warm, chlorinated (200 ppm) water for a few minutes prior to immersion up to the cap in chlorinated (200 ppm) ice water to reduce the likelihood of heat shock. Both warm water and ice water were changed regularly and chlorination level checked prior to each fill.

Initial samples of juice prior to acidification were taken to establish initial microbial counts. Initial samples of acidified juice at each combination of acidifier and were taken to establish initial acidified juice counts. Samples were made in triplicate for each combination of acidifier, pH, container type and sampling point: Time 0, 24 hour, 30 day and 60 day. With

the exception of time 0 samples, all samples were held at room temperature for the first 24 hours and then held at 30ºC for accelerated shelf life testing.

Physical Testing: Samples were tested for pH, soluble solids (ºBrix) and titratable acidity. Samples were taken of initial juice prior to acidification, and at Time 0, 24 hours, 30 days and 60 days. All pH measurement was done using an Orion ROSS Sure-Flow electrode (Thermo Scientific, Hanover Park, IL) connected to an Accumet Basic AB15 pH meter

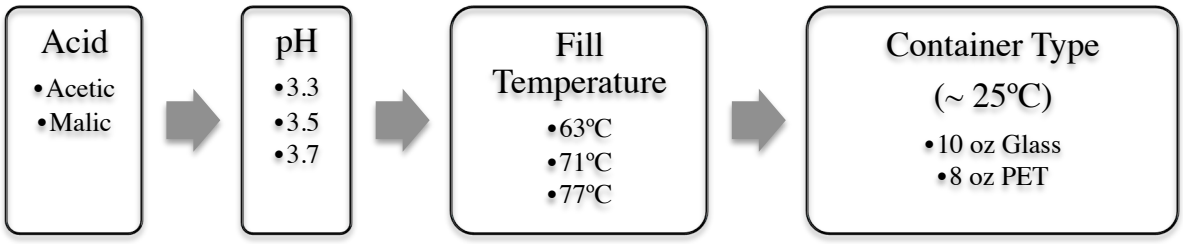


Figure 3.1. Pictorial representation of study design for shelf-stability of apple:carrot juice blend. Four variables representing 36 permutations of acid, pH, fill temperature and container type.

(Fisher Scientific, Pittsburgh, PA) with temperature compensation. Soluble solids were measured using an AUTO ABBE Refractometer (Leica Inc, Buffalo, NY). Titratable acidity was measured using a G20 Compact Titrator with Rondolino (Mettler Toledo, Schwerenbach, Switzerland).

Shelf life Analysis: Initial samples of the juice blend were taken prior to processing for each acid type and pH. Initial samples were tested to establish baseline plate counts for Total Plate Count (Standard Plate Count Media; TPC) and Yeast & Mold (Acidified Potato Dextrose Agar, pH 3.5; YM). Serial dilutions were made in 0.1% sterile peptone water and the dilutions were plated in duplicate. Upon agar solidification, petri dishes were inverted and

incubated for 48 hours at 35ºC ± 2. Colony Forming Units (CFUs) were enumerated using a Quebec Colony Counter and replicate counts were averaged. The same process was used for samples representing each acid, each pH, each fill temperature and each container type (glass or PET) at time 0, 24 hours, 30 days and 60 days.

Cooling Profiles. Since the cooling profiles were compiled to help elucidate results from the original study, the original apple/carrot juice blend was not available. A commercial fruit juice (equal parts Tropicana Farmstand™ Peach Mango and Tropicana Farmstand™ Strawberry Banana fruit & vegetable juice blends, blended) with cloudy appearance indicating high particulate content was obtained from a local supermarket. Extra glass and PET bottles and associated closures from the original study were available and were used to compile the cooling data.

For the first part of the study, rigid thermocouples (Ecklund Harrison Technologies, Inc. Fort Myers, FL) were attached to the center of container caps with a wire running from the thermocouple to a computer running CALSoft 5 software (TechniCAL, Inc., New Orleans LA) to record temperatures over time. Hot juice was filled into room temperature containers, cap/thermocouple combinations were placed on containers, and containers were sealed and tipped on their sides for 2 minutes. After two minutes, containers were placed upright.

For the second part of the study, flexible thermocouples (Ecklund Harrison Technologies, Inc. Fort Myers, FL) were attached to the center of the container caps and bent so that the thermocouple would lay against the inside container side when the cap was affixed to the container. Hot juice was filled into room temperature containers, cap/thermocouple combinations were placed on containers, and containers were sealed and tipped on their sides for 2 minutes. After two minutes, containers were placed right side up. Temperatures were recorded by computer running CALSoft 5 software (TechniCAL, Inc., New Orleans LA).

Results & Discussion. Minor increases in pH were observed at the 24 hour sampling, leading to samples having pH in ranges from 3.30 to 3.35, 3.50 to 3.55 and 3.65 to 3.70, depending on the acid used (data not shown). Increases were common to all samples in a given study condition, and most likely indicate pH equilibration rather than microbial growth since pH was stable after 24 hours within the error of the pH meter throughout the study. The soluble solids (ºBrix) and titratable acidity of the samples remained constant throughout the study (data not shown) even for samples that were noticeably fermenting (3 out of 144 total samples).

