EVALUATION OF COMMERCIAL NONTHERMAL PROCESSING TECHNOLOGIES USED TO INACTIVATE FOODBORNE PATHOGENS IN FRUIT JUICES AND BEVERAGES

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EVALUATING COMMERCIAL NONTHERMAL PROCESSING TECHNOLOGIES USED TO INACTIVATE PATHOGENS IN FRUIT JUICES AND BEVERAGES

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Nonthermal processing technologies have attracted an increasing interest in the food industry for processing foods to ensure food safety in food products and retention of fresh-like qualities. Two important technologies that have been explored for treating fruit juices are the use of chemical inhibitors, such as dimethyl dicarbonate (DMDC), and high-pressure processing (HPP). The efficacy of these technologies against foodborne pathogen inactivation under specific experimental parameters and conditions have been studied. Two concentrations of DMDC (172 and 200 ppm) were evaluated for *Salmonella enterica* and spoilage microbiota inactivation in orange juice. It was found that at both concentrations, a greater than 5-log reduction of *Salmonella* could be achieved at 4°C after 24 hr.

In addition, the effects of high pressure on pathogen inactivation on juices have been investigated, with the focus placed on water processing temperature and product composition, focusing on pH and water activity values. All HPP experiments were conducted at 550 MPa for 1 minute and the juice inoculated with *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. In the study concerning water processing temperatures, experiments were performed at both ambient (20°C) and refrigeration (5°C) temperatures. Results showed that across three juices, inactivation for all pathogens was generally higher at 20°C. Further studies were carried out to understand the effects of pH in product composition. HPP treated juices were

evaluated at initial pH, and at pH values of 4.0, 4.5 and 5.0. The data showed despite acidic conditions (pH <4.6), a 5-log reduction could not be achieved for all pathogens in some juices at pH <4.5. As a counterpart to the study, the effect of water activity on pathogen inactivation was also assessed under acidic and neutral conditions. Two solutes (sodium chloride and fructose) were used to adjust the water activity in a narrow range (0.95 - 0.99). It was found that significantly higher inactivation occurred in solutions at pH 4.5, with NaCl solutions requiring a_w ≥0.96 while fructose solutions requiring a_w ≥0.98 for a 5-log reduction of all pathogens. As a newer technology, HPP has yet to be optimized and streamlined, and estimated costs associated with high pressure treatment have been evaluated and reported.

BIOGRAPHICAL SKETCH

Rebecca Meining Cheng was born in Gainesville, Florida to a family of academics before moving to California at a young age. She spent half of her childhood in the San Francisco Bay Area before her family finally settled down in Irvine, California. An avid cook and baker, Rebecca pursued a Bachelor of Science in Food Science at the University of California, Davis in 2011. At Davis, she explored several facets of Food Science, working in sensory and probiotic labs. In 2013, she had a unique opportunity to work as an undergraduate researcher in a food safety and microbiology lab at the Swiss Federal Institute of Technology (ETH) in Zurich, working on pathogen detection methods. This experience led to her interest and passion to pursue a Ph.D. in food microbiology with a food safety emphasis. In 2015, she was accepted into the Food Science and Technology Ph.D. program at Cornell University under Professor Randy Worobo. She minored in Applied Economics and Management and Food Processing during the course of her graduate study. Her work has focused on evaluating nonthermal technologies and how their parameters affect pathogen inactivation.

This dissertation is dedicated to my family and friends who have supported me throughout my journey.

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CHAPTER 1

INACTIVATION OF SALMONELLA ENTERICA AND SPOILAGE MICROORGANISMS IN ORANGE JUICE TREATED WITH DIMETHYL DICARBONATE (DMDC)*

1.1 Abstract

Salmonella enterica is the pertinent pathogen associated with orange juice products that have resulted in numerous foodborne outbreaks. Although fresh orange juice typically has a pH below 4.0, which inhibits most pathogen growth, S. enterica can survive at low pH for extended periods. Additionally, fresh juice contains spoilage microorganisms such as natural yeasts and molds, which can grow at low pH, and may cause fermentation and product spoilage if left untreated. Numerous *Salmonella* outbreaks linked to fresh orange juice, as well as the burden of product spoilage, have generated increased demand for alternative, non-thermal treatments that can ensure pathogen- and spoilage-free products. In this study, the effect of dimethyl dicarbonate (DMDC) on pathogen and spoilage microorganism inactivation in orange juice has been investigated with two experiments. First, pasteurized orange juice was inoculated with approximately 10^6 –10^7 CFU/ml of five serotypes of *S. enterica* per ml and treated with DMDC to test the effectiveness of inactivation against *Salmonella*. For the fungal spoilage microorganism study, fresh orange juice was held at room temperature to increase natural yeast and mold count to roughly 105–106 CFU/ml, followed with treatment with DMDC. DMDC at two concentrations (172 and 200 ppm) was used, and the tests were carried out at ambient (21 °C ± 3 °C) and refrigeration (4 °C) temperatures. There was a > 5-log reduction of *Salmonella* at 4°C after 24 h at both 172 and 200 ppm of DMDC. For the treatment of fungal spoilage microorganisms, a nearly 5 and 4 log reduction of yeasts and molds was observed at ambient

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temperature and 4 °C, respectively. These results suggest that DMDC is most effective for use under the 4 °C holding conditions to inactivate *S. enterica*, and should be coupled with an additional preservative system for fungal spoilage control to produce safe orange juice that retains fresh quality.

1.2 Introduction

Orange juice is the most consumed juice in the United States, with an average annual consumption rate of 2.7 gallons per person (USFDA, 2017). Traditional orange juice sales have been slowly decreasing due to increasing consumer demand for functional benefits and healthy products, such as vitamin and mineral added, fiber-rich beverages (Mintel, 2017a). Most commercial juices are pasteurized using heat treatment to inactivate pathogens and spoilage microorganisms (Yeom et al., 2000). However, heat treatment of juices can lead to thermal degradation of nutrients, particularly vitamins and antioxidants, and loss of flavor (Jia et al., 1998; Polydera et al., 2004; Vikram et al., 2005). These traits are undesirable and do not keep up with the current trend in the juice industry, where minimally processed or unprocessed products that retain a fresh quality with large nutritional benefits are actively pursued (Mintel, 2017b). However, fresh or unpasteurized juice is highly susceptible to fungal spoilage and may be contaminated with pathogenic bacteria, particularly under conditions without proper processing steps or treatment for controlling microbial growth.

