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Scheff, Deanna S. ***Survival of Salmonella ser.*** **Typhimurium** ***in a Raspberry Beverage Preserved with Organic Acid Treatments***

Abstract

Small beverage processors need low-cost alternatives to pasteurization in order to comply with juice HACCP regulations in the United States. Organic acid-based treatments may be a low-cost and practical means to achieve beverage safety without the capital costs associated with pasteurization. The objective of this study was to assess the efficacy followed by the validation of an organic acid treatment for a 5-log reduction of *Salmonella enterica* subs. *enterica* ser. Typhimurium after challenging an acidified raspberry beverage with the pathogen. The raspberry beverage (25% juice; acidified with citric acid pH = 2.83 ± 0.03) was collected during production from a beverage processor in Wisconsin. Preservative treatments to the beverage included 1) control with no added preservatives; 2) 11 ppm free sulfite and 0.03% (w/v) potassium sorbate; 3) 0.03% (w/v) potassium sorbate; 4) 11 ppm free sulfite; 5) 0.1% (w/v) sodium benzoate; 6) 0.05% (w/v) sodium benzoate and 0.03% (w/v) potassium sorbate. The samples were challenged with a cocktail mixture of *Salmonella* ser. Typhimurium at a level of 7.5 log CFU/mL and were stored at 4 and 22°C and sampled during storage. Populations of *Salmonella* ser. Typhimurium declined during storage in all treatments at both storage temperatures. Presence of the preservative treatments and increased storage temperature had a highly significant effect on increased rates of reduction, also seen in the control. The raspberry beverage containing benzoate and benzoate plus sorbate, significantly reduced populations of *Salmonella* ser. Typhimurium at the fastest rates in beverage stored at 4°C. This study provides evidence that organic acids may be used and validated as treatments to destroy populations of *Salmonella* ser. Typhimurium in beverages.

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Chapter I: Introduction

The United States Centers for Disease Control and Prevention (CDC) estimates that nearly "1 in 6 Americans (48 million) get sick, 128,000 are hospitalized and 3,000 die of foodborne diseases" each year (Centers for Disease Control, 2012a, para. 1). Salmonellosis contributes to 11% of domestically acquired foodborne illness yearly (1,027,561 cases) which is second only to the Norovirus (Centers for Disease Control, 2012a). It is also estimated that for every *Salmonella* case reported there are 29 cases that go undiagnosed. *Salmonella* is the leading foodborne pathogen contributing to foodborne illness requiring hospitalizations, with 35% of diagnosed cases resulting in 19,336 hospitalizations each year in the United States. *Salmonella* is also the leading foodborne pathogen causing deaths resulting from foodborne illness, with 28% of cases (378 deaths yearly) being linked to *Salmonella* as the contributing factor (Centers for Disease Control, 2012a). Foodborne illness can lead to high mortality rates among the "at risk group" which includes infants, children, the elderly, pregnant women, and those suffering from immune disorders.

Between the years of 1995-2005, the CDC reported 21-juice associated outbreaks in the United States. Unpasteurized orange juice was implicated in a multistate outbreak of Salmonellosis in a Florida theme park in 1995. There were 62 confirmed cases of *Salmonella enterica* subs. *enterica* ser. Hartford (*Salmonella* ser. Hartford) and one case of *Salmonella enterica* ser. *enterica* Gaminara (*Salmonella* ser. Gaminara) with a median patient age of 10 years. In 2005, unpasteurized orange juice was associated with another multistate outbreak. There were 152 confirmed cases of *Salmonella enterica* subs. *enterica* ser. Typhimurium (*Salmonella* ser. Typhimurium) and 5 cases of *Salmonella enterica* subs. *enterica* ser. Saintpaul

(*Salmonella* ser. Saintpaul). Both outbreaks were associated with the lack of proper sanitary measures employed by the juice manufacturers.

Current methods employed by the food industry to pasteurize food products include both thermally processed methods as well as non-thermal methods. Roughly, 98% of juice processors across the United States pasteurize their juice products (Vojdani, Beuchat, & Tauze, 2008). This includes high-temperature processing, high-pressure processing, pasteurization, high temperature/short time (HTST), and aseptic processing and packaging. In order to employ these methods high volumes and high capital costs are required. Large beverage manufactures have the capacity for thermally processing their product whereas for very small beverage processors and entrepreneurs this in unattainable due to low production volumes and limited financial resources. These producers either have co-packers to produce their products for them and/or typically use organic acids as a preservative which can be used as an alternative to thermal processing to control the quality and safety of their products.

The most common preservatives employed in beverages are potassium sorbate (sorbate), sodium benzoate (benzoate), and potassium metabisulfite (sulfite). The FDA allows for the use of each of these preservative under the Code of Federal Regulations (CFR) use of Generally Recognized as Safe (GRAS) food additives. However, there have been numerous studies demonstrating sulfite eliciting adverse reactions in asthmatic persons. Sulfite-induced symptoms range from dermatitis, abdominal pain, and flushing of the skin (Vallay & Misso, 2012) and asthmatic symptoms. In some cases, asthmatic symptoms can lead to anaphylactic shock or death in rare case. It is generally accepted that "between 3-10% of adult asthmatics may exhibit adverse reactions to sulphite additives" (Vallay & Misso, 2012, p. 20). Chemical preservatives can be used as an alternative, but limits to the amounts allowed in food products are strictly set.

Benzoate and sorbate, for example, cannot exceed levels of 0.1% in beverages, and must be declared in the ingredient list along with an intended use statement. The use of sulfite is allowed, but concentrations of >10 ppm must be declared on the label along with a warning statement (Vallay & Misso, 2012).

Food trends have shown that there has been an increasing focus and demand for food products without synthetic chemical preservatives and fresher and minimally processed foods (Zinc, 1997). The same trends seen in the United States can be seen throughout the world as well. Between 2004-2005, there was a 28% growth rate of products entering the European marketplace that had labels containing "without preservatives or additives" or "all natural" (El Amin, 2005). Distributors, retailers, and consumers are beginning to demand clean labels with "no added sulfite," "preservative free," or "all natural" claims. "Consumer's aversion to traditional chemical preservatives has left food processors with less flexibility in choosing preservation methods" (Zinc, 1997, p. 467). Sulfites are a proven and effective preservative; they also have health consequences associated with them for a select group of consumers as discussed previously. Beverage manufactures have been forced to find alternatives to sulfite use in beverages. Small manufacturers are currently seeking preservatives to replace sulfites in their products and sorbate and benzoate have been proposed as feasible sulfite replacements with GRAS standing.

Statement of the Problem

In response to numerous outbreaks associated with fruit based beverages, the United States Food and Drug Administration (FDA) require beverage processors to implement HACCP plans in their operations. Pasteurization requires a capital investments and small beverage processors need a low-cost alternative to pasteurization in order to comply with HACCP

regulation put forth from the FDA. In this context, organic acid treatments to juice beverages may be a low-cost and practical alternative to enhance the safety of beverages, while complying with federal regulations. Validated organic acid treatments (authenticated through challenge study methodologies) may be adopted into the processors existing HACCP plan as a critical control point (CCP) to achieve beverage safety.

Purpose of the Study

The objective of this study was to validate an organic acid treatment for an acidified raspberry beverage challenged with 2-strain cocktail of *Salmonella* ser. Typhimurium. The organic acid treatments were compared against potassium metabisulfite treatment, with the intent to also identify sulfite alternatives to enhance the safety and quality of the product.

Assumptions of the Study

An assumption of this study was the consistency of the juice beverages produced yearly at the processing facility of a commercial winery, with respect to consistency in amount of juice added (fresh or from concentrate), sugar added (measured as specific gravity or °Brix), citric acid added (measured as % titratable acidity as tartaric acid) and pH consistency. Sampling of the raspberry beverage occurred during the summer of 2012, but production occurs yearly through the facility on an as needed basis. It is assumed that all production standards are held consistent between batches.

Definition of Terms

Acid resistance. "An exposure of cells for an extended period to an acidic environment (pH 5.0-5.8), enabling them to develop resistance to subsequent exposure to pH≤ 2.5" (Ray & Bhunia, 2008, p. 84).

Acid tolerance response. "A brief exposure of cells to mild acidic environments enabling them to survive subsequent exposure to pH 2.4-4.0" (Ray & Bhunia, 2008, p. 84).

Antimicrobial agent. "Preservatives that prevent the growth of microbes in food" (Ward, 2002, p. 360).

Cross contamination. "The spread [of a bacteria] from a contaminated source – a contaminated food, infected food handler or animal – to other foods or objects in the environment" (Hammack, 2012, p. 15).

Decimal reduction time (D-value). "The time…during which the number of a specific microbial population exposed to a specific temperature is reduced by 90% or 1-log" (Ray & Bhunia, 2008, p. 374).

Differential agar. Differential agar is used to "distinguish one microorganism from one another growing on the same media by their growth characteristics" (Parija, 2009, p. 37).

Enzymatic browning. "The reaction of polyphenol oxidase with oxygen resulting in the production of melanins, brown pigments" (Ward, 2002, p. 277).

Organic acids. An organic acid is an organic compound that exhibits acidic properties. Generally, they are weak acids that do not completely dissociate in water but are soluble in organic solvents and are naturally found in fruits, vegetables, and fermented foods.

Pathogen. "An organism with a demonstrated capacity to cause disease" (Jay, Loessner, & Golden, 2005, p. 536). Pathogens can be bacteria, viruses, or parasites (Centers for Disease Control, 2011).

Pertinent microorganism. "The most resistant microorganism of public health significance that is likely to occur in the juice [beverage]" (Food and Drug Administration, 2012, Title 21 C.F.R. Chapter I Subchapter B Part:120.24a).

Selective agar. "Media that contain substances which inhibit the growth of all but a few bacteria but at the same time facilitate isolation of certain bacteria" (Parija, 2009, p. 27).

Limitations of the Study

A limitation of the experimental procedure was the length between enumeration sampling of *Salmonella* ser. Typhimurium. The death rate of the *Salmonella* ser. Typhimurium occurred rapidly, and closer sampling times may have benefitted the study. A second limitation to the study was source of juice. This research project used juice concentrate as the source of juice in the sample and preparation procedure. Due to lack of fresh berries, a concentrate was selected to minimize variability in growing, harvesting, and storage conditions of the fruit.

Methodology

A raspberry beverage (25% juice) was collected from a small beverage processor in northern Wisconsin. The raspberry samples consisted of water, raspberry concentrate, sugar, and citric acid. The beverage samples were challenged with a two-strain cocktail of *S.* Typhimurium (ATCC 10428, 13311) at a level of approx. 7.5-log cfu/mL followed by enumeration by standard microbiological plating and enrichment methodology. Preservative treatments to beverages included:

1. Control with no added preservatives;
2. 11 ppm free sulfite and 0.03% (w/v) potassium sorbate;
3. 0.03%(w/v) potassium sorbate;
4. 11 ppm free sulfite;
5. 0.1%(w/v) sodium benzoate;
6. 0.05% (w/v) sodium benzoate and 0.03% (w/v) potassium sorbate.

Samples were stored at two treatment temperatures: 4°C and 22°C and sampled on days 0, 1, 2, 3, 5, and 9 following inoculation.

Samples were plated using serial dilutions in buffered peptone water and plated on Xylose Lysine Deoxycholate (XLD) agar. Samples were incubated at 37°C for 24 hours. Following days of growth below detection limit (<10 cfu/mL) on XLD agar, samples were enriched following the United States Food and Drug Administration's Bacteriological Analytical (BAM) Manual.

Chapter II: Literature Review

This chapter begins with an overview of the foodborne illness, salmonellosis. An introduction to the fruit beverage that was assessed for food safety in this study is also discussed. A discussion of microbial safety of fruit beverages and microbial survival mechanism will follow. The chapter will conclude with a discussion on non-thermal methods of destruction of *Salmonella*.