Initial counts for juice are shown in Table 3.2. Average TPC and YM counts are listed in Tables 3.3, 3.4 and 3.5 below. Samples were considered spoiled when counts reached 3log cfu/ml. At these counts, formations of a gelatinous, roughly spherical material could be seen at the interface between the sediment and the serum. These formations were most likely bacterial since the TPC counts were elevated and the YM counts were unchanged in samples exhibiting this type of spoilage. Samples were remarkably consistent in that they first clarified as enzymatic activity increased, then bacteria grew, and finally samples exhibited molds and, in extreme cases, fermentations with gas production. Fermentation was likely caused by yeast since YM counts were elevated for fermenting samples.

Table 3.2. Total Plate Count (TPC) and Yeast and Mold counts (YM) expressed as log cfu/ml for 80:20 apple:carrot juice acidified with either malic or acetic acids.

	Malic Acid		Acetic Acid	
pH	TPC	YM	TPC	YM
3.3	3.00 ± 0.38	2.35 ± 0.47	1.58 ± 0.57	nd[a]
3.5	TNTC	2.15 ± 0.65	TNTC	nd[a]
3.7	TNTC	1.90 ± 0.83	TNTC	2.50±0.50

[a] Four of the six replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. Both the average and standard deviations are calculated using two values. The average is assumed to be less than the calculated average based on the plate count of the clean third plate. Too numerous to count (TNTC) $> 10^6$ cfu/ml.

Table 3.3. Total Plate Count (TPC) and Yeast & Mold counts (YM) expressed as log CFU/ml for 80:20 apple:carrot juice blend filled hot at 63ºC into 10 oz glass or 8 oz PET containers and held hold 2 minutes prior to force cooling.

		Malic Acid - Glass		Malic Acid - PET		Acetic Acid - Glass		Acetic Acid - PET	
pH		TPC	YM	TPC	YM	TPC	YM	TPC	YM
3.3	Time 0	2.95 ± 0.43	-0.301[b]	2.00 ± 0.10	nd[c]	1.69 ± 0.12[a]	nd[c]	1.18 ± 0.31	nd[c]
	24 hours	2.00 ± 0.11	0.700[b]	2.42 ± 0.87	nd[c]	1.61 ± 0.29	nd[c]	1.46 ± 0.08	nd[c]
	Day 30	2.60 ± 0.08	2.74 ± 0.39	1.78 ± 0.18	1.45 ± 0.64	1.61 ± 0.61	nd[c]	1.00[b]	0.700[b]
	Day 60	2.86 ± 1.39	2.85[b]	2.84 ± 1.82	1.44 ± 0.87	0.70[b]	nd[c]	nd[c]	nd[c]
3.5	Time 0	2.10 ± 0.18	nd[c]	1.91 ± 0.13	nd[c]	1.78 ± 0.16	nd[c]	1.87 ± 0.15	nd[c]
	24 hours	2.07 ± 0.04	nd[c]	1.91 ± 0.06	1.00[b]	1.83 ± 0.08	0.70[b]	1.89 ± 0.03	nd[c]
	Day 30	4.56 ± 0.03	4.63 ± 0.01	4.63 ± 0.11	4.62 ± 0.12	0.94 ± 0.34	nd[c]	1.10 ± 0.17	nd[c]
	Day 60	Spoiled	Fermenting	1.67 ± 0.92	1.23 ± 0.64	0.80 ± 0.42	0.700 ± 0.40	0.80 ± 0.42	nd[c]
3.7	Time 0	2.13 ± 0.95	nd[c]	2.17 ± 0.10	nd[c]	1.94 ± 0.11	nd[c]	2.08 ± 0.06	nd[c]
	24 hours	2.12 ± 0.05	nd[c]	1.96 ± 0.13	nd[c]	1.99 ± 0.17	nd[c]	2.02 ± 0.06	nd[c]
	Day 30	3.76 ± 0.16	3.53 ± 0.08	4.01 ± 1.17	4.05 ± 1.06	1.42 ± 0.21	1.30[b]	1.40[b]	nd[c]
	Day 60	3.87 ± 1.98	3.65 ± 2.00	1.33 ± 0.71	1.24 ± 0.72[a]	3.64 ± 1.82	0.700[b]	1.18 ± 0.68	nd[c]

a: One of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. Both the average and standard deviations are calculated using two values. The average is assumed to be less than the calculated average based on the plate count of the clean third plate.

b: Two of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is simply the remaining log value. A standard deviation is not given. The average is assumed to be less than the given average based on the plate counts for the two clean plates.

c: Three of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is given as being less than our detection limits (1.0 log value for the lid samples).

Table 3.4. Total Plate Count (TPC) and Yeast & Mold counts (YM) expressed as log CFU/ml for 80:20 apple:carrot juice blend filled hot at 71ºC into 10 oz glass or 8 oz PET containers and held hold 2 minutes prior to force cooling.