Salmonella is a rod-shaped, Gram-negative bacterium that has been associated with over 1 million foodborne illnesses in the United States, with 19,000 hospitalizations and an estimated 380 deaths annually (CDC, 2012). The genus *Salmonella* is comprised of two species, *Salmonella enterica* and *Salmonella bongori* (Reeves et al., 1989). *S. enterica* is divided into

seven subspecies, including *Salmonella enterica enterica*, the subspecies responsible for most cases of nontyphoidal salmonellosis in humans (Beltran et al., 1988; Uzzau et al., 2000; Winfield and Groisman, 2004). Salmonellosis is an infection caused by the consumption of contaminated water or food, characterized by gastroenteritis, nausea, vomiting, abdominal pain, headaches, elevated body temperature, and non-bloody diarrhea (Chen et al., 2013; Sharma et al., 2001). There have been several outbreaks associated with fresh, unpasteurized orange juice in the past several years, most of which are associated with nontyphoidal *Salmonella* (Butler, 2000; CDC, 1995, 1999; Jain et al., 2009, Krause et al., 2001). To reinforce food safety, the U.S. Food and Drug Administration (FDA) has regulated that juice manufacturers must treat their products to achieve a minimum 5-log reduction for the most pertinent or resistant pathogen of public health concern, or provide a warning label (USFDA, 2001a).

Fresh juice also contains non-pathogenic microorganisms, such as yeasts and molds, which are the primary juice spoilage microorganisms. Unlike pathogens, spoilage microorganisms do not cause harmful effects such as sickness or disease, but produce unwanted characteristics that make the product undesirable for consumption, such as fermentation and off flavors (intVeld, 1996). Microbial spoilage is usually controlled using thermal or nonthermal processing or with food additives as a preservation method (Gabriel, 2015).

Dimethyl dicarbonate (DMDC) is a microbial control agent that has been used primarily in wine preservation, as it inactivates yeast (Delfini et al., 2002; Bartowsky, 2009). DMDC, a colorless liquid, controls microbial growth by inactivating enzymes through protein modification via methoxycarbonylation of enzymes, and thus cell death (Bartowsky, 2009). The FDA approved its use in wines in 1988, with the maximum level permitted set at 250 ppm (USFDA, 2001b). Studies have shown that DMDC is useful as an alternative processing treatment in fruit

juices, such as apple and citrus, to inactivate pathogenic microorganisms (Assatarakul, 2017; Basaran-Akgul et al., 2009; Whitney et al., 2008; Williams et al., 2005). However, its use as the sole pathogen inactivating and fungal spoilage reducing agent in fresh orange juice has not been extensively studied.

In this work, we have investigated the non-thermal treatment of orange juice by DMDC and measured the effectiveness of the agent to achieve a 5-log reduction of pathogens in unpasteurized juice. To assess the efficiency of the process, the microbial tests were carried out at both ambient (21°C ± 3°C) and cold (4°C) temperatures. Five strains of *S. enterica* isolated from juice were used for determining the efficacy of DMDC. We also investigated the effect of DMDC on natural fungal spoilage microorganisms under similar conditions. This study was conducted to determine the effects of DMDC as a viable measure for controlling microbial growth.

1.3 Materials and Methods

1.3.1 Orange juice and bacterial strains

Two types of orange juice were used for this study. Pasteurized and unpasteurized orange juices were purchased from the local supermarket (Wegmans, Ithaca, NY). Both were divided into 50 mL aliquots and frozen at -20°C. Frozen samples were brought to the targeted temperature (4°C or 21°C) before use, depending on the conditions specified in the study. Orange juices were evaluated for pH and °Brix values. The pH values ranged from 3.65 – 3.79 and the °Brix values ranged from 10.07 – 11.47.

Five strains of *S. enterica* ATCC 8324, ATCC 10717, Hartford H0778, ATCC 14028, ATCC 8387 of serotypes Gaminara, Rubislaw, Hartford, Typhimurium, and Montevideo,

respectively, were employed for this work (obtained from M.E. Parish of the University of Florida). These strains were isolated from juices and maintained in frozen culture at -80°C. Each serotype was streaked out on Brain Heart Infusion (BHI) Agar (Sigma-Aldrich, St. Louis, MO) from the frozen stocks and maintained by restreaking on fresh agar monthly.

1.3.2 Preparation of DMDC

DMDC (Velcorin™, 99.8%; LANXESS, Pittsburgh, PA) solution was prepared by a 1:4 dilution in 100% ethyl alcohol to yield a stock solution with a concentration of 312.5 x 10^3 parts per million (ppm). Specifically, 200 µL of fresh DMDC were added to 600 µl of ethyl alcohol to achieve a 1:4 dilution. Aliquots of 28 µl and 32 µl of DMDC stock solution were added to 50 mL orange juice to reach final concentrations 172 ppm and 200 ppm, respectively. The DMDC treatments of 172 and 200 ppm were selected as levels that are more commonly employed by the juice and beverage industry, and recommended by the commercial manufacturer of DMDC to ensure that levels of DMDC are not higher than regulatory limits due to the variability of the DMDC dosing apparatus.

1.3.3 Orange juice preparation and Salmonella inoculation

Salmonella serotypes were streaked out and grown on Brain Heart Infusion (BHI) plates (Difco, Detroit, MI). Single colonies were used to inoculate 5 mL BHI liquid media and were grown overnight at 37°C, tilted and shaking at 150 rpm for 10 – 12 hrs. 500 µl of each serotype was mixed to form a cocktail mixture of five *Salmonella* serotypes. 500 µl of the cocktail mixture was added to 50 ml of pasteurized orange juice to achieve a starting bacterial

concentration of 10^7 CFU/ml. Pasteurized orange juice was used to study the effects of DMDC on *Salmonella* without competing microorganisms.

DMDC was added to achieve concentrations of 172 ppm and 200 ppm. Orange juice samples containing *Salmonella* without DMDC were used as controls. Samples were incubated at both 4°C and 21 ± 3 °C. Samples were collected at 0, 1, 2, 4, 6, 24, 48, 72, and 96 hrs. Samples were then serial diluted in phosphate buffered saline (PBS). 100 µl of serial dilutions were spread plated on Standard Plate Count (SPC) and Xylose Lysine Deoxycholate (XLD) agar plates (Sigma-Aldrich, St. Louis, MO). The plates were incubated at 37°C for 48 hrs, and then enumerated to determine the CFU/ml at each time point. Each of the enumerations for the respective media were averaged and converted into log numbers. The *Salmonella* inoculation and DMDC experiments were conducted in triplicate.