Salmonellosis

"Foodborne infection occurs from the consumption of food or water, contaminated with pathogenic enteric bacteria and viruses" (Ray & Bhunia, 2008, p. 283). It is estimated that between 10-10^5 viable cells are needed to contract an infection, depending on the pathogen. An infectious does for Shigella *spp.* can be as little as 10 organisms, but for *Salmonella* it is estimated at least >10^5 organisms are needed (Kothary & Babu, 2007). The pathogenic cells from the contaminated food move through the gastric acid barrier in the stomach, on to the intestinal tract of the individual. From this point, they are able to reproduce and excrete toxin(s) in the body. General symptoms include nausea, diarrhea, and abdominal pain. Most individuals affected by foodborne infection recover within a week by increasing fluid intake. Hospitalization occurs if infection spreads past the intestinal tract (Ray & Bhunia, 2008).

According to the CDC, "when two or more people get the same illness from the same contaminated food or drink, the event is called a foodborne outbreak. Illnesses that are not part of outbreaks are called 'sporadic'" (Centers for Disease Control, 2011, para. 1). Government and public health officials investigate outbreaks to locate the source as a way to control the outbreak and stop the spread of the disease. In the United States, salmonellosis contributes to 11% of domestically acquired foodborne illnesses yearly, which is second to the Norovirus,

which is attributed to 58% of all foodborne illnesses occurring yearly (Centers for Disease Control, 2012a). *Salmonella* is also the leading pathogen contributing to hospitalizations and deaths each year, 35% and 28% respectively. In 2012 alone, *Salmonella* outbreaks were linked to ground beef, chicken, mangoes, dog food, and many other food products. Food safety related outbreak pose both a public health concern as well as a business concern for food manufacturers and distributors. A report published by the United States Department of Agriculture: Economics Research Services (USDA-ERS) in 1996 estimates that cost of human illness due to foodborne bacteria was $2.9-6.7 billion (United States Department of Agriculture Economic Research Service, 1996). This cost can be attributed to items such as loss of production at work, loss of wages, hospitalization costs, loss of manufactured food products, and lawsuit costs.

Pathogenesis of *Salmonella*. *Salmonella* is a motile, rod-shaped, gram-negative bacterium. The *Salmonella* genus is divided into two species that are able to cause disease in humans: *Salmonella enterica* and *Salmonella bongori*. In this thesis, a serotype of the subspecies *Salmonella enterica* subs. *enterica* was assessed for food safety in a raspberry beverage system. The family *Salmonella enterica* is further divided into six subspecies. These species consist of (Hammack, 2012):

1. *S. enterica* subsp. *enterica*
2. *S. enterica* subsp. *salamae*
3. *S. enterica* subsp. *arizonae*
4. *S. enterica* subsp. *diarizonae*
5. *S. enterica* subsp. *houtenae*
6. *S. enterica* subsp. *indica*

Each subspecies can be further divided into serotypes based on their antigenic and flagellar properties. As of 2007, there were 2,579 serotypes classified by the CDC (Hammack, 2012, p. 12). Each serotype has a unique name as well as a common name. For example, *Salmonella enterica* subsp. *enterica* ser. Typhimurium is commonly referred to as *Salmonella* ser. Typhimurium or *S.* Typhimurium *(*Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000).

Salmonellosis is an infection stemming from the consumption of food contaminated with the bacterium *Salmonella* (Centers for Disease Control, 2012d). *Salmonella* is a natural inhabitant of the gastrointestinal tracts of animals as well as the intestinal tract of humans (Ray & Bhunia, 2008). *Salmonella* can also be found naturally in the environment such as in the soil, water, animal feces, insects, and on kitchen/factory surfaces. Salmonellosis is transferred by the fecal-oral route. It may be also transferred from person-to-person, but is less rare. Salmonellosis can present itself as one of four manifestations: (a) nontyphoidal; (b) true typhoid fever; (c) *Salmonella* gastroenteritis; (d) asymptomatic carriage. Each syndrome is accompanied by typical *Salmonella* strains (Hui, Gorham, Murrell, & Cliver, 1994). In order to be pathogenic *Salmonella* strains must possess specific virulence factors such as (a) ability to invade cells; (b) a complete lipopolysaccharide coat; (c) the ability to replicate intracellularly; and (d) possibly the development of toxin(s) (Giannella, 1996). The severity of illness has a direct correlation to dosage, serotype, age, and health status of the infected individual. Salmonellosis affects all genders and ages. Those most at risk are the young, the elderly, and those suffering with a weakened immune system.

Upon consumption of contaminated food sources, the pathogen begins to colonize in the large and small intestine. The pathogen adheres to the mucosal membrane in the intestines and invades the mucosal cells. From this point, the pathogen begins to multiply intracellularly in the

epithelial cells. The pathogen can begin to spread throughout the body via the systemic circulation. The areas targeted in the body depend on the strain of *Salmonella* and the status of the human body's defense system. Typically, the pathogen does not leave the GI tract. The reproduction of the pathogen in the epithelial cells eventually leads the lysis of the cells, and inflammation and severe edema occurs at the site of infection. The damage to surrounding tissues triggers the release of prostaglandins and thereby increasing the cyclic adenosine monophosphate (cAMP) levels in the mucosal cells. This interacts with the electrolyte balance of the mucosal cells. The rise of cAMP levels prevents uptake of sodium (Na^+) ions and releases chloride (Cl^-) ions into the intestinal tract. Since electrolyte balance is essential for fluid flow, this initiates fluid loss in the intestinal tract resulting in diarrhea (Ray & Bhunia, 2008). The schematic of the pathogenesis of salmonellosis is diagramed in Figure 1 below.

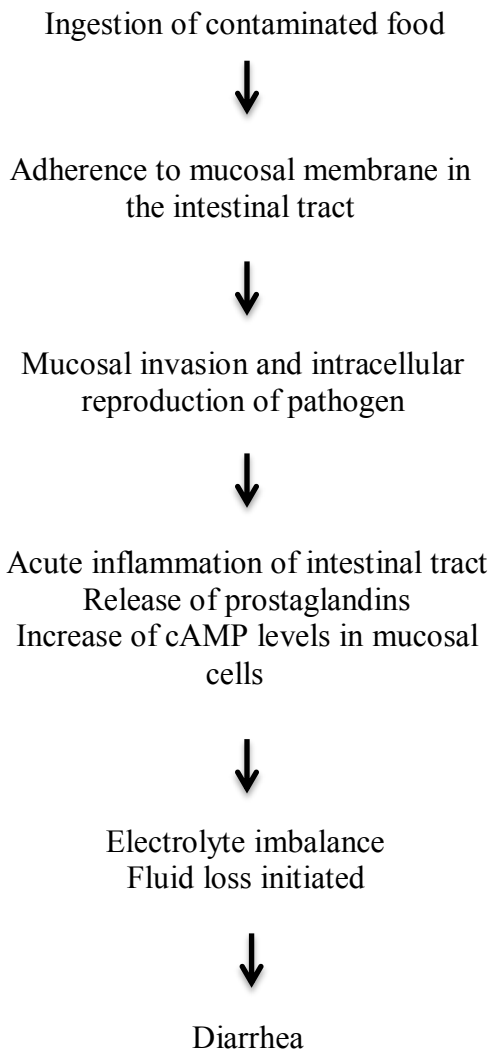

Figure 1. Pathogenesis of salmonellosis

It should be noted that each case of salmonellosis varies, and not all infected individuals display identical symptoms.

Food sources of *Salmonella.* *Salmonella* is ubiquitous in nature. It can be found in environmental sources or intestinal tracts of animals and humans. *Salmonella enterica* subs. *enterica* ser. Enteritidis can be found inside of eggshells, from the infected ovaries of chickens. *Salmonella enterica* subs. *enterica* ser. Typhi and *Salmonella enterica* subs. *enterica* ser.

Paratyphi are found only in human hosts and are spread by person-to-person contact. The various other strains are found outside on the food (or water) source. Out of thousands of *Salmonella* strains, *S.* Typhimurium has been linked with 20% of the total amount of cases of salmonellosis being reported.

Foods of animal origin have long been associated with salmonellosis. Between 1973 and 1987, there were 790 outbreaks associated with salmonellosis in the United States. The top food categories were beef (77 outbreaks), dairy products (50), turkey (36), chicken (30), and pork (50) (Ray & Bhunia, 2008). These food sources were contaminated either directly or indirectly with fecal matter from carrier animals. Recently, fresh produce such as spring greens, cantaloupes, cucumbers, and mangoes have been a source of outbreaks by the *Salmonella* genus.

It should be noted that the main cause of an outbreak can be linked back to cross contamination. Cross contamination can occur at any point in the processing line, from the field cultivation to the final packaging of the food product. A recent example of a Salmonellosis outbreak associated with cross contamination due lack of Good Manufacturing Practices (GMPs) at the processing site was the 2012 peanut butter outbreak that affected 42 people.

Signs and symptoms of infection by *Salmonella*. "Human salmonellosis is different than the nontyphoidal salmonellosis cause by serotypes *Salmonella* ser. Typhi and *Salmonella* ser. Paratyphi" (Ray & Bhunia, 2008, p. 286). Human salmonellosis infections generally develop within 6-72 hours after infection. An infectious dose consists of $>10^5$ cells. The dose needed to cause an infection is related to the age, health of individual, and strain of *Salmonella* (Hammack, 2012). Common symptoms can range from adnominal cramps, nausea, fever, vomiting, and diarrhea, but not all individuals have the same symptoms or severity of symptoms.

Most infected persons recover within 4-7 days with the first 1-2 days presenting the most severe symptoms.

Most salmonellosis infections are brushed off as the 24-hour stomach flu and no further action is taken. Generally, those who become infected and seek medical attention are treated without the need for hospitalization (Centers for Disease Control, 2012d). Complications lie in lack of electrolyte balance and dehydration due to diarrhea and vomiting. The patients may need intravenous fluids to revert dehydration and to restore the body's natural electrolyte balance. These symptoms can lead to death in young, elderly, and immunocompromised groups if not promptly treated. The need to administer antibiotics is not necessary for a *Salmonella* infection, except for rare occasions. Antibiotics would be required in situations in which the *Salmonella* infection spreads outside of the intestines. In this case, severe consequences can occur such as blood poisoning (septicemia) or infection of internal organs and/or joints. Strong antibiotics such as ampicillin or ciprofloxacin would be administered (Centers for Disease Control, 2012d). An additional symptom that occurs in 2% of cases is the incidence of reactive arthritis, or arthritis from immune reaction to the infection. This may present itself in 3-4 weeks after initial infection. Reactive arthritis symptoms include joint inflammation, urethritis, uveitis, or conjunctivitis (Hammack, 2012).

Diagnosis and treatment for Salmonellosis. Salmonellosis cases can only be diagnosed based on laboratory testing. Stool samples from the infected persons are analyzed for the presence of *Salmonella* by culturing the stool samples using gram-negative enrichment cultivation. Once a positive identification is made, further testing can be used to determine genera and species type to find the "fingerprint" of the strain causing the foodborne pathogen.

From this point, other strains of salmonellosis can be analyzed to determine if there is a link between diagnoses from two (or more) cases.

A multiplex polymerase chain reaction, (PCR) can also be employed in the clinical setting to determine the presence of *Salmonella*. PCR methodology is a more sensitive, efficient, and faster method than stool cultivation alone. A study conducted by Chiu and Ou (1996) determined that the multiplex PCR method had a 95% efficient rating as compared to 60% efficiency rating of culture alone when analyzing stool samples of 57 children with confirmed cases of Salmonellosis. Rapid genetic identification is only possible for roughly 100 *Salmonella* subspecies and serovar strains from a pure culture. The remaining 2,400 plus strains needs to be identified by traditional methodology (Hammack, 2012). Nearly all of the *Salmonella* serovars contain *inv* genes, which is what allows for the invasion of cells in the body. Only five serovars are known to contain specific virulence plasmids that contain the *spv* gene. These include the serovars Typhimurium, Choleraesuis, Dublin, Enteritidis, and Gallinarum-Pullorum (Chiu & Ou, 1996). Since these serovars are most commonly isolated strains from positive stool samples, rapid methods of identification have been created. The remaining 2,000 plus stains are typically cultured in the traditional fashion. Chiu and Ou (1996) reported accurate results from a rapid test method based on PCR methodology with amplifiers for both the *inv* and *spv* genes of *Salmonella* serovars.