		Malic Acid - Glass		Malic Acid - PET		Acetic Acid - Glass		Acetic Acid - PET	
pH		TPC	YM	TPC	YM	TPC	YM	TPC	YM
3.3	Time 0	1.88 ± 0.76	nd[c]	1.32 ± 0.15	nd[c]	0.90 ± 0.17	nd[c]	0.80 ± 0.17	nd[c]
	24 hours	1.40 ± 0.20	0.70[b]	1.29 ± 0.11	nd[c]	1.12 ± 0.10	nd[c]	0.700[b]	nd[c]
	Day 30	3.30 ± 0.43	nd[c]	1.19 ± 0.43	0.70[b]	0.70 ± 0.00[a]	0.70[b]	0.80 ± 0.17	0.700[b]
	Day 60	2.33 ± 1.42	2.10[b]	1.30[b]	2.59 ± 1.83	1.40[b]	0.70[b]	0.70[b]	nd[c]
3.5	Time 0	1.89 ± 0.21	nd[c]	1.92 ± 0.22	nd[c]	1.36 ± 0.10	nd[c]	0.86 ± 0.28	nd[c]
	24 hours	1.48 ± 0.15	nd[c]	1.24 ± 0.47	nd[c]	1.52 ± 0.22	nd[c]	1.06 ± 0.10	nd[c]
	Day 30	3.41 ± 0.34	1.78 ± 1.52	1.77 ± 0.66	2.40[b]	1.39 ± 0.09	nd[c]	1.02 ± 0.28	nd[c]
	Day 60	3.19 ± 2.11	0.90 ± 0.47	1.10 ± 0.67	1.54[b]	3.38 ± 1.69	nd[c]	1.30[b]	nd[c]
3.7	Time 0	2.07 ± 1.26	0.00[b]	1.61 ± 0.24	0.60 ± 0.10	1.48 ± 0.16	nd[c]	1.02 ± 0.28	nd[c]
	24 hours	1.53 ± 0.13	nd[c]	1.52 ± 0.48	nd[c]	1.55 ± 0.22	nd[c]	1.49 ± 0.20	nd[c]
	Day 30	3.00 ± 1.50	1.00[b]	1.98 ± 1.10	3.12[b]	0.96 ± 0.24	0.70[b]	0.70 ± 0.00[a]	1.00[b]
	Day 60	1.55 ± 1.10	0.70[b]	0.85 ± 0.5[a]	1.60[b]	3.63 ± 1.81	nd[c]	1.84 ± 1.5	1.95[b]

a: One of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. Both the average and standard deviations are calculated using two values. The average is assumed to be less than the calculated average based on the plate count of the clean third plate.

b: Two of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is simply the remaining log value. A standard deviation is not given. The average is assumed to be less than the given average based on the plate counts for the two clean plates.

c: Three of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is given as being less than our detection limits (1.0 log value for the lid samples).

Table 3.5. Total Plate Count (TPC) and Yeast & Mold counts (YM) expressed as log CFU/ml for 80:20 apple:carrot juice blend filled hot at 77ºC into 10 oz glass or 8 oz PET containers and held hold 2 minutes prior to force cooling.

		Malic Acid - Glass		Malic Acid - PET		Acetic Acid - Glass		Acetic Acid - PET	
pH		TPC	YM	TPC	YM	TPC	YM	TPC	YM
3.3	Time 0	1.26 ± 0.61	nd[c]	1.02 ± 0.28	nd[c]	0.700[b]	nd[c]	0.700[b]	nd[c]
	24 hours	1.34 ± 0.19	nd[c]	0.96 ± 0.24	nd[c]	0.70 ± 0.00[a]	nd[c]	0.94 ± 0.34	nd[c]
	Day 30	3.96 ± 0.21	2.18 ± 2.09	1.24 ± 0.34	nd[c]	1.00 ± 0.00[a]	nd[c]	0.70 ± 0.00[a]	0.70 ± 0.00[a]
	Day 60	2.48 ± 1.84	nd[c]	nd[c]	nd[c]	0.80 ± 0.42	nd[c]	1.05[b]	nd[c]
3.5	Time 0	1.94 ± 1.19	-0.301[b]	1.83 ± 0.76	nd[c]	1.39 ± 0.12[a]	nd[c]	1.15 ± 0.21[a]	nd[c]
	24 hours	1.38 ± 0.33	0.70[b]	1.23 ± 0.21	nd[c]	1.00 ± 0.43	nd[c]	1.33 ± 0.21	0.70[b]
	Day 30	3.29 ± 0.82	0.70[b]	1.48 ± 0.96	2.53[b]	1.14 ± 0.42	nd[c]	0.70 ± 0.00	nd[c]
	Day 60	4.04 ± 2.10	0.70 ± 0.40[a]	2.36[b]	2.90[b]	3.00 ± 1.55	nd[c]	nd[c]	nd[c]
3.7	Time 0	1.32 ± 0.15	0.301[b]	1.18 ± 0.00[a]	nd[c]	1.16 ± 0.15	nd[c]	1.47 ± 0.07	nd[c]
	24 hours	1.42 ± 0.10	nd[c]	1.29 ± 0.11	nd[c]	1.42 ± 0.05	nd[c]	0.94 ± 0.34	nd[c]
	Day 30	4.02 ± 0.57	3.89[b]	1.30[b]	nd[c]	1.22 ± 0.24	nd[c]	1.00 ± 0.00[a]	0.70[b]
	Day 60	2.08 ± 1.50*	0.70[b]	0.90 ±0.53	nd[c]	3.61 ± 1.80	nd[c]	0.70[b]	nd[c]

a: One of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. Both the average and standard deviations are calculated using two values. The average is assumed to be less than the calculated average based on the plate count of the clean third plate.

b: Two of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is simply the remaining log value. A standard deviation is not given. The average is assumed to be less than the given average based on the plate counts for the two clean plates.

c: Three of the replicates resulted in a zero plate count and therefore could not be used in the mathematical calculation of a geometric average and standard deviation. The average is given as being less than our detection limits (1.0 log value for the lid samples).