1.3.4 Orange juice preparation and fungal spoilage growth

Fresh, unpasteurized orange juice was left at an ambient temperature of 21°C overnight (12-16 hrs) to simulate temperature abuse and to achieve natural microbiota growth of $10^5 – 10^6$ CFU/ml on acidified potato dextrose agar (aPDA) (Sigma-Aldrich, St. Louis, MO) and Standard Plate Count Agar (SPC) (Sigma-Aldrich, St. Louis, MO). DMDC was added to achieve the concentrations of 172 ppm and 200 ppm. Temperature abused orange juice without DMDC were used as controls. Samples were incubated at both 4°C and 21 ± 3 °C. Samples were collected at 0, 1, 2, 4, 6, 24, 48, 72, and 96 hrs. Samples were then serial diluted in PBS. 100 µl of serial dilutions were spread plated on SPC and aPDA. The plates were incubated at 30°C for 48 hrs, and then enumerated to determine the CFU/ml at each time point. Each of the enumerations for

the respective media were averaged and converted into log numbers. Fungal spoilage and DMDC experiments were conducted in triplicate.

1.3.5 Statistical analysis

The statistical software R (R Core Team, Vienna, Austria) and package lme4 were used to fit linear mixed effects regression models. Means and post-hoc comparisons were estimated from the model using the lsmeans package. Significant relationships and analysis were determined based on initial populations and at time points where the DMDC reaction had been fully exhausted. CFU/ml per time point were converted to log CFU/ml and averaged with the standard deviation. Due to the method of plating, the lowest observable counts are recorded at 10 CFU/ml.

Table 1.1. Effect of different concentrations of DMDC on *Salmonella enterica* and spoilage microorganisms at 4°C and 21°C.

	4°C			21°C		
	0 ppm DMDC	172 ppm DMDC	200 ppm DMDC	0 ppm DMDC	172 ppm DMDC	200 ppm DMDC
Salmonella enterica (log CFU/ml)						
0 hr	*7.66*	*7.65*	*7.70*	*7.69*	*7.74*	*7.74*
1 hr	7.66	5.55	5.15	7.74	5.31	4.48
2 hr	7.63	4.42	3.12	7.75	4.41	4.00
4 hr	7.67	4.10	3.55	7.73	4.25	4.11
6 hr	7.65	4.00	3.44	7.66	4.09	4.12
24 hr	*7.67*	***2.19***	***1.52***	*7.58*	***4.00***	***3.98***
48 hr	7.55	2.31	1.44	7.19	3.72	3.84
72 hr	7.23	2.27	1.43	7.06	3.81	3.81
96 hr	7.40	2.47	1.63	6.90	3.58	3.77

Yeast and Mold (log CFU/ml, aPDA)						
0 hr	*6.84*	*6.58*	*6.67*	*5.82*	*5.93*	*5.82*
0 hr*	NA	***3.83***	***3.32***	NA	ND	***1.00***
1 hr	6.76	3.72	3.31	6.13	ND	1.00
2 hr	6.72	3.27	3.19	6.19	ND	ND
4 hr	6.68	3.26	3.20	6.55	1.00	ND
6 hr	6.83	3.41	3.29	6.67	1.14	ND
24 hr	6.38	3.74	3.51	6.52	2.65	1.75
48 hr	6.23	3.66	3.60	6.80	5.98	4.40
72 hr	5.05	2.86	2.79	7.09	6.99	6.69
96 hr	4.99	2.83	2.78	7.33	6.82	6.85
Yeast and Mold (log CFU/ml, SPC)						
0 hr	7.16	6.94	7.20	6.25	6.33	6.14
0 hr*	NA	5.31	4.68	NA	1.53	1.23
1 hr	7.19	4.13	3.94	6.40	1.52	1.39
2 hr	7.15	3.85	3.77	6.44	1.39	1.39
4 hr	7.09	3.85	3.64	6.69	1.25	1.30
6 hr	7.18	3.79	3.73	6.88	1.65	1.58
24 hr	7.08	3.98	3.96	6.66	2.82	2.17
48 hr	6.75	4.03	4.04	6.78	6.06	4.56
72 hr	5.41	3.14	3.12	7.25	7.05	6.95
96 hr	5.32	3.22	3.27	7.37	6.88	6.97

ND: not detected, NA: not applicable, *: sampled immediately after treatment, *italic*: point where significance was compared, **bold**: significant within the condition ($p \leq 0.01$)

1.4 Results

1.4.1 Reduction of Salmonella in orange juice

Salmonella strains were grown and inoculated into orange juice to approximately 10^7 CFU/ml and dosed with DMDC to determine the effect on the microbial population. Experiments were performed at ambient (21 ± 3 °C) and refrigeration (4°C) temperatures. The bacterial populations under each experimental condition were enumerated and converted to log numbers (Table 1.1). Results show that at the 24-hour time period at 4°C, the orange juice treated with 172 ppm and 200 ppm DMDC achieved a 5.46 and 6.2 log reduction, respectively (Figure 1.1). After 24 hours, the bacterial population increased by less than 1 log from 48 to 96 hours. The decimal reduction time (D-value), the time required to reduce the microbial population by 90% (or 1 log), was calculated for both concentrations and were found to be 37.10 min at 172 ppm and 26.16 min at 200 ppm.

At the ambient temperature for the 24-hour time period, the 172 ppm and 200 ppm treated samples achieved a 3.74 and 3.76 log reduction, respectively (Figure 1.1). The bacterial population continued to decrease by less than 1 log up to 96 hours for both DMDC concentrations. D values were again calculated and were found to be 35.98 min at 172 ppm, and 33.42 min at 200 ppm.

For both temperature conditions, the initial bacterial populations of all samples had no significant differences. However, at 24 hours, concentrations 172 and 200 ppm were found to be significantly different ($p \leq 0.01$) from the control (0 ppm), but not from each other.