Conventional isolation and testing methods for commercial sampling from possible contamination points, includes a pre-enrichment in nutrient broth, followed by selective enrichment in broths, and then streaking on selective-differential agar media. This method can be found in the Food and Drug Administration (FDA) Bacterial Analytical Manual (BAM). The BAM contains specific methods of enrichment of environmental sources or foods suspected of

contamination. Conventional methods require 4-6 days for results. Recently, rapid test methods have been made available to reduce the turnaround time. Rapid methods require 1-2 days. They are meant as a screening tool and positive results are to be followed up by conventional methods.

Recent outbreaks associated with *Salmonella* in food products. During the summer of 2012, (June-September) 42 people in the United States became infected, and 10 were hospitalized, with an outbreak strain of *Salmonella enterica* subs. *enterica* ser. Bredeney (*Salmonella* ser. Bredeney) from the consumption of Trader Joe's Valencia Peanut Butter manufactured by Sunland, Inc. The outbreak covered 20 different states in the United States. Ages of infection ranged from <1 year-79 years of age with a median age of 7 years. A FDA document showed that since 2009 Sunland, Inc. knowingly had distributed lots of peanut butter that tested positive for *Salmonella* (Centers for Disease Control, 2012b).

In a recent inspection (2012), the FDA showed five finished products that tested positive for *Salmonella* yet the manufacturer's in house testing returned negative for the presence of *Salmonella* in these same finished products. Additional 28 environmental locations also tested positive for *Salmonella*. The FDA cited the company for failure to clean the production and packaging lines each time a positive sample returned. There were also no records that these areas were ever cleaned between the 2009 and 2012 audits. There were also multiple counts of environmental infractions assessed on the company. Investigation by the FDA showed that Sunland, Inc. had allowed open trailers of in-shelled peanuts to be stored at their facility where contamination from the environment, birds, insects, and mammals was likely to occur. The company was also cited for not closing off processing rooms, allowing access by animals, rodents, and insects. FDA inspection noted signs of bird excrement in the facility. On November 26th 2012, the FDA suspended Sunland, Inc.'s food facility. This prohibited the

company from distributing interstate or intrastate commerce due to the faulty sanitation and food protection program (Centers for Disease Control, 2012b).

The year 2012 also saw a multistate outbreak of two strains of *Salmonella* linked to cantaloupes. These two strains included *Salmonella* ser. Typhimurium and *Salmonella enterica* subs. *enterica* ser. Newport, both of which were epidemiologically linked to the same outbreak. The outbreak occurred throughout July and September and included 24 different states, mainly in the eastern part of the United States. There were 261 individuals affected (228 from *Salmonella* ser. Typhimurium and 33 from *Salmonella ser.* Newport), with 94 individuals hospitalized and 3 confirmed deaths. The age of infected individuals ranged from 1 to 100 years old with a median age of 47 years. Through epidemiological, laboratory testing, and trace back investigations, the FDA found the source of the outbreak to Chamberlain Farms Produce, Inc. of Owensville, Indiana (Centers for Disease Control, 2012c).

FDA inspections at the growing fields and packing house showed several food safety infractions. Environmental samples from the growing fields showed 16 samples positive for *Salmonella* and 4 of which contained the outbreak strain. Swabs taken from the packinghouse also tested positive for the outbreak strains of *Salmonella*. During the inspection period, the FDA inspector noted several unsanitary procedures including; accumulation of organic material on food contact surfaces, standing water containing algae growth, bird excrement in rafters above food contact surfaces, and buildup of debris under conveyer belt. These unsanitary conditions may have been the causative factor for the contamination of cantaloupes with the pathogen. Following the inspection results, Chamberlin Farms Produce, Inc. voluntarily initiated a recall of their product from retail distribution. The company also removed watermelons from the marketplace in September after inspections showed the presence of *Salmonella*. Following

the outbreaks, the FDA issued a warning letter to the company which required corrective actions, and if not implemented would have resulted in severe consequences for the company including suspension of distribution.

Prevention and control of Salmonellosis. The Centers for Disease Control and Prevention recommends several steps for the prevention of salmonellosis (Centers for Disease Control, 2010). These included the following advisory to consumers:

1. Do not consume unpasteurized milk or other dairy products.
2. Cook poultry, ground beef, and eggs thoroughly.
3. Wash hands, work surfaces, and utensils with soap and water immediately after they have been in contact with raw meat or poultry.
4. Keep raw foods away from cooked foods.
5. Avoid direct or indirect contact between reptiles and infants or other immunocompromised persons.
6. Those with salmonellosis should not prepare food or pour water for others until their diarrhea has been resolved.
7. Wash hands after contact with animal feces or after using the bathroom.
8. Chill foods promptly after serving (within 2 hours).

Following these simple steps will significantly reduce an individual's chance of becoming ill with salmonellosis. The same precautionary steps provided for individuals should also be applied in the manufacturing and distribution processes of food products. Food manufactures are required to follow a Hazard Analysis Critical Control Point (HACCP) plan of action, which also includes Good Manufacturing Practices (GMP's). These are regulations put forth by the government to ensure safe preparation, processing, packaging, and transportation of food

products before consumption by the consumer. HACCP and GMP plans are designed to prevent any potential contamination of food product by pathogens of concern. Whether food is being prepared in a commercial facility or in a home kitchen, following these recommendations will reduce the likely hood of contracting a foodborne illness.

Fruit Beverages

Production of spritz beverage. A spritz is a carbonated juice beverage that may or may not be alcohol based. Typically, spritz beverages are manufactured by small beverage processors that take advantage of local fresh-grown berries. If seasonal fruit yields are low, a concentrated fruit stock may be used such as raspberry or blueberry concentrate, as an alternative source of the fresh fruit. If using fresh fruit, the fresh fruit is pressed and the juice is either used the same day of pressing or frozen until needed for production. The general formula for a fruit beverage formulation to make spritz is as follows:

- Water
- Fresh Fruit (or concentrate)
- Sugar
- Citric Acid
- Potassium Sorbate
- Sodium Metabisulfite
- Pectinase
- Carbon Dioxide

The production of the beverage is accomplished by mixing the desired fruit with water, followed by the addition of sugar (a sweetener). This is followed by addition of citric acid, potassium sorbate, sodium metabisulfite, and pectinase. Citric acid is added to preserve the freshness and

color of the juice product. It also gives a characteristic mouth feel to the beverage. Potassium sorbate and sodium metabisulfite are added to prevent enzymatic browning during processing and storage. They are also added as antimicrobial and antifungal agents to increase the safety of the beverage. Pectinase is added to help break down cell walls and increase the volume of juice extracted. Pectinase is added to each batch regardless of whether fresh juice or concentrate is used in production. In addition, pectinase also aids in lowering the viscosity of the juice thus keeping it from becoming gummy and thick. Pectinase also reduces the cloudiness of the juice, making it significantly more eye appealing to consumers. Following the addition of all ingredients, producers perform quality control checks on the product to ensure all processing standards are met. Quality control tests included specific gravity, pH, % TA (measured as tartaric acid), and SO_2 levels in the beverage. Once the beverage meets the quality standards, the product is filtered and stored in carbonating tanks under refrigeration for approximately two days before bottling.

After storage in a refrigerator, samples are ready for bottling. Samples are bottled using a closed system to prevent any cross-contamination. The fruit beverage is bottled in 12oz. glass bottles topped with a metal twist cap. All bottles are rinsed with a sanitation wash for approximately one minute before filling. The fruit beverage is passed through a 0.45µm filter, into a bulk-carbonating tank before filling a sanitized bottle. The bottles are then placed in the refrigerator for long-term storage and selling in the marketplace.

Microbiological Safety of Fruit Beverages Against *Salmonella*

"Outbreaks of illnesses associated with consumption of fruit juice have been a growing public health problem since the early 1990s" (Vojdani et al., 2008, p. 356). The FDA has responded to these growing health concerns requiring beverage processors to implement a

science based hazard management control system such as HACCP, whereby the potential hazards are identified, and critical control points (CCPs) are integrated within the processing systems as effective interventions to maintain safety.

Outbreaks of *Salmonella* in fresh juice. Between the years of 1995-2005, the CDC reported 21-juice associated outbreaks. Ten of these cases implicated apple cider, 8 implicated orange juice, and 3 were listed as "other fruit juice." *Salmonella* accounted for five outbreaks, resulting in 710 (52%) illnesses, and 94 hospitalizations (63%) (Vojdani et al., 2008).

In 1995, unpasteurized orange juice caused and outbreak of salmonellosis in a theme park in Orlando, Florida. There were 62 confirmed cases of *Salmonella* ser. Hartford infections and one case of *Salmonella* ser. Gaminara. It was estimated however, that only 1-5% of actual cases were reported and that the actual number of cases was estimated to be between 1240-6200 (Cook et al., 1998). Those infected with salmonellosis were from 21 different states and visited the theme parks between May and June of 1995. The range of individuals infected was between 1-63 years old and a median age of 10 years. Through traceback from state and government officials, it was determined that the source of the outbreak came from unpasteurized orange juice that was sold/served at the theme park. Twenty-nine of the cases could be tied to a "character breakfast" as the source of the outbreak. The theme park received shipments of orange juice from a local processor in Florida. Shipments occurred every other day and juice was used within 2-3 days of delivery (Cook et al., 1998).

Onsite inspection of the orange juice processor indicated several deficiencies. One deficiency noted was the lack of closure between the outside environment and the processing room. There were cracks in the walls and ceilings, which allowed for frogs access to the processing room and frogs were present around processing equipment during inspections. Also

noted was the presence of bird excrement in the same room. The inspection also documented the lack of proper sanitary cleanup of equipment, evident by the buildup of precipitate on the inside of the juice-pressing machine. Inspections of opened and unopened containers of orange juice were also examined. "All juice samples tested contained *coli*forms and *Escherichia coli (E. coli)*" (Cook et al., 1998, p. 1506). *Salmonella* was isolated in 10 out of 12 juice containers. Though there were a number of positive samples for *Salmonella* and other *coli*forms, the original reservoir source of the *Salmonella* outbreak was unknown. Two plausible explanations were purposed by Cook et al. The first being the introduction of *Salmonella* into the facility by amphibians, such as the frogs present in the processing room. The facility lacked closure from the outside environment and thus increased the likely hood of an environmental contamination from an outside source. The second likely scenario was harvesting methodology of the growers. Oranges are "frequently harvested after being dropped to the ground" and thus the exterior poses the possibility of environmental contamination through animal feces, contaminated ground/surface water, or manure type fertilizer (Cook et al., 1998, p. 1507). The lack of sanitary measures can result in cross-contamination of the subsequent processing equipment and food products.

Following these investigations, the theme park decided to serve only pasteurized orange juice throughout its facilities. In addition, the Florida Department of Citrus sanctioned new regulations for production of unpasteurized orange juice. The Florida Department of Citrus banned the use of oranges picked from the ground for use in producing fresh orange juice. They also required implementing the washing of fruit with an acid (or equivalent) wash, rinsing of fruit with a hypochlorite (or equivalent bactericide), complete closure of the processing room,

routine microbiological surveillance of juice, and required processors to implement a GMP and HACCP programs.