Samples acidified with malic acid were less stable than those acidified with acetic acid, as expected. Samples acidified with acetic acid and hot-filled into PET bottles were stable throughout the study, regardless of pH or fill temperature. Samples acidified to 3.3 with acetic acid were the only samples unquestionably stable in glass, and were stable regardless of fill temperature. Samples acidified to 3.3 with malic acid were stable when filled into PET at 77ºC; samples filled at 63ºC show TPC counts approaching spoilage levels, and those filled at 71ºC show the beginnings of YM growth. Samples acidified to 3.5 and 3.7 with malic acid and filled at 63ºC spoiled in PET by the day 30 sampling. These juices (3.5 and 3.7, 63ºC) showed decreasing counts by day 60 sampling, although this most likely reflects die off due to nutrient depletion during initial spoilage. Samples acidified to 3.5 and 3.7 with malic acid and filled at 71ºC showed increasing YM counts by day 60 sampling and while these counts were not high enough to indicate spoilage at the sampling time, the increase of counts indicate, at the least, spoiling of some samples and the likelihood of fairly immediate future spoilage had the study continued. The juice was very turbid (2230 NTU) and it is likely that the turbidity provided a shield to microorganisms in the juice that made the 63ºC, and even the 71ºC fill temperatures problematic.

As demonstrated in Tables 3.2, 3.3 and 3.4, data in all other instances was remarkably inconsistent. Samples acidified with malic acid and filled at 71ºC in PET were stable over the study for pH 3.3, but both pH 3.5 and 3.7 showed rising YM counts. Samples acidified with malic acid and filled at 77ºC in PET were stable with the exception of samples acidified to 3.5, one of which showed increasing YM counts, which calls into question the stability results for juices acidified with malic acid to pH 3.7. Juices acidified with malic acid and filled into glass spoiled regardless of fill temperature or pH. Juices acidified with acetic acid to pH 3.7 and filled into glass also spoiled regardless of fill temperature. Contrary to expectation, juices acidified with acetic acid pH 3.5 filled at 63ºC did not spoil while those filled at 71and 77ºC did.

The data may be explained by a number of factors that, in conjunction, led to counter-intuitive and contradictory results. It is likely that one main factor was the initial microbial load of the fresh-pressed juice. The apple juice was pressed from old (8-12 month) apples. Numerous apples in the crate showed considerable visual spoilage (mold and/or rot), and while those apples were avoided, it is likely that spoilage microorganisms were rife even on visually sound fruit. As a root vegetable, carrots are more contaminated than bush or tree fruits since the carrots are in direct contact with soil (13). Torres-Vitale et.al. isolated coliform bacteria, fecal coliforms, diarrhoeagenic *E. coli, E. coli* and *Salmonella* from raw carrot juice purchased from restaurants in Mexico (32). Shredded carrots tested soon after processing showed high (10^4-10^6 log CFU/ml) counts of lactic acid bacteria (LAB), yeast and mold, and coliforms according to a review by Nguyen-the and Carlin; both LAB and yeasts play a main role in spoilage of shredded carrot (20). Patterson et.al. identified spoilage microorganisms in carrot juice to be primarily Gram-positive bacteria; and noted the generally high initial microbial load (22).

We had aimed for very high microbial loads in order to produce a worst-case scenario system for the study. We may have been too successful, maintaining a microbial load that overwhelmed the system, particularly in juice acidified with malic acid, and particularly in glass containers, further explored below. Study results indicate that a sanitation step is needed for fresh carrots; washing in cold water is not enough. Use of good-quality apples is also necessary. While use of good quality ingredients that are properly sanitized is indicated by the use of Good Manufacturing Practices (21CFR110) and repeated more clearly in Juice HACCP (21CFR120) regulations, we wanted to identify parameters that would yield a stable product even in extreme circumstances.

For the most part, samples filled into glass performed worse than samples filled into PET containers, which outcome was contrary to expectation. Rough estimations of weights of container versus weight of product were made, suggesting an almost 1:1 ratio of container