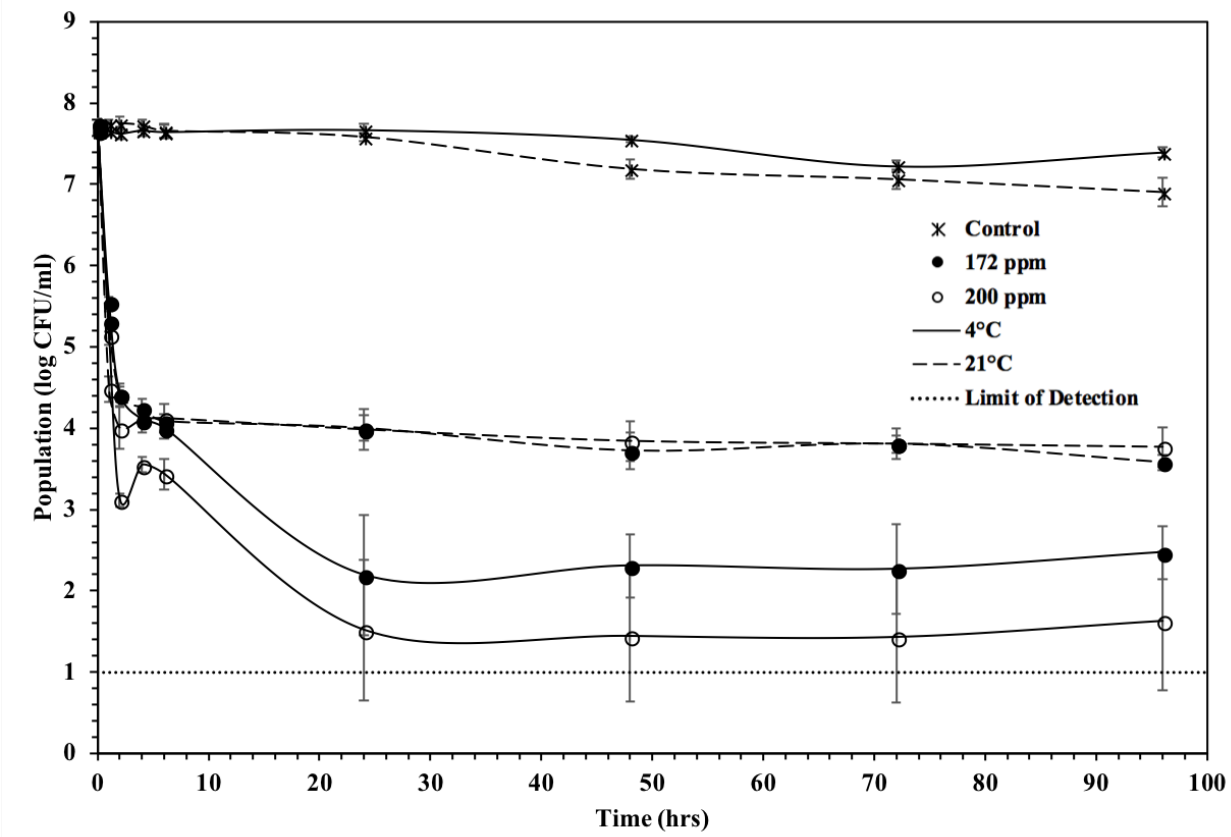

Figure 1.1. Population of *Salmonella* in pasteurized orange juice treated with DMDC at 4°C (refrigeration temperature) and 21 ± 3°C (ambient temperature).

1.4.2 Reduction of yeasts and molds in temperature abused orange juice

DMDC was also used to treat orange juice samples that had high counts of fungal spoilage microorganisms associated with fresh or unpasteurized orange juice. Initial microbial counts were approximately 10^5 – 10^6 CFU/ml and showed no significant difference. At 4°C, the untreated control showed a decreasing population of more than 1.85 log (aPDA) and 1.84 log (SPC) after 96 hours. For DMDC treated samples, total counts were taken immediately after treatment, which yielded 2.75 and 3.35 log reductions (aPDA) and 1.63 and 2.52 log reductions (SPC) at 172 and 200 ppm, respectively (Figures 1.2 & 1.3). At this time point, the populations of the treated samples were significantly different ($p \leq 0.01$) from the untreated population, as well as each other. Microbial counts continued to decrease for the extended period by 96 hours,

leading to a 3.75 and 3.89 log reduction (aPDA) and 3.72 and 3.93 log reductions (SPC) for 172 ppm and 200 ppm, respectively.

At ambient temperature, the microbial counts of the untreated control gradually increased by more than 1 log after 96 hours. Immediately after the addition of DMDC at ambient temperature, the microbial load was reduced to levels at or below the limit of detection, yielding 4.82 and greater than 4.93 log reductions (aPDA) and 4.80 and 4.91 log reductions (SPC) for 200 ppm and 172 ppm, respectively (Figures 1.2 & 1.3). The CFU/ml for both concentrations at this time point were found to only be significantly different ($p \leq 0.01$) from the initial population. Additionally, the microbial population remained at less than 10^1 CFU/ml from immediately after treatment with 172 ppm DMDC, up to the 4-hour time point. The population then increased by 5.82 log numbers from 4 to 96 hours. At 200 ppm, the microbial population dropped to $< 10^1$ CFU/ml within 2 to 6 hours. The population then increased by 5.85 log numbers from 24 to 96 hours. By 96 hours, DMDC treated samples at both concentrations had microbial counts greater than the initial population count.