Another recent case (2005) involved a multistate outbreak of *Salmonella* ser. Typhimurium and *Salmonella* ser. Saintpaul in unpasteurized orange juice. Between May and July 2005, 152 cases of *Salmonella* ser. Typhimurium and five cases of *Salmonella* ser. Saintpaul that were reported. The outbreak occurred in 23 different states in the United States. Patients infected with the *Salmonella* ser. Typhimurium strain ranged between 6 months to 78 years, with a median age of 23 years. Patients infected with the *Salmonella* ser. Saintpaul stain ranged between 4-7 years with a median age of 5 years. Following investigations by state and federal agencies, the Orchid Island Juice Company was implicated as the source of the outbreak. Though there was not a definite source of contamination, it was theorized that the fruit used may have been contaminated in the orchard and the pathogen may have survived the washing and waxing operations. A second mode of contamination may be the internalization of the *Salmonella* bacteria in the flesh of damaged fruit, in which case external washing and waxing may have had no effect. In either case, the orange juice was not pasteurized or treated with any additive or method to reduce pathogens, and was labeled as "all natural fresh-squeezed" orange juice (Jain et al., 2009).

The FDA conducted a routine investigation of the processing facility to determine the source or cause of the contamination. The FDA noted that the juice processing company lacked compliance with juice HACCP regulations, which came in full effect in 2004. According to the juice HACCP regulations, juice processors are required to demonstrate a 5-log pathogen reduction of their product. However, the procedure used does not have to be a FDA certified method, the company must internally validate their process methodology. The juice company's

control testing on their orange juice product frequently tested positive for generic *E. coli*. These positive results indicate that the sanitization method employed by the company was inferior and corrective action needed to be undertaken to correct the problem. There was no evidence that the company had ever employed corrective actions. Another documented fault by the Orchid Island Juice Company was the lack of required sanitation monitoring records as well as conducting insufficient cleaning procedures for equipment used in processing (Jain et al., 2009).

Federal Food Safety Regulations

Hazard Analysis and Critical Control Point (HACCP) system. The regulatory requirement for juice processors to implement a juice HACCP plan, in 2001, by the United States FDA was a direct result of the number and severity of juice outbreaks associated with pathogenic bacteria including *Salmonella* and *E. coli* (Chikthimmah, LaBorde, & Beelman, 2003). HACCP was designed as a system that should "lead to the production of microbiologically safe foods by analyzing for the hazards of raw materials" (Jay, Loessner, & Golden, 2005, p. 497). The approach of the HACCP system emphasizes the quality of ingredients of a food product and ensures all processing methods are controlled, which results in a safe product. Before implementing a HACCP system, prerequisite programs need to be implemented successfully. These prerequisite programs include current good manufacturing practices for personnel, buildings and grounds, sanitary operations, equipment, and production and process controls. GMP's for personnel includes disease control, personnel cleanliness, wearing suitable outer garments, removing all unsecured jewelry and other objects, and education and training of employees. Building and facility GMP's included maintenance of roads and yards, waste treatment and disposal, grounds control, and adequately draining areas that may provide breeding places for pests. The FDA's GMP programs also includes guidelines

for sanitary operations for cleanliness of all components of a facility, use of safe cleaning substances, pest control, food and non-food contact surfaces, and storage and handling of cleaned equipment and utensils. Following successful implementation of GMP's a HACCP can be put in place. A HACCP plan consists of seven principles (Jay et al., 2005, p. 499):

1. Assess the hazards and risks associated with the growing, harvesting, raw materials, ingredients, processing, manufacturing, distribution, marketing, preparation, and consumption of the food in question.
2. Determine the Critical Control Points (CCPs) required to control the identified hazards.
3. Establish the critical limits that must be met at each identified CCP.
4. Establish a system to monitor each CCP.
5. Establish corrective actions to be taken when there is a deviation identified by monitoring a given CCP.
6. Establish procedures for verification that the HACCP system is working correctly.
7. Establish effective record-keeping systems that document the HACCP plan.

When the HACCP regulation for fresh juice beverages was released in 2001, it mandated the application of HACCP to manufacturers of fruit and vegetable juices. HACCP regulations went into effect on January 22, 2002 for most manufactures. Very small manufactures with total annual sales of <$500,000, had until January 20, 2004 to implement a HACCP program. As of 2013, HACCP regulations are in effect for all processors producing a fruit based beverage despite annual sales, number of employees, and amount of juice produced.

The United States Food and Drug Administration's Code of Federal Regulations HACCP system applies to "any juice sold as such or used as an ingredient in beverages shall be processed

in accordance with the requirements of [Title 21 C.F.R. Chapter 1 Subchapter B Part 120]" (Hazard Analysis Critical Control Point, 2012). Juice is defined as "the aqueous liquid expressed or extracted from one or more fruits or vegetables…or any concentrates of such liquid" (Hazard Analysis Critical Control Point, 2012). Therefore, any processor using juice as an ingredient or sold as a juice is required to have a HACCP system implemented.

In addition to a HACCP system, processors also need to conform to current Good Manufacturing Practices (GMP's) and Sanitation Standard Operating Procedures (SSOP's). GMP's involve the determination of whether the "facilities, methods, practices, and controls used to process juice are safe" (Hazard Analysis Critical Control Point, 2012). These include wearing gloves when handling food for human consumption, wearing hairnets, beard covers, or hats when applicable, and removing unsecured jewelry or other objects that may fall into food products during processing. Manufacturers are required to implement a SSOP that "addresses sanitation conditions and practices before, during, and after processing" (Hazard Analysis Critical Control Point, 2012). Types of SSOP's would include exclusion of pests from processing area, proper storage, and labeling of any toxic chemicals that are present in the facility, and maintenance of toilet and hand washing facilities.

Following successful implementation of GMP's and SSOP's, each juice processor "shall develop…a written hazard analysis to determine whether there are food hazards that are reasonably likely to occur" (Hazard Analysis Critical Control Point, 2012). A HACCP plan applies those seven principles as discussed earlier. A processor will create a plan that includes identifying food hazards and critical control points (points where food hazards can be introduced), creating process control measures to ensure safety of the juice product, and an action plan for when a critical control point fails or a hazard is identified. A failure to implement

a validate HACCP system "shall render the juice products of that processor adulterated" (Hazard Analysis Critical Control Point, 2012). Once a HACCP plan has been validated and introduced, the HACCP plan shall be re-validated at least once in a 12-month period. This is to ensure the processor is following their HACCP plan and the juice being produced is free of food safety hazards. As part of the HACCP system, juice processors are required to demonstrate a minimum 5-log (100,000-fold) reduction of the pertinent foodborne pathogen for a period at least as long as the shelf life of the juice product.

FDA food additive regulations. A food additive is defined by the Code of Federal Regulations as " all substances…intended use of which results or may reasonably be expected to results, directly or indirectly, either in their becoming a component of food or otherwise affecting the characteristics of food" if such substance GRAS listed (Food Additives, 2012). This includes any substance added to a food product at any point in the processing line from production, to packaging, to storage of a food product. Food additives can either be direct or indirect additives. A direct additive are those "that are added to a food for a specific purpose in that food" (Food Additives, 2012). Indirect additives are those "that become part of the food in trace amounts due to its packaging, storage, or other handling" (Food Additives, 2012). The FDA has stringent guidelines as to amounts and types of food additives allowed in a food product. There are two groups of food additives allowed in food products: Group 1: GRAS (generally recognized as safe) substances and Group 2: Food additives with prior approval (before 1958). Those additives that meet these requirements may be added to food at appropriate levels. Those that do not meet these requirements must undergo "pre-market" approval. In order to market a new food additive, "a manufacturer or other sponsor must first petition the FDA for its approval" (Food Additives, 2012). Included in the petition is evidence provided by the manufacture that the new food

additive is safe in the way that it will be used. When reviewing new food additive petitions the FDA considers four key attributes (Food Additives, 2012):

1. Composition and properties of the substance
2. The amount that would typically be consumed
3. Immediate and long-term health effects
4. Various safety factors

Since there is no 100% certainty any food substance can be risk free a standard of safety has been established by the FDA: "Reasonable Certainty of No Harm." This is left to the manufactures to demonstrate that under normal use conditions, even those with a safety margin, no increase in harm will come to the individual when consuming this food additive.

Two of the organic preservatives in the current study are all listed as GRAS substances: sodium metabisulfite and potassium sorbate. Sodium benzoate is listed as a direct food substance affirmed as generally recognized as safe. "Sodium metabisulfite is generally recognized as safe when it is used in accordance with good manufacturing practices (GMP's)" (Substances Generally Recognized As Safe, 2013). The use of sodium metabisulfite is prohibited to be used in meats, foods recognized as a source of vitamin B_1, and on raw fruits and vegetables (such as fresh salad bars). Potassium sorbate is generally recognized as safe, when used in accordance with GMP's. The amount of sorbate in a food product must not exceed 0.3% as sorbic acid. Sodium benzoate is a direct food additive with GRAS standing. The amount of sodium benzoate used in a food product must not exceed GMP's or 0.1% as sodium benzoate.

Mechanisms for the Survival of Foodborne Pathogens

Salmonella is a pathogenic bacterium that grows at moderate temperatures, moderate pH, and cannot fix carbon (heterotroph). *Salmonella* needs inorganic salts containing phosphorus,

sulfur and nitrogen, a source of cations and an organic substrate for carbon and energy (Hui et al., 1994). Changes in pH, temperature, and concentration of salts and solutes impedes the growth of the pathogen and evokes and environmental stress response. Changes to the environment of the bacterium are the basis to inhibit or destroy the pathogen in food or beverage sources.

Thermal sensitivity. "*Salmonella* are killed rapidly by modest heat treatments" or conventional household cooking methods (Hui et al., 1994, p. 268). This may not be the case if the *Salmonella* load is unusually high or the serotype is abnormally heat resistant. The higher the amounts of microorganisms present in a food source the higher its degree of heat resistance (Jay et al., 2005). It has been proposed that *Salmonella* poses an adaptive mechanism to combat heat stress. When *Salmonella* is exposed to moderately high temperatures, the bacterium is able to synthesize new stress proteins. The initiation of stress proteins are from specific polypeptides of sigma factors (σ). When *Salmonella* is placed under a heat stressed environment, the rpoH gene is activated to synthesized σ^{32} protein. The synthesized proteins combine with RNA polymerase to complete the RNA polymerase enzyme. This enzyme can further bind with a promoter for the heat-shock gene family, resulting in the synthesis of heat-shock proteins on the outer cell wall. These proteins can directly increase the heat resistance of the microorganism (Ray & Bhunia, 2008, p. 85). The same mechanistic approach is used to explain the acid resistance of some bacteria, but the difference lies in the specific σ-factor that becomes activated within the microorganism.

The ability of *Salmonella* to grow and thrive in acidic conditions is dependent on the growth [storage] temperature of the medium [juice beverage] (Alvarez-Ordonez, Fernandez, Bernardo, & Lopez, 2010). Alvarez-Ordonez et al. have demonstrated the effect that *Salmonella*

grown at 10°C have a reduced acid tolerance when compared to these grown at 30°C (2010). Yang, Teo, Bang, and Yuk have also demonstrated that there was a significant decrease in decimal reduction time of both mango and pineapple juice of acid-adapted *Salmonella* than the non-adapted control cells (2012). This suggests that the *Salmonella* strains are more sensitive to thermal treatments after acid adaptation. In a production sense, considerably less heat will be required to achieve "pasteurization" of high acid foods.

Following heat treatments, bacteria cells have "shown loss of permeability and increased sensitivity to some compounds to which they are normally resistant" (Ray & Bhunia, 2008). A study conducted in 2003 conducted by Chikthimmah, LaBorde, and Beelman, demonstrated the effectiveness of storage temperature on *E. coli*O157:H7 destruction in apple juice preserved with organic acids (2003). Changing the storage temperature of apple cider from 15-25°C significantly increases the destruction rate of *E. coli* O157:H7 (Chikthimmah et al., 2003). It has been theorized that at higher temperatures, there is an increase in fluidity of the cellular membrane which by allows for rapid diffusion of organic acids into the cytoplasm of a bacteria. The undissociated preservatives begin to dissociate resulting in a change in the internal pH of the cell. This results in the death of *Salmonella* cells due to irreversible injuries to cell membrane, cell wall, and DNA and RNA structure.