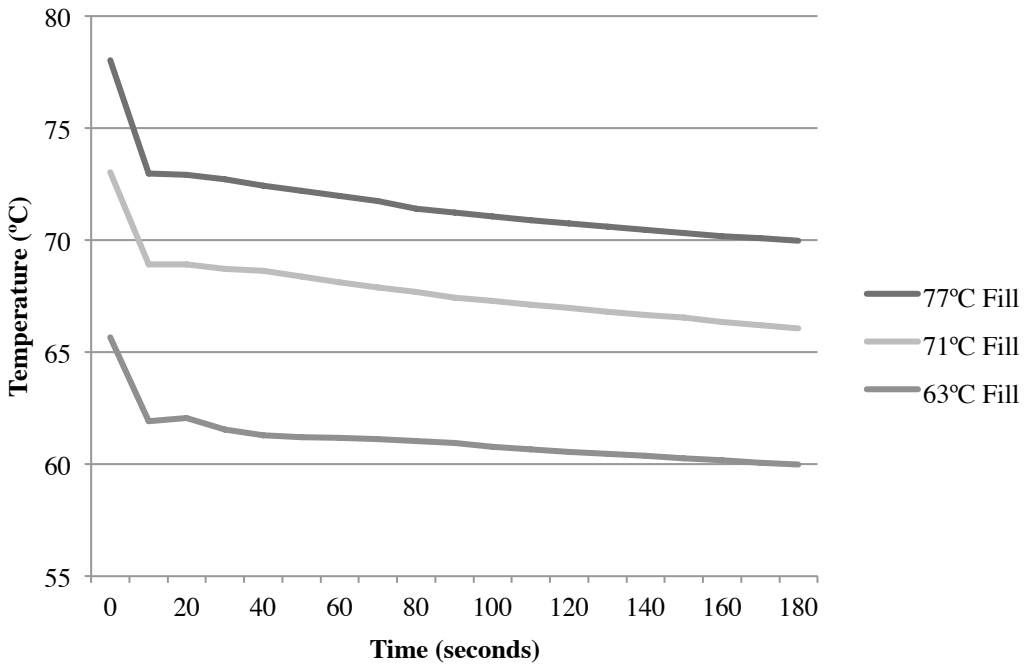
weight to product weight for glass versus about 1:8 ratio of container weight to product weight for PET. Since the containers were held at ambient temperature, the ratios above mean that the product filled into glass containers had considerably more material to warm up than product filled into PET containers. Cooling profiles were run using purchased fruit juice in the same room-temperature 10 oz glass and 8 oz PET containers in order to determine if heat loss after fill could help explain these results. Figure 3.2 shows cooling profiles for glass and PET containers with the thermocouple placed in the center of the container. Figure 3.3 shows cooling profiles for thermocouples placed along the container side. Table 3.6 shows lethality achieved over a 2 minute hold time based on temperatures recorded at the side of the containers and at the center of the containers. Lethality was calculated in two ways: with a reference temperature (Tref) of 93ºC and a z value of 8.8ºC as suggested by Pflug for shelf stable acidified foods (23); and with a Tref of 71ºC and z value of 5.3ºCF as suggested by Mazzotta for pasteurization of fruit juices with a pH < 4.0 (17).

Table 3.6. Lethality (in equivalent min) for 8 oz PET and 10 oz Glass containers, calculated based on temperatures recorded over 2 minutes at container side and container center, and using two sets of Reference Temperature (Tref) values and corresponding z values.

Fill	PET				Glass			
Temperature	Side T1	Side T2	Center T1	Center T2	Side T1	Side T2	Center T1	Center T2
63ºC	0.001	0.001	0.001	0.001	0.000	0.000	0.001	0.001
71ºC	0.004	0.795	0.009	2.878	0.002	0.432	0.005	1.040
77ºC	0.019	10.677	0.022	13.458	0.007	3.821	0.013	6.928

T1: Total lethality over 2 min hold time, Tref = 93ºC; z = 8.8ºC (23)
T2: Total lethality over 2 min hold time, Tref = 71ºC; z = 5.3ºC (17)

a)



b)