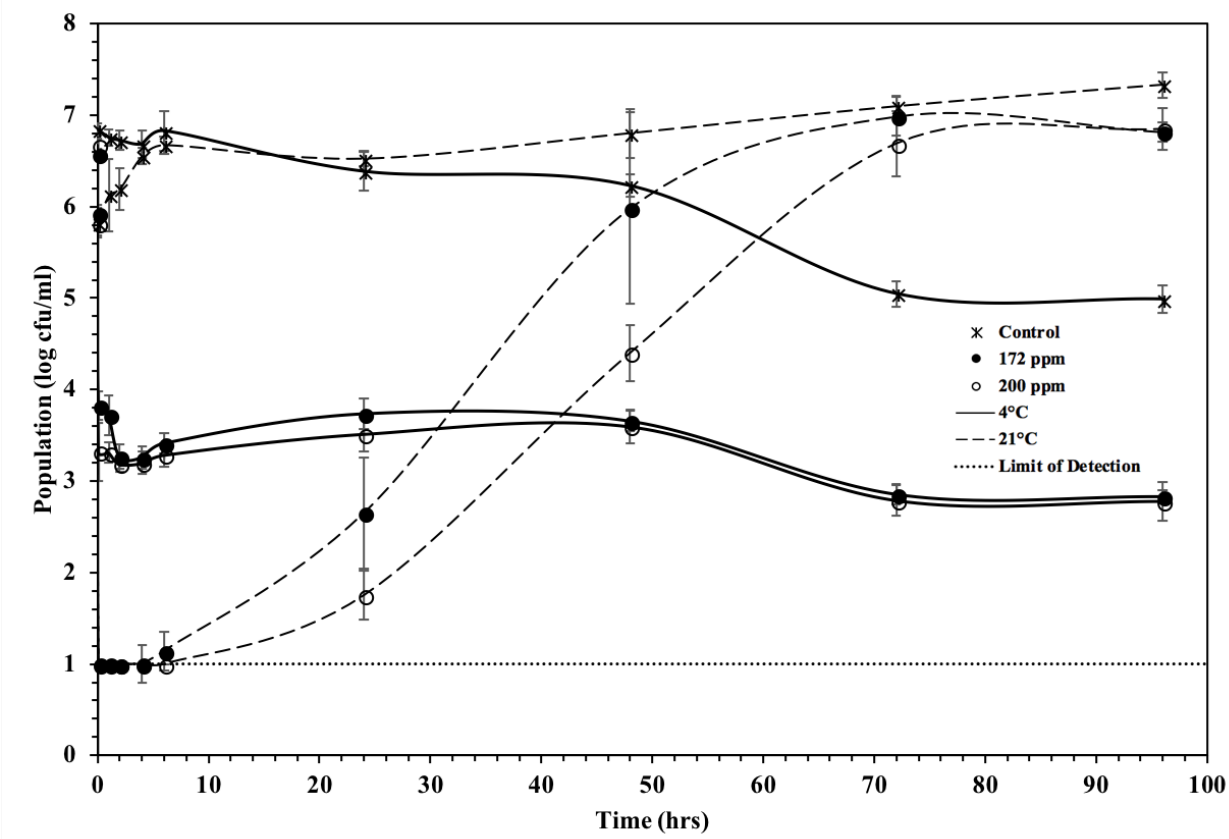

Figure 1.2. Population of fungal spoilage (aPDA) in unpasteurized orange juice treated with DMDC at 4°C (refrigeration temperature) and 21 ± 3°C (ambient temperature).

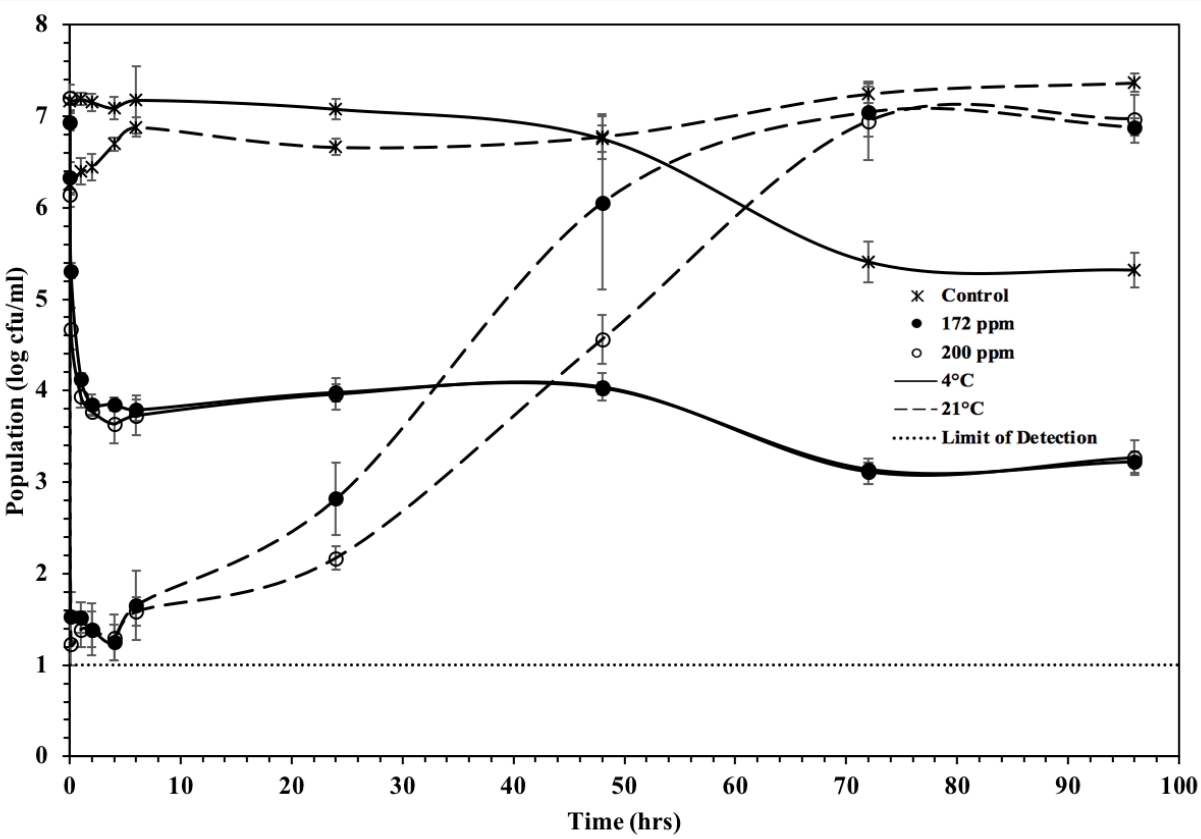

Figure 1.3. Population of microbial spoilage (SPC) in unpasteurized orange juice treated with DMDC at 4°C (refrigeration temperature) and 21 ± 3°C (ambient temperature).

1.5 Discussion

Salmonella has been a health concern for unpasteurized orange juice since 1995, especially in the wake of salmonellosis outbreaks (Danyluk et al., 2012). Prior to these events, orange juice was considered a low risk beverage given low pathogen growth and the high level of safety due to low pH conditions (Parish et al., 1997). However, one study has found that at high concentrations, *Salmonella* can survive in pH as low as 3.5 up to 27 days, and even longer at a higher pH (Parish et al., 1997). Unpasteurized juice is generally stored and kept up to 10 – 16 days at 4.4°C, which is prone to the risk of foodborne illness (Fellers, 1988).

As the juice industry trend has shifted towards nontraditional and pasteurized juices, there is an increasing interest in alternative processing measures to achieve a safe, fresh product.

DMDC, previously listed as a yeast inhibitor in wine, is currently defined by FDA as a microbial control agent for use as a supplemental safety control in beverages (USFDA, 2001). DMDC is known to inactivate yeast and has been reported to be effective at inactivating molds and bacteria (Golden et al., 2005). DMDC reacts rapidly and easily with certain compounds, such as imidazoles or amines, which contain functional groups commonly found in amino acids. The reaction results in methylation of proteins containing such groups, which leads to active site blocking and conformational changes that can cause enzyme inhibition, and eventually, cell death (Golden et al., 2005). At the end of the reaction, DMDC is hydrolyzed into carbon dioxide and methanol, in sufficiently low levels that is deemed acceptable by the FDA, and in accordance with the maximum DMDC concentration regulations (USFDA, 2001).