Acid tolerance and the response mechanism. Most foodborne pathogens such as *E. coli* O157:H7 and *Salmonella* are susceptible to low pH environments such as 4.5 (Ray & Bhunia, 2008). At low pH, the microorganisms are also more susceptible to antimicrobial treatments such as organic acids and thermal treatments. Numerous studies have shown that foodborne pathogens, such as *Salmonella*, possess mechanistic adaptations to acidic environments allowing them to cope and survive in a low pH. In a study by Hui et al. (1994),

Salmonella cells cultured at pH values in the range of 5.5-6.0, had enhanced (100-1000 times) survival in severely acidic conditions, pH value of 3.3. This phenomenon is referred to as the acid tolerance response (ATR) mechanism. The mechanism employed by bacteria to adapt to an acidic environment is similar to the mechanism described previously for thermal tolerance. This impact of acid tolerance response mechanisms on food processing has not been well studied and is recommended by the author of this study for further research. Beverage producers may have to avoid any possible pre-exposure of potential bacterial contaminants of hurdle treatments is employed to destroy the pathogenic strains.

Non-thermal Methods for the Destruction of Foodborne Pathogens

Many small beverage manufactures need low cost alternatives to pasteurization to comply with Federal juice HACCP regulations. Organic acid preservatives in combination with low pH have shown to be effective antimicrobial measures against the growth and destruction of *Salmonella*.

Organic acid preservatives. Three common preservative or organic acids used in beverages are sodium benzoate, potassium sorbate, and sodium metabisulfite. Sodium benzoate is effective against yeast and bacteria, at a pH 2.5-4.0. Potassium sorbate is effective against mold and yeast, at a pH of <6.5 (Ward, 2002). A study conducted to determine the antimicrobial effects of organic acids against enterohemorrhagic bacterial strains (*E. coli* O157:H7) found the most effective treatments were sulfite>benzoic acid>sorbic acid (Lu, Breidt, Perez-Diaz, & Osborne, 2011). Organic acids are theorized to work by diffusion across the cellular membrane of a bacterium. This results in the undissociated molecules to dissociate and lower the internal pH of the bacteria, which interrupts the metabolic activity of the cell, disrupts pH homeostasis, and nutrient transport (Lu et al., 2011). This in culmination leads to cellular death. Therefore,

the pH of the medium and the pK_a of the organic acid ultimately determine the effectiveness of the organic acid. Lu et al., provides an additional mechanistic theory based on the study conducted to determine the effect of organic acids under anaerobic conditions. Lu et al. purposed that sulfite was most effective due to its ability to break the disulfide bonds with in the cell of a gram-negative bacterium like *Salmonella* or *E. coli* O157:H7. This results in conformation changes in the bacteria's enzymes and metabolic profile (Lu et al., 2011). Benzoates ability to destroy pathogen growth was attributed to its ability to attenuate anaerobic glycolysis, which ultimately results in ATP depletion within the cell. (Lu et al., 1994). Sorbate was deemed to affect the sulfhydryl enzymes in the cell. The current study does an assessment of these preservatives in a juice beverage system challenged with *Salmonella* ser. Typhimurium.

Effect of low pH of pathogen growth and survival. "The pH of foods is important because it can limit the ability of microorganism to grow, cause spoilage, and in some cases produce toxins" (Russel & Gould, 1991, p. 22). Ray and Bhunia in their textbook, report that the pH range of growth for molds is 1.5-9.0, yeast: 2.0-8.5, gram-positive bacteria: 4.0-8.5, and gram-negative bacteria: 4.5-9.0 (2008). It should be noted however, that individual species and serotypes might differ than reported values due to acid-resistance and tolerance response mechanisms. Controlling the pH of a food product works by controlling the flow of hydrogen ions (H^+) in the food medium and pathogen host. Undissociated organic acid molecules, such as citric acid and sodium benzoate, can enter the cell membrane of a *Salmonella* bacterium. Upon entering the cell, the molecule (benzoate, sorbate, or sulfite) can dissociate into its constituents. This in turn increases the concentration of H^+ ions in the cell. The increase of H^+ ions results in the increased activity of the cytoplasm. The reduction of pH inhibits the cellular process resulting in microbial death. If the low pH is maintained for an extended period, irreversible

damage to the pathogen cell occurs, leading to cell death. (Russell & Gould, 1991). Chikthimmah et al. demonstrated a significant ($p<0.05$) decrease in the destruction time for *E. coli* O157:H7 when the pH of apple cider was reduced from a pH of 4.7 to 3.2.

The hurdle concept. The hurdle concept employs the use of multiple factors or techniques in combination to control microorganisms in foods. This may be accomplished by employing pH, chemical and organic preservatives, and temperature synergistically to prevent/destroy microbial loads in a food product. The theory is based on the scientific idea that when two or more treatments are used in combination, the combined effect is more effective that if each treatment was used separately and can be used at a lower treatment level (Ray & Bhunia, 2008).

In the research conducted in this study, the hurdle concept is employed as a non-thermal alternative to pasteurization of a fruit beverage produced in a commercial facility. Using the theories discussed in this chapter, the fruit beverage is presented with three hurdles used in combination as a preventative measure against microbial spoilage. These hurdles include an intrinsic factor (low pH), a processing factor (storage temperature), and an extrinsic factor (organic acids). All three hurdles in combination provide unique barriers in which a pathogenic bacterium needs to overcome for survival or growth.

Chapter III: Methodology

The objective of this study was to validate an organic acid treatment for an acidified juice beverage challenged with *Salmonella* ser. Typhimurium. The preservative treatments were compared against potassium metabisulfite with the intent to develop a sulfite alternative to enhance the microbial safety of a raspberry beverage.

Pilot Study for Juice Variety Determination

Fresh, unpasteurized, and unpreserved raspberry, blueberry, and black currant juice beverages were obtained from a commercial beverage processor in northern Wisconsin. Beverage samples were prepared according to processor formulations (3.79L of water; 94.5mL of fruit concentrate; sugar and citric acid). Sugar and citric acid was added based on the final product attaining a specific gravity of 1.045 and percent titratable acidity, measured as tartaric acid equivalent between 7.4-8.0%. The characteristics of each of the juice beverages are shown in Table 1.

Table 1

Specific Gravity, % Titratable Acidity, and pH Values of Three Juice Beverage Formulations

Juice Beverage Type	Specific Gravity	% Titratable Acidity	pH
Raspberry	1.045	7.6	2.90
Blueberry	1.045	7.4	3.04
Black Current	1.045	8.0	2.88

The prepared juice beverages were stored in sealed 1–gallon plastic containers (HDPE or No. 2 plastic) and kept frozen (-18°C) until further use. Two days prior to each experiment, a 1–gallon container was removed from the freezer and allowed to thaw completely at 4°C for 48 hours.

A *Salmonella* ser. Typhimurium strain, obtained from the culture collection of the University of Wisconsin – Stout Biology department, was used for the pilot study. An isolated colony was selected and transferred to 10mL of sterilized tryptic soy broth (BD Difco) with 0.85% yeast extract, TSBYE, (BD Difco) (AMSCO Lab 250 Steam Sterilizer, 121°C, 15 minutes), vortexed for 5 seconds, and incubated (VWR Incubator, Model 1565) for 24 hrs. at 37°C. A 100µL-aliquot of inoculated broth was transferred into subsequent 10 mL of sterilized TSBYE, vortexed for 5 sec, and incubated for 24 hrs. at 37°C. The bacterial suspension was centrifuged at 500 X *g* for 10 minutes. The supernant was poured off and the pellet was resuspended in 1mL of 0.85% sodium chloride (Fisher Chemical, Fair Lawn, NJ). The final solution contained approximately 7.5-log CFU/mL of *Salmonella* ser. Typhimurium. The suspension was vortexed for 5sec. before inoculation of each juice beverage.

Juice samples were inoculated with 1 mL of *Salmonella* ser. Typhimurium inoculum and were promptly plated on xylose lysine deoxycholate, XLD, (BD Difco) in order to determine initial inoculum loads. Inoculum levels for all trials were determined by serial dilutions of the beverage samples in buffered peptone water (Oxoid). Next, 100 µL aliquots were spread plated onto XLD agar plates followed by incubation at 37 ± 2°C for 24 hours. *Salmonella* ser. Typhimurium colonies appear as large, glassy black colonies. Enumeration of *Salmonella* ser. Typhimurium at 0, 1, 2, 3, 4, 6, 8, and 11 days. All samples were run in duplicate.

Sample Selection and Description

Fresh, unpasteurized, and unpreserved raspberry beverage was made at the production facility of a commercial processor in northern Wisconsin. Raspberry beverage samples were prepared by adding 94.5 mL of raspberry concentrate (9-1 ratio of concentrate to water based on manufacturer's recommendations) to 1 gallon of water, creating approximately 25% juice

beverage. Raspberry beverage samples were prepared in triplicates. Sugar (332.40 ± 11.52 grams) and citric acid (14.39 ± 0.09 grams) was added to each sample to achieve end product specifications, set forth by the beverage manufacture. Following the addition of all ingredients the specific gravity, pH, and percent titratable acidity (measured as tartaric acid equivalents) were measured to ensure product specifications. Specific gravity was adjusted with additional sugar, and percent titratable acidity and pH were adjusted with citric acid additions. The final specific gravity for samples was 1.045 ± 0.001, percent titratable acidity was 8.6 ± 0.1, and the pH was 2.83 ± 0.03, shown in Table 2.

Table 2

Specific Gravity, % Titratable Acidy, and pH Values of Three Raspberry Beverage Formulations

Sample	Specific Gravity	% Titratable Acidity	pH
Juice 1	1.044	8.7	2.86
Juice 2	1.044	8.5	2.8
Juice 3	1.046	8.6	2.84

The resulting samples were stored in sealed 1–gallon plastic containers and frozen (-18°C) until further use. Two days prior to each experiment, a 1–gallon container was removed from the freezer and allowed to thaw completely at 4°C for 48–hours.

Data Collection Methodology

***Salmonella* ser. *Typhimurium* strain preparation.** The preparation of *Salmonella* ser. Typhimurium was adapted from Chikthimmah, LaBorde, and Beelman (2003). Two *Salmonella* ser. Typhimurium strains were obtained from the American Type Culture Collection, 14028 (isolated from animal tissue) and 13311 (isolated from animal tissue). *Salmonella* ser.

Typhimurium cultures were stored at 4°C on Luria-Bertani, LB, (BD Difco) agar. Fresh samples were prepared three days prior to use.

Salmonella ser. Typhimurium inocula (both strains) were prepared by streaking a sterilized pre-poured LB agar plate followed by incubation for 24 hrs. at 37°C. An isolated colony was selected and transferred to a sterilized centrifuge tube, with 10mL of sterilized tryptic soy broth (BD Difco) with 0.85% yeast extract, TSBYE, (BD Difco), and vortexed for 5 seconds prior to incubation at 37°C for 24 hours. 100µL-aliquot of inoculated and incubated broth was transferred to sterilized centrifuge tubes containing 10 mL of sterilized TSBYE, vortexed for 5 seconds, and incubated at 37°C for 24 hours. Following incubation, the bacterial suspension was centrifuged at 3000 X *g* for 20 minutes in order to form a pellet containing the concentrated *Salmonella* ser. Typhimurium. Following centrifugation, the supernant was poured off and the pellet was resuspended in 10mL of 0.85% sodium chloride. The suspensions of the two strains were combined to make the cocktail strain, which was used to inoculate filter sterilized juice beverage samples. The final solution contained approximately 7.5-log CFU/mL of *Salmonella* ser. Typhimurium.