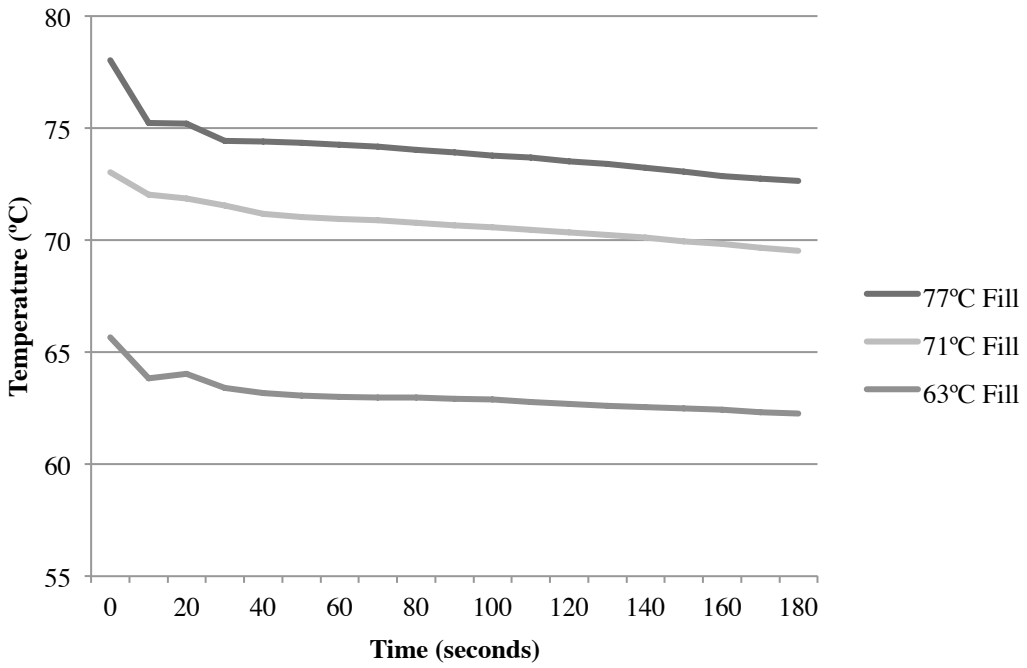
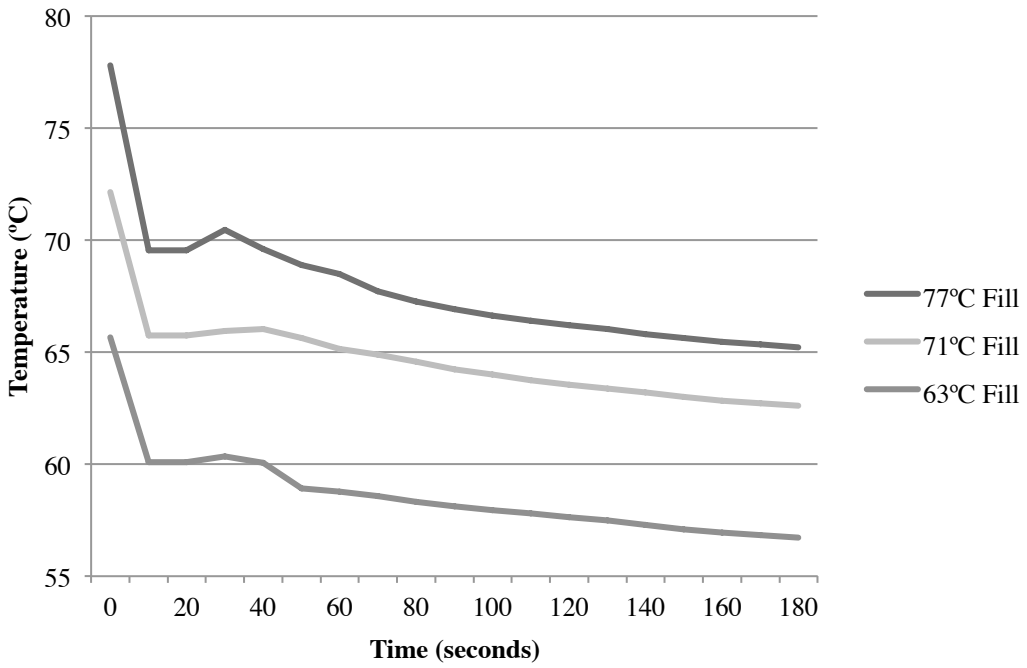


Figure 3.2 Cooling profile at the container center for commercial fruit juice filled at 63, 71 or 77ºC into a)10 oz glass containers and b) 8 oz. PET containers.

a)



b)

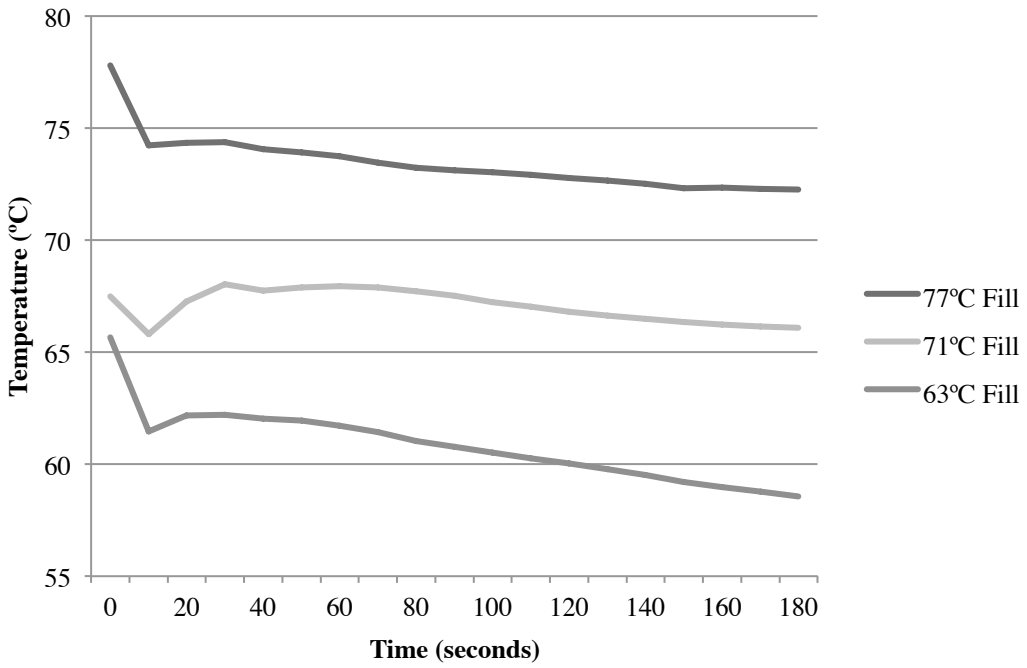


Figure 3.3 Cooling profile at the container side for commercial fruit juice filled at 63, 71 or 77ºC into a)10 oz glass containers and b) 8 oz. PET containers.