In this study, pasteurized and unpasteurized orange juices were used to determine the effects of DMDC on pathogenic and spoilage microorganisms. It was found that the two concentrations of DMDC used were successful in inactivating *Salmonella* at 4°C, achieving a 5-log reduction. Both non-selective and selective growth media were used to enumerate plate counts for to avoid any discrepancies of a selective growth medium. Results of using a non-selective medium were similar to that of the selective medium. However, it was found that due to the stress conditions the selective medium created, the *Salmonella* inoculated plates needed to be incubated for a longer period of time (48 hrs) to recover injured, but viable colonies for a complete total plate count. For the tests concerning yeasts and molds, DMDC was shown to have an immediate effect on inactivating yeast and mold growth. Due to the acidic environment of orange juice and the comparable colony counts from both SPC and aPDA in Table 1.1, the most common microorganisms in the temperature-abused juice were determined to be yeast. Results indicated that the residual yeast population left after treatment rapidly increased back to its

original population over several days. Once the DMDC reaction reaches completion, the products left in the juice are carbon dioxide and methanol, which are not capable of controlling microbial growth at low concentrations and allows for repopulation of microorganisms if left under desirable growth conditions.

As DMDC is nonspecific and temperature dependent, it will react with proteins found in solution, whether they are from the microorganism or juice itself, and the rate of reaction will increase with higher temperatures (Golden et al., 2005). The results suggest that DMDC should be applied to room temperature juice for a faster rate of inactivation. However, to inhibit bacterial and fungal growth, the juice should be stored at cold temperatures, which slows growth. It should be noted that DMDC is highly effective in small doses and does not have to be labeled on juice products as an additive since it is considered a processing aid (Basaran-Akgul et al., 2009).

In conclusion, this study has shown that DMDC is effective at achieving a 5-log reduction in *Salmonella* at both 172 and 200 ppm, with a holding time of more than 24 hrs at 4°C. DMDC is also effective at inactivating yeasts in a relatively short amount of time, thus potentially extending the shelf life of fresh juice. Given the challenges often met in the control of spoilage microorganisms, especially regarding fresh juices, DMDC appears to be ideal and most effective if used in conjunction with other processing steps, either as a pre-step, with an additional preservation system, or another non-thermal processing method, such as high-pressure processing. This alternative method would provide an additional microbial safety measure and quality control for fresh, unpasteurized juice producers to control for both pathogenic and spoilage microorganisms.

1.6 Acknowledgements

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CHAPTER 2

EFFECTS OF WATER PROCESSING TEMPERATURE ON FOODBORNE PATHOGEN INACTIVATION IN FRUIT JUICES USING HIGH PRESSURE PROCESSING (HPP)

2.1 Abstract

High pressure processing (HPP) is a non-thermal processing method used to inactivate pathogens and preserve the fresh qualities in certain food products. The juice industry has seen a shift towards functional and nutritional juices, which has led to an exploration of alternative non-thermal processes that ensure desirable qualities as well as guarantee the safety of the food. HPP is an important method to this trend, where the main factors that can be controlled, including time, pressure, and temperature, remain to be systematically characterized. This study was carried out to investigate the effects of water processing temperature on HPP and pathogen inactivation. Three fruit juices (apple, orange, and grape) were chosen for the study and processed under two temperatures to determine the microbial reduction on key pathogens. Five strains of each pathogen (*E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*) were grown and separately inoculated into apple, orange, and grape juice (pH 4.5) at 5°C and 20°C. Samples were processed by HPP for 1 minute at 550 MPa using water temperatures corresponding to the holding temperature (5°C or 20°C) and immediately plated on selective media. It was found that in apple juice, only *Listeria* could achieve a greater than 5-log reduction at 5°C, while all pathogens tested achieved a 5-log reduction at 20°C. In orange juice, there was a greater than 5-log reduction of *Salmonella* and *Listeria* at both temperatures. However, in grape juice, only *Listeria* achieved a greater than 5-log reduction under all testing conditions. The results suggest that the processing temperature plays a significant role in pathogen

inactivation by HPP and various pressure tolerances of the tested pathogens have been demonstrated. The outcome of the study provides additional information for establishing microbial safe harbors for HPP treatment of foods, and to assist juice processors to guarantee the safety of their HPP processed juices.

2.2 Introduction

The current trend of the juice industry is moving away from traditional juices and toward products with functional benefits from a variety of fruits and vegetables, that retain fresh qualities and characteristics. Most commercial juices are pasteurized, thermally processed to kill any pathogenic bacteria as a means to ensure their safety. The U.S. Food & Drug Administration (FDA) has regulated that all processes to inactivate pathogens must achieve a 5-log reduction (USFDA, 2004). Thermal processing, or pasteurization, has long been studied, with published research on creating general guidelines and safe harbors for juice pasteurization. The FDA recommends that juices with a pH of less than 4.0 to be pasteurized at 71.1°C (160°F) for 3-6 seconds to ensure a 5-log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* (Mak et al., 2001; Mazzotta, 2001). However, thermal pasteurization can cause undesirable product composition changes, such as loss of vitamins, flavor, color, and general nutritional values (Hogan et al., 2005; Huang et al., 2017). The consumer-based demand for fresh and functional food products has then been a driving force for alternative processing treatments to ensure food safety.

High pressure processing (HPP) relies on water pressure to inactivate microorganisms. It is a non-thermal or cold process, and mostly utilized for products that could be damaged or impacted by high heat treatments. Unlike thermal processing, HPP has minimal

effect on desirable qualities found in a fresh-like product, retaining vitamins, flavor and color compounds (Georget et al., 2015; Oey et al., 2008b). High pressure processing, when compared to thermal processing, is relatively understudied, which necessitates additional studies to guarantee safe parameters for product processing. Currently, HPP has been utilized to process products such as guacamole, salad dressings, jams, and fruit and vegetable juices and beverages. Products that have been high pressure processed must be kept in cold storage after processing, necessitating HP processors to maintain their facilities at refrigeration temperatures (Balasubramaniam et al., 2016; Martínez-Monteagudo and Balasubramaniam, 2016). HPP does not inactivate all enzymatic activity, and studies have shown that some HPP products must be stored at refrigerated temperatures or risk enzymatic browning (Oey et al., 2008a). Therefore, storage at low temperatures serves the purpose to control both enzymatic properties of the product and slow or inhibit any microbial growth (IFT/FDA, 2003). For most pathogenic bacteria, ambient temperatures are favorable for rapid growth, as most require minimum growth temperatures of 10°C (50°F) (USFDA, 2019). Microorganisms also have an optimum pH range required for growth, and at low pH (< pH 4.6) most microbial growth is inhibited (Beales, 2004). Storing acidic products at refrigerated temperatures helps to control both of these issues.