Challenge of raspberry beverage treatments with *Salmonella* ser. Typhimurium. Raspberry beverage samples were acquired from a beverage processor in northern Wisconsin. Two hundred milliliters of sample were membrane filtered for sterility (Thermo Scientific, 156-4020) at 0.2 µm (SFCA, membrane), under aseptic conditions in a bio safety cabinet (Labconco, Class II, Type A2). 200 mL of beverage samples were added into 500-mL sterile glass bottles (Wheaton). The experimental treatments were:

1) raspberry beverage (RB) control;
2) RB with 11ppm free sulfite and 0.03% (w/v) potassium sorbate (RB + Su + So);

3) RB with 0.03% (w/v) potassium sorbate (RB + So);
4) RB with 11 ppm free sulfite (RB + Su);
5) RB with 0.1% (w/v) sodium benzoate (RB + Ben);
6) RB with 0.5% (w/v) sodium benzoate and 0.03% (w/v) potassium sorbate (RB + Ben + So).

Control beverage samples had no added preservatives. The suppliers for the sulfite, sorbate, and benzoate were Fisher BioReagent, Acros, and Aldrich respectively.

Sorbate and Benzoate levels were measured on a weight per volume basis. Sulfite levels were quantified by the aspiration method of determination. First, 20 mL of the raspberry beverage sample was mixed with 0.3% hydrogen peroxide and 25% phosphoric acid in a test tube. An air bubbler was added and bubbled for 15 minutes. Following sample aspiration, the sample was titrated with 0.1M sodium hydroxide using a SO_2 indicator solution, which turns from purple to green when the titration is complete. The amount of sodium hydroxide needed for the titration is then used to calculate free SO_2 levels in the raspberry beverage (11ppm free SO_2). 11ppm of free SO_2 was used based on the pH of the beverage.

Following preservative addition to containers, the bottles were mixed fully to dissolve added preservatives. After addition of preservatives, samples were allowed to stand at room temperature 60 ± 5 minutes prior to inoculation. The juice beverage samples (200mL) were inoculated with 1ml of each *Salmonella* ser. Typhimurium cocktail strain inoculum, and swirled for 15 seconds before enumeration of *Salmonella* ser. Typhimurium populations on day 0 (initial load). Samples were stored in capped bottles (Wheaton) at either 4.0°C or at room temperature (22°C) for the duration of the testing period. Treatments were tested in triplicate.

Enumeration of *Salmonella* ser. Typhimurium. Inoculum levels for all trials were determined by serially diluting the juice samples in buffered peptone water (BPW, Oxoid). Spread plates were prepared from 100µL aliquots onto XLD agar plates followed by incubation at 37 ± 2°C for 24 hours. One-micro liter aliquot of trial control (non-*Salmonella* inoculated) sample was plated on LB agar in duplicate followed by incubation at 37 ± 2°C for 24 hours. *Salmonella* ser. Typhimurium colonies appear large, glassy black colonies. Enumeration for *Salmonella* ser. Typhimurium followed at the following days 0, 1, 2, 3, 5, and 9 days of storage at either 4°C or 22°C.

Enrichment of raspberry beverage samples for the enumeration of *Salmonella* ser. Typhimurium. The enrichment of samples for the qualitative detections of *Salmonella* ser. Typhimurium was conducted following the United States Food and Drug Administration's (FDA) Bacterial Analytical Manual (BAM) for *Salmonella* (Andrews, Jacobson, and Hammack, 2011). The method described for unpasteurized apple cider with a low microbial load in the BAM was used in this study. Beverage treatment samples were subject to enrichment following negative growth on XLD agar plates.

25mL of sample was aseptically added to 225mL of sterilized universal preenrichment broth, UPB, (BD Difco) in a 500mL screw-capped jar. The flask was swirled, loosely capped, and allowed to stand at room temperature for 60 ± 5 minutes. The flask was incubated at 35°C for 24 ± 2 hours.

Following incubation, 0.1mL of juice sample was transferred to 10mL of sterilized Rappaport-Vassiliadis (RV) broth (BD Difco), vortexed, and incubated at 42 ± 2°C for 24 ± 2 hours. Simultaneously, 1.0mL of juice sample was added to 10mL of Tetrathionate (TT) broth (Acumedia), vortexed, and incubated at 35 ± 2°C for 24 ± 2 hours.

Following incubation, each broth was vortexed and a 3 mm loopful of sample (TT and RC) was streaked for isolation across a pre-poured XLD agar plate. Plates were incubated at 35 ± 2°C for 24 ± 2 hours then examined for the presence of colonies. Positive samples resulted in large, glossy, and black colonies.

Data Analysis

All experimental treatments were conducted in triplicate. Analysis of variance and means separation was conducted using the General Linear Model (GLM) using Minitab® 16.1.0 software (Minitab In.). Microsoft® Excel (Microsoft Corporation, Cambridge, Massachusetts, USA) was used to generate graphical data and trend lines when required. Pairwise comparisons were conducted between treatments at the same storage temperature. Pairwise comparisons were also conducted within treatments stored at different storage temperatures.

Limitations

A limitation of the experimental procedure was the length of time between enumerations of *Salmonella*. The rapid destruction of *Salmonella* would be better demonstrated by sampling at shorter intervals, such as 0, 12 hrs., 24 hrs., 36 hrs., 48 hrs., 3 d, 4 d, 5 d, 6 d, 7 d, 8 d, and 9 d. This would conceptually give a deeper understanding into the destructive power of each treatment.

Chapter IV: Results

This experiment was conducted to determine the effect of organic acid treatments on the destruction rate of a cocktail strain of *Salmonella* ser. Typhimurium in a raspberry fruit beverage (acidified with citric acid). The objective of the research was to assess the developed organic acid treatments for microbial food safety and also to determine if any of the treatments may be effective as sulfite replacements in the raspberry beverage.

Food Safety Pilot Study; Assessment of Raspberry, Blueberry, and Black Current Beverages

Fresh, unpasteurized, raspberry, blueberry, and black current juice beverages were obtained from a commercial beverage processor in northern Wisconsin. Samples were challenged with a strain of *Salmonella* ser. Typhimurium (obtained from the culture collection of the University of Wisconsin-Stout) in order to determine which variety of juice beverage was most resistant to the pathogenic bacteria. Samples obtained had an average specific gravity of 1.045 (±0.0), % titratable acidity of 7.67 (±0.14), and pH of 2.97 (±0.09). Samples were held at 4°C and sampled on 0, 1, 2, 3, 4, 6, 8, 11, 14 days. The results of the pilot study can be seen in Figure 2 below.

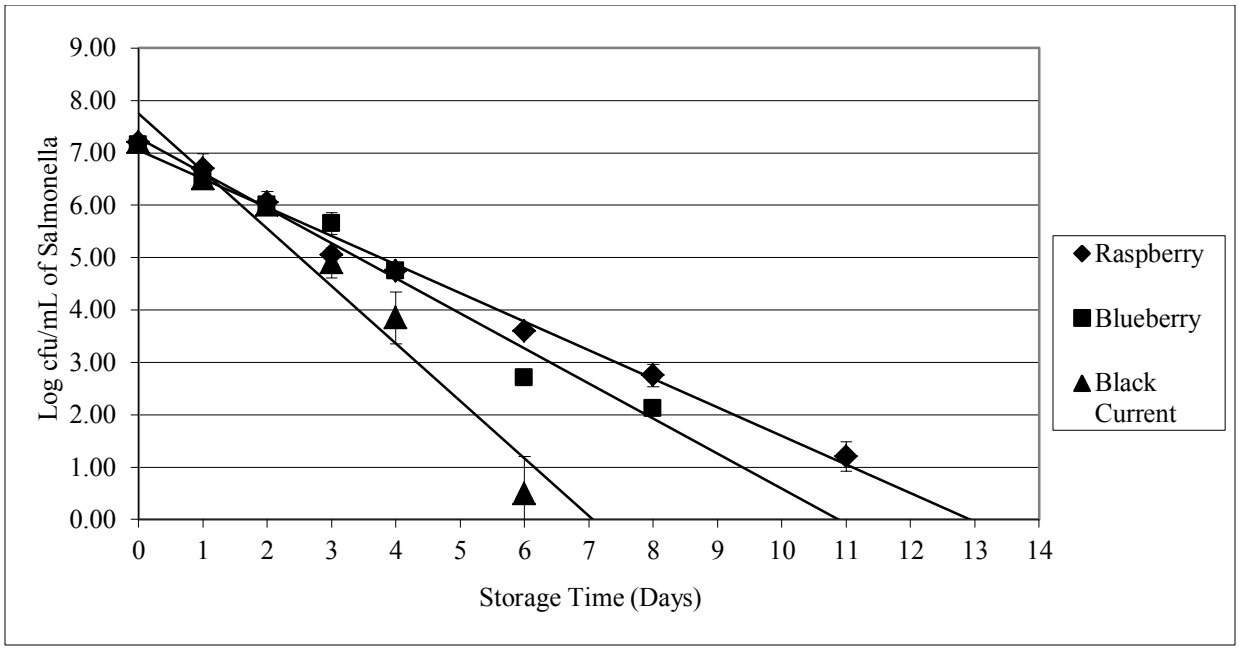

Figure 2. Destruction rate of *Salmonella* ser. Typhimurium in three juice varieties stored at 4°C.

As can be seen in Figure 2, *Salmonella* ser. Typhimurium showed a slower rate of reduction in the raspberry beverage. Therefore, a further study on organic acid treatment development was conducted in a raspberry beverage.

Effect of Organic Acid Treatments on the Destruction of *Salmonella* ser. Typhimurium at 4°C Storage Temperature

Populations of *Salmonella* declined during storage at 4°C for all treatments. The organic acid treatments had a significant effect in increasing the rate of destruction of *Salmonella* ser. *Typhimurium*. All samples showed no growth on XLD agar plates after day 9 (Table 3). The control sample showed the least effect, while samples containing benzoate was the most effective against *Salmonella* ser. Typhimurium. Table 3 below, depicts the declining rates of *Salmonella* ser. Typhimurium survival.

Table 3

Survival of Salmonella ser. Typhimurium in a Raspberry Juice Beverage Stored at 4°C with or without the Addition of Organic Acids

Time (days)	*Salmonella* populations (Log CFU/mL)											
	RB		RB + Su+ So		RB + So		RB + Su		RB + Ben		RB + Ben + So	
	XLD[2]	Enrich.[3]	XLD[2]	Enrich.[3]	XLD[2]	Enrich.[3]	XLD[2]	Enrich[3].	XLD[2]	Enrich.[3]	XLD[2]	Enrich.[3]
0	7.55	+	7.55	+	7.55	+	7.55	+	7.55	+	7.55	+
1	6.46	+	4.70	+	6.32	+	5.19	+	ND[1]	+	1.58	+
2	5.98	+	1.29	+	5.23	+	2.74	+	ND[1]	+	ND[1]	+
3	5.08	+	ND[1]	+	3.65	+	ND[1]	+	ND[1]	+	ND[1]	+
5	2.55	+	ND[1]	+	ND[1]	+	ND[1]	+	ND[1]	+	ND[1]	+
9	ND[1]	+	ND[1]	-	ND[1]	-	ND[1]	-	ND[1]	-	ND[1]	-
13	ND[1]	-										

[1]ND, not detectable (<10 CFU/mL)

[2]Colony counts after enumeration and plating on XLD agar

[3]Qalitivate enrichment on XLD agar (+ growth on XLD, - no growth on XLD)

Table 3 shows an average starting with a microbial load of 7.55±0.21 CFU/mL. A 5 log reduction was achieved in all treatments. D-values were calculated for each treatment, Figure 3 (shown later), and can be seen in Table 5 in the subsequent results discussed below. The D-values for treatments 5 and 6 were the lowest, which corresponds to the rapid destruction rate, while treatment 1 had the highest D-value. There was an extended period of enriched samples being positive for *Salmonella* ser. Typhimurium in each treatment, which is indicative bacterial injury.

Effect of Organic Acid Treatments on the Destruction of *Salmonella* ser. Typhimurium at 22°C Storage Temperature

Populations of *Salmonella* ser. Typhimurium declined during storage at 22°C for all treatments. The organic acid treatments had a significant effect in increasing the rate of

destruction of *Salmonella* ser. Typhimurium as compared to the control sample. The organic acid treatments had a significant effect on the increasing rate of reduction of *Salmonella* ser. Typhimurium species ($p<0.05$), and complete destruction was seen in all samples. Table 4 below depicts the declining rates of *Salmonella* ser. Typhimurium below.