The initial temperature of the glass bottles themselves was about 14ºC, and about 15ºC for PET bottles. Initial heat loss for glass was about 5–10ºC; for PET it was about 3–5ºC. For juices filled at 71 and 77ºC, initial heat loss suggests bottle treatments closer to 63 and 68ºC respectively. Using a Tref of 93ºC and a z value of 8.8ºC, numbers used by Pflug to calculate lethality resulting in commercial sterility of acidified foods, none of the fill temperatures yield an adequate lethality (0.1 minutes for pH <3.9) (23). Mazzotta proposed a thermal treatment equating to 3 seconds (0.05 minutes) at 71ºC for a 5log reduction in *E.coli*, *L.monocytogenes* and *Salmonella* for acidic fruit juices at pH < 4.0 (17). If Mazzotta's parameters are applied, neither PET nor glass achieved pasteurization at a 63ºC fill. However, lethality is achieved, and safety is met, for PET at a fill temperature of 71ºC, and for both PET and glass at fill temperature of 77ºC. In fact, lethality achieved at 77ºC is so far in excess of that required, that commercial sterility would not be surprising. Certainly the TPC and YM counts after 60 days in juice acidified with acetic acid and filled into PET, together with the initial cooling study lethality values, suggest achievement of shelf stability, although Mazzotta calculates values only for pasteurization.

Although the cooling studies present rough estimates of the cooling profiles during the experiment, temperature loss and lethality after 2 minutes (corresponding to hold time) suggest containers themselves received only minimal thermal treatment and that the hold times were inadequate resulting in spoilage. Regarding the 63ºC juice fill temperature sample, juice in the cooling studies was filled, in actuality, at temperatures above 65ºC resulting in an in-bottle hold time of seconds at or above 63ºC. The juice in the original study remained at 63ºC long enough for the juice to pass through the pasteurization unit – a matter of seconds and was filled immediately, achieving seconds, at most, of hold time in-bottle of at least 63ºC. The FDA requires a hold time of five minutes at 63ºC for a 5-log reduction in *E. coli*. When the heating time in the pasteurizer is added to the probable thermal treatment as suggested by

the cooling profiles, product in this study did not receive even the minimum recognized pasteurization for *E. coli*, much less a hot fill that could be expected to yield a stable product. Calculated lethality, both by the Pflug (23) and the Mazzotta (17) methods, over a 2-minute hot hold confirms this. New York State allows pasteurization at 71.1ºC for 6 seconds to ensure a 5-log reduction in *E. coli* in juice (8). The cooling study data, using the Mazzotta method to calculate lethality, indicates that at the 71 and 77ºC fills into PET, lethality was achieved for *E. coli*, *L. monocytogenes* and *Salmonella*. Based on cooling study data, juice filled at 71ºC into glass was pasteurized depending on where the temperature was measured: lethality calculated based on temperature measured at the side suggests the thermal process was not quite adequate; lethality a the center of the glass container surpassed the 0.05 minutes required.

Both methods of calculating lethality suggest that even if all spoilage microorganisms were killed or inactivated by heating the juice, any microorganisms in the containers and closures themselves may have survived the heat treatment, particularly in glass, and particularly at the 63 and 71ºC fill temperatures. When added to differences in microbial stability between juices acidified with acetic vs. malic acids, the stability of juice in PET regardless of fill temperature or pH was most likely also a factor of the acid used to acidify the system: acetic acid at the concentrations needed to achieve pH 3.3 worked; juice acidified with malic acid to pH 3.3 showed less reliable results. Juice blend acidified with acetic acid, even to pH 3.7, and packed into PET was stable over the course of the study suggesting that, together with the lower initial heat loss in PET, acetic acid itself holds antimicrobial properties as suggested by Hsiao and Siebert (11) and found in numerous studies regarding the efficacy of acetic acid against pathogens (15, 23, 24). Results of this study also coincide with the findings of Hsiao and Siebert (11) and Buchanan and Edelson (5) that malic is a weaker acid than acetic in terms of antimicrobial action.

Conclusion. Acetic acid was a better acidifier than malic acid in terms of its abilities to inhibit microbial growth. In an 80:20 apple:carrot juice blend with acetic acid as the acidifier, juice was stable at pH values of 3.3, 3.5 and 3.7 stored at 30ºC for 60 days (equivalent to 120 days at ambient temperature) when hot filled into 8 oz. PET at 63, 71 or 77ºC; juice acidified with acetic acid to 3.3 was stable when filled into 10 oz. glass. The results of this study were inconclusive regarding the validity of fill temperature, suggesting that container type has as much to do with product safety and stability as product ingredients. Discrepancies in shelf life study data for 10 oz glass containers, and for apple:carrot juice filled into PET and acidified with malic acid may be a result of rapid initial cooling as the hot juice hit room temperature containers, and/or initial microbial load from the juice blend. The cooling studies suggest that at minimum, product should receive complete pasteurization prior to fill for refrigerated products. For shelf stable products, product should be pasteurized prior to fill, containers must be maintained pathogen-free by strict adherence to Good Manufacturing Practices as stated in 21CFR110 and the product should contain preservatives to control any spoilage microorganisms coming in on the product or the containers.