Temperature is one of the main parameters that can be adjusted and controlled during the HP process. As a nonthermal process, products can be treated by HPP at ambient and refrigerated temperatures (Lado and Yousef, 2002). Low temperatures can slow or inhibit bacteria growth. However, at mild or ambient temperatures, bacterial growth and toxin production can proliferate (Lado and Yousef, 2002). Commercial HPP products are typically processed at refrigerated temperatures. There are several studies regarding the efficacy of HPP against pathogens that have been conducted at ambient temperatures (Alpas et al., 2000; Dogan

and Erkmen, 2004; Hiremath and Ramaswamy, 2012; Patterson et al., 1995; Ramaswamy et al., 2008; Shigehisa et al., 1991). However, as previously mentioned, most commercial HP processors treat their products at refrigerated temperatures.

The purpose of this study is to determine if HPP water processing temperature in a commercial scale HP processing unit has an effect on pathogen inactivation and the differences in pathogen pressure tolerance at two treatment temperatures if these effects exist. This study was carried out in three fruit juices (apple, orange, and grape). These juices were inoculated with three pertinent pathogens, *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* and processed at 5°C (refrigeration temperatures) and 20°C (ambient temperatures) for 1 minute at 550 MPa.

2.3 Materials and methods

2.3.1 Juice and bacterial strains

Three types of shelf stable juice were used for this study. Apple (Wegmans, Geneva, NY), orange (Walmart, Geneva, NY), and white grape juice (Wegmans, Geneva, NY) were purchased from local supermarkets. Juices were stored at ambient temperatures before brought to the targeted temperature (5°C or 20°C) before use, depending on the conditions specified in the study. All juices were evaluated for °Brix and water activity values (Table 2.2).

Five strains or serotypes of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* were used for this work. The strains, serotypes and origins are shown in Table 2.1. Strains and serotypes were streaked out on Tryptic Soy Agar (TSA) (Alpha Biosciences, Baltimore, MD) from frozen cultures maintained at -80°C and re-streaked on fresh agar monthly.

Table 2.1. List of strain or serotype of each pathogen with origin.

Pathogen	Strain or serotype	Origin	Lab code
E. coli O157:H7	C7927	Human isolate, apple cider linked to an outbreak (date of outbreak unavailable)	E1
	ATCC 43890	Human isolate, date of outbreak unavailable	E2
	ATCC 43894	Human isolate, date of outbreak unavailable	E3
	ATCC 43889	Human isolate, date of outbreak unavailable	E4
	ATCC 35150	Human isolate, date of outbreak unavailable	E5
Salmonella enterica	Hartford H0778	Orange juice, US outbreak in 1995	S1
	Typhimurium FSL R9-5494	Orange juice, multistate US outbreak in 2005	S2
	Muenchen FSL R9-5498	Alfalfa sprouts, multistate US outbreak in 2016	S3
	Javiana FSL R9-5273	Tomatoes, multistate US outbreak in 2002	S4
	Enteriditis FSL-R9-5505	Beans sprouts, multistate US outbreak in 2014	S5
Listeria monocytogenes	Lineage I, serotype 4b FSL J1-108	Coleslaw, US outbreak in 1981	L1
	Lineage I, serotype 4d FSL J1-107	Coleslaw, US outbreak in 1981	L2
	Lineage II, serotype 1/2a FSL R9-0506	Cantaloupe, US outbreak in 2011	L3
	FSL R9-5411	Caramel Apple, multistate US outbreak 2014-2015	L4
	FSL R9-5506	Packaged Salad, multistate US outbreak in 2016	L5

2.3.2 High pressure processing specifications

A commercial scale 55L Hiperbaric High Pressure Processing machine was used to conduct this experiment (Hiperbaric, Burgos, Spain). Water processing temperature was adjusted to either 5 ± 3°C or 20 ± 3°C. Samples were processed at 550 MPa (79,991 psi) for 1 minute.

2.3.3 Juice preparation and pathogen inoculation

Pathogen strains and serotypes were streaked out and grown on TSA plates. Single colonies were used to inoculate 5 ml Tryptic Soy Broth (TSB) (Alpha Biosciences, Baltimore, MD) and were grown overnight (20 ± 2 hr) at 35°C, tilted and shaking at 175 RPM. 1 ml of each serotype was mixed to form a cocktail mixture for each pathogen.

Each juice was adjusted to pH 4.50 using 50% w/w sodium hydroxide and aliquoted into 4 oz. bottles. Each bottle was then adjusted to the targeted processing temperature of 5 ± 3°C or 20 ± 3°C before inoculated with 1 ml of the desired pathogen cocktail to achieve a starting bacterial concentration of 10^7 CFU/ml. Bottles were then capped and sealed. The samples to be

processed were then placed in a plastic bag filled with calcium hypochlorite solution (300 ppm) at the required temperature. The hypochlorite solution was used as a means to prevent contamination of the HPP unit in the event of a container breach of the pathogen inoculated samples. The bags were sealed using a vacuum sealer and sealed again in an additional bag. Two bottles per pathogen per experiment condition were prepared – one control sample and one sample to be processed. The control sample was plated after inoculation to obtain the initial counts. Samples were serial diluted in Bacto™ Peptone (Becton Dickinson, Sparks, MD). One milliliter of serial dilutions was plated using the pour plate method with the corresponding selective media. Violet Red Bile Agar (Alpha Biosciences, Baltimore, MD) was used for *E. coli* detection, Bismuth Sulfite Agar (Becton Dickinson, Sparks, MD) was used for *Salmonella*, and Oxford Listeria Agar (Alpha Biosciences, Baltimore, MD) with Modified Oxford Antimicrobic Supplement (Becton Dickinson, Sparks, MD) was used for *Listeria* enumeration. Samples that were high pressure processed were plated using the same method for the control samples. The plates were incubated at 35°C for 48 hr and then enumerated to determine the CFU/mL, averaged and converted into log numbers. Three biological replicates were conducted per juice, pathogen, and experimental processing condition.