Table 4

Survival of Salmonella ser. Typhimurium in a Raspberry Juice Beverage Stored at 22°C with or without the Addition of Organic Acids

	***Salmonella* populations (Log CFU/mL) at 22°C**											
Time (days)	**RB, Control**		**RB+So+Su**		**RB+So**		**RB+Su**		**RB+Ben**		**RB+Ben+So**	
	XLD[2]	**Enrich.[3]**	**XLD[2]**	**Enrich.[3]**	**XLD[2]**	**Enrich.[3]**	**XLD[2]**	**Enrich.[3]**	**XLD[2]**	**Enrich.[3]**	**XLD[2]**	**Enrich.[3]**
0	7.74	+	7.74	+	7.74	+	7.74	+	7.74	+	7.74	+
1	6.31	+	0.92	+	5.27	+	0.82	+	1.56	+	0.38	+
2	5.62	+	ND[1]	+	4.36	+	ND[1]	-	ND[1]	-	ND[1]	-
3	5.39	+	ND[1]	-	3.31	+						
5	4.62	+			ND[1]	+						
8	0.30	+			ND[1]	-						
9	ND[1]	+										
11	ND[1]	-										

[1]ND, not detectable (<10 CFU/mL)

[2]Colony counts after enumeration and plating on XLD agar

[3]Qalitivate counts after enrichment

There was no significant difference between samples containing sulfite (treatments 2, 4) and samples containing benzoate (treatments 5, 6). When enumerated on XLD agar, these four treatments were the most powerful against *Salmonella* ser. Typhimurium. A 5 log reduction was achieved in all treatments. D-values for each treatment were also calculated using the formula in Figure 3 (shown later) and reported in Table 5. Treatments 2, 4, 5, 6 had significantly lower D-values as compared to the other treatments. As reported earlier in treatments held at 4°C, there was an extended period of enriched samples being positive for *Salmonella,* which is indicative of bacterial injury in the raspberry beverage. Samples held at 22°C, showed a reduced presence of

injured bacterial cells, up to three days following negative growth on XLD agar, as compared to five days seen in samples held at 4°C.

Effect of Treatments on D-Values

Following the enumeration and enrichment of *Salmonella* samples, D-values were calculated for each treatment. D values were determined by plotting the survival of *Salmonella* versus storage time (tables 3 and 4). D values can then be calculated using the following formula:

$$D_T = \frac{t}{log_{10}x - log_{10}y} = \frac{-1}{slope}$$

Formula 1. Formula used for calculating D-values.

Where D_T = t minutes, and T represents the temperature and t represents time in minutes. In the formula x and y represent microbial counts before and after exposing the microbe to temperature (T) for t minutes. D values will give a representation of the destructive power of each treatment against *Salmonella* ser. Typhimurium.

Storage temperature had a significant effect on the destruction of *Salmonella* ser. Typhimurium in raspberry beverages with organic acid treatments. Storage at higher temperatures (22°C) resulted in an increased rate of reduction of *Salmonella* ser. Typhimurium. Table 5 below describes the statistical findings of the calculated D-values for each treatment.

Table 5

Calculated D-values for Salmonella ser. Typhimurium for Various Treatments and Temperatures

Treatment	Calculated D-Values (days)	
	4C	22C
1. RB	1.032^{A1}	1.682^{A2}
2. RB + So + Su	0.326^{C1}	0.152^{C2}
3. RB + So	0.722^{B1}	0.646^{B1}
4. RB + Su	0.389^{C1}	0.172^{C2}
5. RB + Ben	0.128^{D1}	0.216^{C1}
6. RB + Ben + So	0.170^{D1}	0.174^{C1}

^{ABCD} values with different letters within a column indicate a significant difference between samples

^{12} values with different numbers between columns indicates a significant difference between samples

In both storage temperatures the control juice had a significantly higher D-value than all other treatments ($p<0.05$). At 4°C, juice with benzoate had a significantly smaller D-value than all other treatments except the benzoate and sorbate combination ($p<0.05$). This is not the same for the juice being stored at 22°C. Juice with sorbate and sulfite, sulfite, benzoate, and benzoate and sorbate all had statistically similar d-values, ($p>0.05$). The decrease in D-value for sorbate plus sulfite and sulfite treatments is significantly different between the two storage temperatures. The increase in d-values for the juice with sulfite derivatives can be attributed to the increase in temperature. Yet, the temperature had no effect on benzoate and benzoate plus sorbate treatments.

Chapter V: Discussion

In the experiment conducted on the destruction of *Salmonella* ser. Typhimurium in a raspberry juice beverage a reduction of *Salmonella* ser. Typhimurium was seen in all treatments, including the control samples. In addition, a 5-log reduction was seen in all treatments. Those treatments, which included organic acids, reduced the *Salmonella* ser. Typhimurium counts significantly faster than that of the control treatment alone. An increase in the storage temperature (22°C) significantly increased the rate of destruction of *Salmonella* ser. Typhimurium in all treatments, including the treatment with sulfite.

Effect of Organic Acids on *Salmonella* ser. Typhimurium Destruction

At 4°C, benzoate and benzoate plus sorbate, were the most effective organic acid treatments. At 22°C, there was no significant difference between organic acid treatments. All treatments were more effective than the control at reducing populations of *Salmonella* ser. Typhimurium. The current manufacturing procedures require refrigeration of the juice product for 2-days prior to bottling. Thus the strength of organic acids are as follows (ranked from most effective to least effective) benzoate and benzoate plus sorbate > sulfite > sulfite plus sorbate > sorbate. The results of this study are in agreement with that of Hossain, Islam and Islam (2011). Their study on the effect of organic acids on the shelf life of tomato juice determined that sodium benzoate had a significantly higher effect than potassium metabisulfite and potassium sorbate on the total viable counts present in the tomato juice. Another study by Lu, Breidt, Jr., Perez-Diaz, and Osborne (2011) demonstrated that the most effective organic acid to destroy populations of *E. coli* O157:H7 was sulfite > benzoic acid > sorbic acid in non-heat processed acidified vegetables. In consensus, each of these studies concluded that the tested organic acid treatments are effective in reducing populations of microorganisms. Each of the organic acid treatments

had a greater effect than the control (no organic acid treatment) or alternate weak organic acids (malic acid, citric acid, fumaric acid).

Benzoate and benzoate plus sorbate were the only treatments in which *Salmonella* ser. Typhimurium was undetected by enumeration on selective XLD agar with D-values less than one day. Among the organic acid treatments evaluated, these treatments may be recommended for food safety of raspberry juice beverages, with the caveat that the presence of *Salmonella* ser. Typhimurium is still present during enrichment of treated samples. This is an indication that the *Salmonella* ser. Typhimurium cells underwent injury and demonstrated recovery in the enrichment process.

The enhanced survival of *Salmonella* ser. Typhimurium in the raspberry beverage system, compared to serial dilution plating, may be attributed to select acid tolerance mechanism of *Salmonella.* The low temperature and acidic environment may be invoking an ATR, which increases the length of time viable *Salmonella* cells are able to persist under harsh conditions. The mechanism of ATR is discussed below in a forthcoming section.

Effect of Treatments at 4° and 22° C Storage Temperatures

Increasing the storage temperature had a significant effect on increasing the rate of destruction of *Salmonella* ser. Typhimurium in a raspberry beverage with the organic acid treatments. This result is consistent with previous studies (Chikthimmah et al., 2003; Comes & Beelman, 2002), demonstrating increased rates of reduction of *E. coli* O157:H7 at higher storage temperatures. A study conducted by Alvarez-Ordonez et al., (2010), also demonstrated that an increase in storage temperatures significantly increase the D-values in cultured *Salmonella* ser. Typhimurium. Alvarez-Ordonez inoculated sterile Brain Heart Infusion (BHI) with approximately 3-log CFU/mL of *Salmonella* ser. Typhimurium in non-acidified and acidified

conditions at stored at various temperatures. The results concluded that acid-adapted cells were more resistant to lethal pH conditions that non-acid adapted cells at four incubation temperatures (10, 25, 37, 45°C) (Alvarez-Ordonez, 2010). In the current study, an increase in storage temperature significantly reduced microbial loads and D-values for treatments that incorporated sulfite. The treatments containing: sorbate, benzoate, benzoate plus sorbate, and the control, showed no significant difference between storage temperatures.

Higher storage temperatures increase cellular membrane fluidity, thus allowing the organic acids to more easily diffuse into the cytoplasm of *Salmonella* ser. Typhimurium. Upon entering the cytoplasm, the organic acids begin to dissociate within the cell and lower the internal pH of the bacteria. When treatments were compared with the control, all treatments showed significant temperature effects. The D-values for *Salmonella* ser. Typhimurium at each of the treatment conditions at the higher storage temperature was less than 2-days. This indicates that *Salmonella* ser. Typhimurium underwent irreversible damage at higher temperatures.

Acid Tolerance of *Salmonella* ser. Typhimurium

Salmonella poses a unique ATR mechanism that allows for its survival in acidic environments. Numerous studies have been conducted to demonstrate and model the ATR of *Salmonella* ser. Typhimurium. It has been proposed in acidic environments pH<3.0, that *Salmonella* ser. Typhimurium cells have the ability to maintain internal pH of the cytoplasm. The cell may be able to maintain internal pH for a period of time at mildly acidic conditions (Greenacre, Brocklehurst, Waspe, Wilson, & Wilson, 2003). ATR was not observed when cells were challenged at pH 3.0. The results of this study showed that each of the organic acid treatments had a destructive effect against *Salmonella* ser. Typhimurium, at various rates (tables

3 and 4). Additionally, the control samples showed complete destruction of *Salmonella* ser. Typhimurium.

Injured *Salmonella* ser. Typhimurium Survival at 4°C

Enrichment of raspberry beverage samples indicated the presence of injured *Salmonella* ser. Typhimurium in the beverage after initial indications (enumeration) showed zero growth (Tables 3 and 4). XLD agar provides a harsh environment for microbes in which to grow, due to its selective and differential nature. The length of time between zero counts on XLD and negative results for enrichment was longer for samples held at 4°C than for samples held at 22°C. This would indicate the lower storage temperature is functioning as a protective mechanism for the *Salmonella* ser. Typhimurium. In storage at 22°C, this was not evident. Acidic environments can alter the intracellular metabolic actives of a bacterium such as cellular signals and nutrient transport (Meng et al. as cited in Lu et al., 2011). Inoculating *Salmonella* ser. Typhimurium into such a system could allow undissociated organic acids to diffuse through the cellular membrane into the cell's cytoplasm, yet the susceptibility of *Salmonella* ser. Typhimurium to such intrusion is dependent on the growth temperature of the medium (Alvarez-Ordonez, 2011). The most acid-resistant cells are those that are grown at their optimum temperature and decrease when diverging from their optimum temperature (Alvarez-Ordonez, 2011). Our results are consistent with those seen by Alvarez-Ordonez. The samples stored at 22°C showed a decreased in the length between zero counts on XLD and negative results for enrichment whereas those stored at 4°C showed an increased length.

As a result of these analyses a question raised was were 100% death *Salmonella* ser. Typhimurium occurs. Is it determined by enumeration or by enrichment? Enumeration gives a quantitative estimate in which all viable cells are dead. Enrichment is a qualitative measure of

the viability of cells in the sample. A positive outcome of an enrichment assay can be a result of one viable cell, while enumeration needs at least 10 cells. The fact that enrichment shows positive presence may be due to *Salmonella* ser. Typhimurium recovering from injury due to the acidic environment. It may also be interpreted as small population of acid adapted cells entering the viable but nonculturable state (VBNC). VBNC bacteria fail to grow and colonize when inoculated in growth media, such as XLD, but still poses metabolic activities that indicate their presence and their ability to retain pathogenicity (Zeng et al., 2012). Zeng et al. (2012) recently conducted a study that demonstrated the ability of *Salmonella* Typhi to be induced into a VBNC state. *Salmonella* Typhi was challenged with copper ion stimulation and low storage temperatures, in order to induce a VBNC state. A VBNC state was determined based on glucose consumption as well as nitrogen consumption throughout storage. By this method, both temperature and copper ion stimulation demonstrated *Salmonella* Typhi had entered the VBNC state by reductions in glucose and nitrogen content as compared to control samples. Following the results of this study, further analysis on the interface of *Salmonella* ser. Typhimurium injury or VBNC in the raspberry beverage system is recommended for further research.