Moreover, it seems likely that the fresh juice had such a large microbial load that it overwhelmed the system, even when filled at 77ºC. Future efforts to repeat this study should use better quality apples. Retorted or aseptically packaged carrot juice could be used to minimize the microbial load contributed by carrot juice. However, aseptic or retorted carrot juice is not usually used in practice by the small-scale processors working with the New York State Food Venture Center. Even if small-scale processors could get these juices, the juices may meet a great deal of resistance from processors who are aiming for a fresher, less "processed" product. Research into fill temperatures, acidification and container type which aim to help small-scale processors may be better advised to sanitize fresh produce prior to

juicing rather than using aseptic or retorted juices in order to better reflect the system as it is in the small-scale processing environment.

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CHAPTER 4

CONCLUSIONS AND FURTHER WORK

The work in this thesis was done to begin to address questions regarding process parameters that we have received from clients of the New York State Food Venture Center (NYSFVC). Clients need scheduled processes for products ranging from acidified sauces, salsas, and pickles to dessert sauces like hot fudge sauce to meet state and/or federal regulation. Increasingly, process authority approval is given against a backdrop of regulatory scrutiny, and must take increased regulatory requirements into account. The work in this thesis seeks to begin addressing production scenarios that either have large amounts of anecdotal support (pickled egg production) or have great client interest (studying the appropriateness of various fill temperatures and containers). This research will provide published references that the U.S Food and Drug Administration (FDA) will accept as support for processing procedures that produce safe food.

Novel methods for evaluating lid contamination were developed for the Pickled Egg Challenge Study. To our knowledge, lid inoculation is not common practice, nor have we seen potential methods for lid inoculation, data demonstrating results of challenging lids, or even acknowledgement that the lid of a container should be part of a challenge study. When reviewing FDA form 2541a, which processors must fill out to register a shelf stable, acidified or low acid product with the FDA, FDA officials have been increasingly interested in how the headspace and underside of a product's lid are treated, and the officials are requiring evidence that a product process addresses headspace and under side of lid. The concern is based on a scenario in which contamination from an untreated lid or headspace can contaminate the product once it is packed. By extending the pickled egg challenge study to the lids as well as the eggs, we have demonstrated that the processes we suggest are adequate to eliminate

pathogens on product lids with minimal contact between lid and brine. Notably, the lid/brine contact occurred with initial brine at full strength (2.5% acetic acid or 5% acetic acid) rather than after egg equilibration. If processors rely on product jostling during shipping for lid treatment, it is possible that the brine will have a much lower strength. In this case, challenge studies would need to reflect lid/brine contact only after equilibration when brine strength is lowest.

Determining a method for lid inoculation was a challenge since lids have a small surface area and cannot be sampled as product can by removing sample aseptically from the jar. We decided to designate spots for inoculation and sample one spot at each sampling point, returning the lid to the jar after sampling. We could not inoculate the entire lid because efforts to sample the lid would have potentially contaminated the entire inoculated site. There was a risk of contamination of additional spots during sampling since the surface area was so small, but the risk was much less than if the entire lid had been inoculated. The only other alternative would have been to make a separate sample jar for each sampling point, seven sample jars for each replicate. This would have been time and cost prohibitive, requiring much more product as well as jars and personnel time.

For the study of acidification and stability, we wanted to address questions from clients of the NYSFVC regarding the acid used as an acidifier, fill temperatures at various pH values, and allowable container types. While the study confirmed existing research designating acetic acid as having antimicrobial properties, conversations with small processors have suggested studying fumaric and phosphoric acids as well. Both of these acids are used frequently in the small-scale, rather than cottage scale, processing industry. The efficacy of fumaric and phosphoric acids against acetic acid is interesting to contemplate, and would be useful to study. Neither fumaric nor phosphoric acid are generally contemplated by NYSFVC clients as an option for reasons including access to these acids and having to state

their inclusion in the ingredient list on a product label. However, research into these two acids would benefit larger food businesses.

Of direct interest for the cottage-scale food processing businesses dealt with most often by the NYSFVC, is the conundrum of acidified hot sauce. The pH values of these products range from 3.3 to 4.2, yet processors want to be able to fill them into the five ounce glass woozy bottle with plastic top typical of other hot sauce products. This combination of bottle and cap does not provide a hermetic seal as required by 21CFR114. Cooling profiles of the glass, analysis of use of various acidifiers and various pHs, and analysis of a variety of fill temperatures would be useful. The acidification and stability study discussed in this thesis has applicable parameters, however study of a glass bottle with plastic lid, as well as pHs between 3.7 and 4.2 would be useful. This study would entail both challenge studies and stability studies in order to satisfy requirements of FDA and provide published support for process authorities in general.

A much broader area of research is that of processing parameters for water activity controlled foods such as hot fudge sauce. We have relied on a correlation between established parameters for acidified foods to provide safe parameters for water activity controlled foods, but there is little literature regarding processing parameters for water activity controlled foods which was developed directly with these foods. Traditionally, there has been little concern about fudge sauce or caramel sauce, however recent outbreaks of *Salmonella* in peanut butter begin to challenge long held assumptions and make study of water activity controlled foods timely and important.