Conclusions

The use of organic acids significantly reduced populations of *Salmonella* ser. Typhimurium in a raspberry juice beverage at a faster rate than that of the control treatment. The results of this study may be used by beverage processors to implement critical control points and critical limits in order to enhance food safety in their products.

Among all the preservative treatments tested, those containing benzoate had the most lethal effect against *Salmonella.* For the juice beverage stored 4°C, treatments with benzoate and benzoate plus sorbate were the most effective. There was no difference between organic acids at

22°C, except sorbate alone (treatment 3). Sulfite in the current product may be replaced by benzoate in the future, however further testing for yeast, molds, color deterioration, and consumer taste testing is recommended prior to this change. This research provides evidence for non-thermal alternatives to pasteurization when pasteurization is not feasible due to capital costs.

Recommendations

Based on the research conducted in this study, the recommendations for future research include:

1. The effect of carbonation on the destruction rate of *Salmonella* ser. Typhimurium was not addressed in this research. Carbonation may reduce the pH of the juice, due to the formation of carbonic acid. A study to determine how CO_2 gas addition will affect the influence of organic acids on *Salmonella* ser. Typhimurium survival is recommended.
2. A study of the effect of organic acids on the shelf life of the juice beverage is recommended. The current process uses sorbate and sulfite, in combination, to preserve the color, taste, and antimicrobial properties associated with their use. Switching the preservative levels as well as type, may have an effect on the color and taste of the juice beverage. By conducting a study that incorporates each treatment in sterile and closed containers over an extended period (6 months – 1 year), and through Accelerated Shelf Life testing (ASLT), the change in the physical and chemical properties of the juice beverage can be assessed. Using this in combination with consumer taste tests, would present clear indications on how each organic acids affects color and taste of a juice beverage.

3. The survival of yeast and molds was not addressed in this study. Mold and yeast have been shown to survive in low pH environments. A challenge studies with characterized strains of yeasts and molds will be useful to optimize the process. .
4. A study on the injury vs. death kinetics of *Salmonella* ser. Typhimurium in a low acid fruit product could be an additional experiment. In the current challenge study conducted at 4°C, the presence of *Salmonella* ser. Typhimurium was detected by enrichment for nearly a week after plating on XLD indicated the absence of the bacteria. This is an indication of injury and extended survival of the pathogen in the juice beverage. Determining the changes to the first order death kinetics in the injured phase (possible tailing effect) would be a relevant study. An adaption of the most probable number (MPN) technique using RV broth or TT broth as a means to quantify the number of *Salmonella* ser. Typhimurium cells in the injured state during storage will provide information on the death kinetics that occur in the injured phase. This would provide for valid conclusions and explanations as to the tailing effect seen in the current study.

References

Alvarez-Ordones, A., Fernandez, A., Bernardo, A., & Lopez, M. (2010). Acid tolerance in *Salmonella* Typhimurium induced by culturing in the presence of organic acids at different growth temperatures. *Food Microbiology, 27,* 44-49. doi:10.1016/j.fm.2009.07.015.

Andrews, W. H., Jacobson, A., & Hammack, T. S. (2011). *Salmonella. in the bacteriological Analytical manual* (Chapter 5). Retrieved from: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070149.htm.

Comes, J. E., & Beelman, R. B. (2002). Addition of fumaric acid and sodium benzoate as al alternative method to achieve a 5-log reduction of *Escherichia coli* O157:H7 populations in apple cider. *Journal of Food Protection, 65*, 476-483.

Centers for Disease Control and Prevention. (2010). *Salmonella prevention.* Retrieved from: http://www.cdc.gov/*Salmonella*/general/prevention.html

Centers for Disease Control and Prevention. (2011). *Investigating foodborne outbreaks*. Retrieved from: http://www.cdc.gov/outbreaknet/investigations/

Centers for Disease Control and Prevention. (2012a). *Centers for Disease Control 2011 estimates: Findings.* Retrieved from: http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html

Centers for Disease Control and Prevention. (2012b). *Multistate outbreak of Salmonella Bredeney infections like to peanut butter manufactured by Sunland, Inc.* Retrieved from: http://www.cdc.gov/*Salmonella*/bredeney-09-12/index.html

Centers for Disease Control and Prevention. (2012c). *Multistate outbreak of Salmonella Typhimurium and Salmonella Newport infections linked to cantaloupe.* Retrieved from: http://www.cdc.gov/*Salmonella*/Typhimurium -cantaloupe-08-12/index.html

Centers for Disease Control and Prevention. (2012d). *Salmonella*. Retrieved from: http://www.cdc.gov/*Salmonella*/general/index.html

Chikthimmah, N., LaBorde, L. F., & Beelman, R. B. (2003). Critical factors affecting the destruction of *Escherichia coli* O157:H7 in apple cider treated with fumaric acid and sodium benzoate. *Journal of Food Science, 68*(4), 1438-1442.

Cook, K. A., Dobbs, T. E., Hlady, G. W., Wells, J. G., Barrett, T. J., Puhr, N. D., Lancette, G. A., Bodager, D. W., Toth, B. L., Genese, C. A., Highsmith, A. K., Pilot, K. E., Finelli, L., & Swerdlow, D. L. (1998). Outbreak of *Salmonella* serotype Hartford infections associated with unpasteurized orange juice. *The Journal of the American Medical Association, 280*(17), 1504-1509.

Danyluk, M. D., Goodrich-Schneider, R. M., Schneider, K. R., Harris, L. J., & Worobo, R. W. (2012). *Outbreaks of foodborne disease associated with fruit and vegetable juices,* 1922-2010. Retrieved from: https://edis.ifas.ufl.edu/fs188

Giannella, R. A. (1996). *Salmonella*. In S. Baron (Ed.), *Medical microbiology* (4th ed). Available from http://www.ncbi.nlm.nih.gov/books/NBK8435/#top

Greenacre, E. J., Brocklehurst, C. R., Wilson, D. R., & Wilson, P. R. G. (2003). *Salmonella enterica* serovar Typhimurium and *Listeria* Monocytogenes acid tolerance response induced by organic acids at 20°C: Optimization and modeling. *Applied and Environmental Microbiology, 69*(7), 3945-3951. doi:10.1128/AEM.69.7.3945-3951.2003

Hammack, T. (2012). *Salmonella* species. In K.A. Lampel, S. Al-Khaldi, & S. M. Cahill, (Eds.), *Bad bug book: Foodborne pathogenic microorganisms and natural toxins* (2nd ed.; pp.12-16). Retrieved from: http://www.fda.gov/downloads/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/UCM297627.pdf

Hazard analysis and critical control roint (HACCP) systems, 21 C.F.R. pt. 120 (2012).

Hossain, N., Islam, F., & Islam, N. (2011). Effect of chemical additives on the shelf life of tomato juice. *American Journal of Food Technology, 6*(10), 914-923. doi:10.3923/ajft2011.914.923

Hui, Y.H., Gorham, J. R., Murrell, K. D., & Cliver, D. O. (Eds.; 1994). *Salmonella*. *Foodborne disease handbook: Diseases caused by bacteria* (Vol. 1). New York, NY: Marcel Dekker, Inc.

Jain, S., Bidol, S. A., Austin, J. L., Berl, E., Elson, F., LeMaile-Williams, M., Deasy III, M., Moll, M. E., Rea, V., Vojdani, J. D., Yu, P. A., Hoekstra, R. M., Braden, C. R., & Lynch, M. F. (2009). Multistate outbreak of *Salmonella* Typhimurium and Saintpaul infections associated with unpasteurized orange juice – United States, 2005. *Clinical Infectious Disease, 48*, 1065-1071. doi:10.1086/597397

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). *Modern food microbiology* (7th ed.). New York, NY: Springer.

Kothary, M. H., & Babu, U. S. (2007). Infectious dose of foodborne pathogens in volunteers: A review. *Journal of Food Safety, 21*(1), 49-68. doi:10.1111/j.1745-4565.2001.tb00307.x

Lu, H. J., Breidt, Jr. F., Perez-Diaz, I., & Osborne, J. A. (2011). Antimicrobial effects of weak acids on the survival of *Escherichia coli* O157:H7 under anaerobic conditions. *Journal of Food Protection, 74*(6), 893-898. doi:10.4318/0362-028X.JFP-10-404

Parija, S. C. (2009). *Textbook of microbiology and immunology.* Haryana, India: Elsevier

Ray, B., & Bhunia, A. (2008). *Fundamental food microbiology* (4th ed.) Boca Raton, FL: Taylor & Francis Group.

Russel, N. J., & Gould, G. W. (Eds.; 1991). *Food preservatives.* New York, NY: Blackie and Sons Ltd.

Substances generally recognized as safe. 21 C.F.R. pt. 182 (2013)

United States Department of Agriculture Economic Research Service. (1996). *Bacterial foodborne diseases: Medical costs and productivity losses.* (Economic Report No. 741). Washington, DC: U. S. Government Printing Office.

United States Food and Drug Administration. (2012a). *Current good manufacturing practice in manufacturing, packaging, and food-packaging material.* Retrieved from: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR&searchPath=Title+21%2FChapter+I%2FSubchapter+B%2FPart+110&oldPath=Title+21%2FChapter+I%2FSubchapter+B%2FPart+120%2FSubpart+A&isCollapsed=true&selectedYearFrom=2012&ycord=1273. 21 C.F.R. pt. 110.1-110.10

United States Food and Drug Administration. (2012b). *Food additives*. (Retrieved from: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR&searchPath=Title+21%2FChapter+I%2FSubchapter+B%2FPart+170&oldPath=Title+21%2FChapter+I%2FSubchapter+B%2FPart+110&isCollapsed=true&selectedYearFrom=2012&ycord=2153. 21C.F.R. pt. 170.3

United States Food and Drug Administration. (2012c). *Hazard analysis critical control (HACCP) systems.* Retrieved from: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR&searchPath=Title+21%2FChapter+I%2FSubchapter+B%2FPart+120&oldPath=Title+21%2FChapter+I%2FSubchapter+B&isCollapsed=true&selectedYearFrom=2012&ycord=1512. 21 C.F.R. pt. 120.1-120.25

United States Food and Drug Administration. (2013). *Overview of food ingredients, additives, and colors.* Retrieved from: http://www.fda.gov/Food/Ingredients PackagingLabeling/FoodAdditivesIngredients/ucm094211.htm

Vallay, H., & Misso, N. L. (2012). Adverse reactions to the sulphite additives. *Gastroenterology and Hepatology From Bed to Bench, 5*(1), 16-23.

Vojdani, J. D., Beuchat, L. R., & Tauxe, R. V. (2008). Juice-associate outbreaks of human illness in the United States, 1995-2005. *Journal of Food Protection, 71*(2), 356-364.

Ward, J. (2007). *Principles of food science*. Tinley Park, IL: The Gooheart-Willcox Company, Inc.

Yang, Y., Teo, J. H., Bang, W. & Yuk, H. (2012). Changes in acid and heat resistance of *Salmonella* Newport and *Salmonella* Saintpaul stored in mango and pineapple juices. *Journal of Food Safety, 32,* 311-317. doi:10.111/j. 174565.2012.00382.x

Zeng, B., Zhao, G., Cao. X., Yang, Z., Want. C., & Hou, L. (2012). Formation and resuscitation of viable but nonculturable *Salmonella typhi*. *BioMed Research International, 2013,* 1-7.