2.3.4 Statistical analysis

The statistical software R (R Core Team, Vienna, Austria) and package lme4 were used to fit linear mixed effects regression models. Means and post-hoc comparisons were estimated from the model using the lsmeans package. Significant relationships and analysis were determined based on the log reductions derived from initial control populations and after processing at each temperature. CFU/ml were converted to log CFU/ml and averaged with the

standard deviation. Due to the method of plating, the lowest observable counts are recorded at 1 CFU/ml.

2.4 Results

Table 2.2. Average °Brix and water activity (a_w) measurements of each juice adjusted to 4.5 pH and the effect of HPP water processing temperature on pathogen inactivation, represented by pathogen reduction averages and standard deviation. HPP was conducted at 550 MPa for 1 min.

Apple Juice	*°Brix*	a_w
	11.8	0.9875
Pathogen Reduction (log CFU/mL)	*5°C*	*20°C*
E. coli O157:H7	**2.54** ± 0.53	**6.66** ± 0.52
Salmonella enterica	4.90 ± 0.44	5.73 ± 0.04
Listeria monocytogenes	7.06 ± 0.06	7.02 ± 0.06
Orange Juice	*°Brix*	a_w
	11.7	0.9901
Pathogen Reduction (log CFU/mL)	*5°C*	*20°C*
E. coli O157:H7	2.92 ± 0.63	3.09 ± 0.22
Salmonella enterica	5.10 ± 0.73	5.34 ± 0.15
Listeria monocytogenes	7.18 ± 0.16	7.27 ± 0.07
Grape Juice	*°Brix*	a_w
	16.1	0.9763
Pathogen Reduction (log CFU/mL)	*5°C*	*20°C*
E. coli O157:H7	**1.15** ± 0.73	**4.65** ± 0.33
Salmonella enterica	**1.77** ± 0.06	**4.23** ± 0.28
Listeria monocytogenes	7.10 ± 0.10	7.19 ± 0.02

Bold: significantly different within the condition ($p \leq 0.01$)

2.4.1 Reduction of pathogens in apple juice

All strains were grown and inoculated into juice to approximately 10^7 CFU/ml and then high pressure processed at 550 MPa for 1 min. Experiments were performed at refrigeration (5 ± 3°C) and ambient (20 ± 3°C) temperatures. Before HPP, juice samples were measured for °Brix and water activity (Table 2). Results show that when inoculated apple juice is high pressure processed at 5°C, log reductions of 2.54, 4.90, and 7.06 were found for *E. coli*, *Salmonella*, and *Listeria* respectively, while log reductions of 6.66, 5.73, and 7.02 were achieved at 20°C (Figure 2.1).

Comparing the two processing temperatures, there were no significant differences found between the log reductions in *Salmonella* and *Listeria* at either temperature in the tested apple juice. However, the log reductions of *E. coli* were found to be significantly different ($p \leq 0.01$) when compared at 5°C against 20°C.

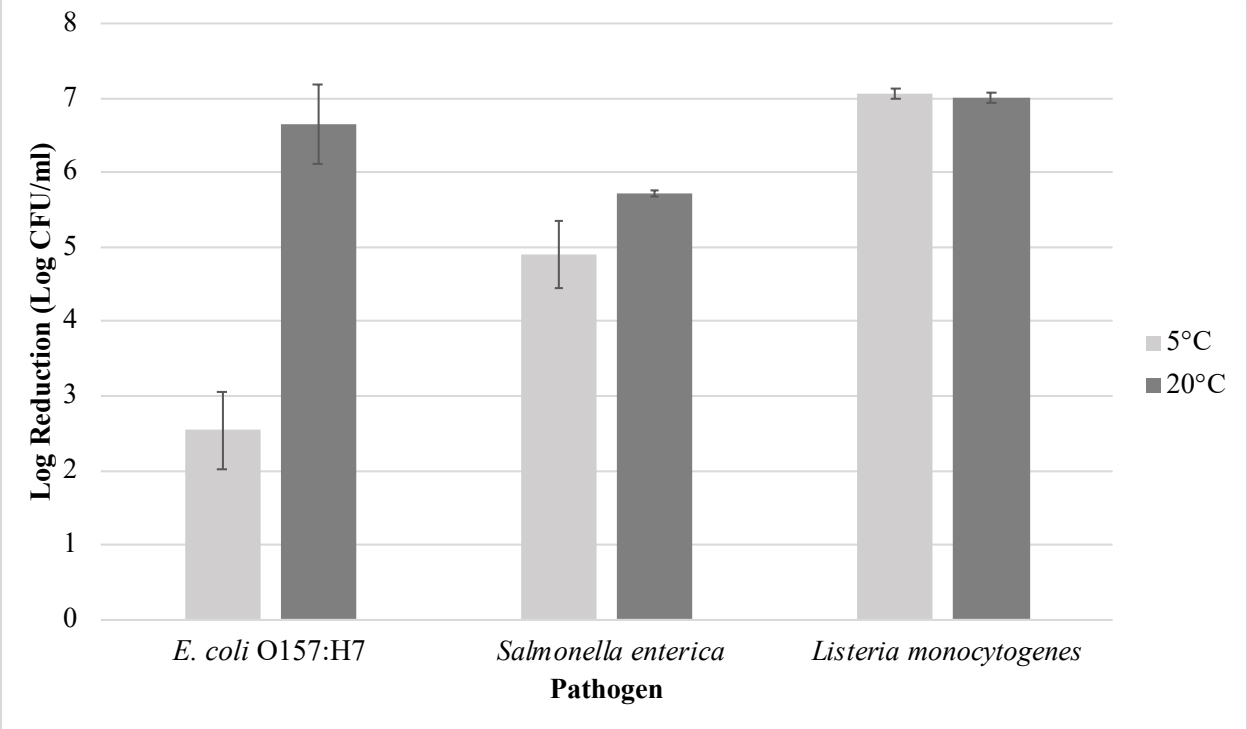

Figure 2.1. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* due to HPP at 550 MPa for 1 min, at 5°C (refrigeration temperature) and 20°C

(ambient temperature) in apple juice adjusted to 4.5 pH. Error bars represent standard deviation of the log reductions averages.

2.4.2 Reduction of pathogens in orange juice

The same experimental conditions were repeated in all three types of juice. The results for orange juice show log reductions of 2.92, 5.10 and 7.18 at 5°C and 3.09, 5.34, and 7.27 at 20°C for *E. coli*, *Salmonella*, and *Listeria*, respectively (Figure 2.2). For all three pathogens, there were no significant differences found between the two processing temperatures.

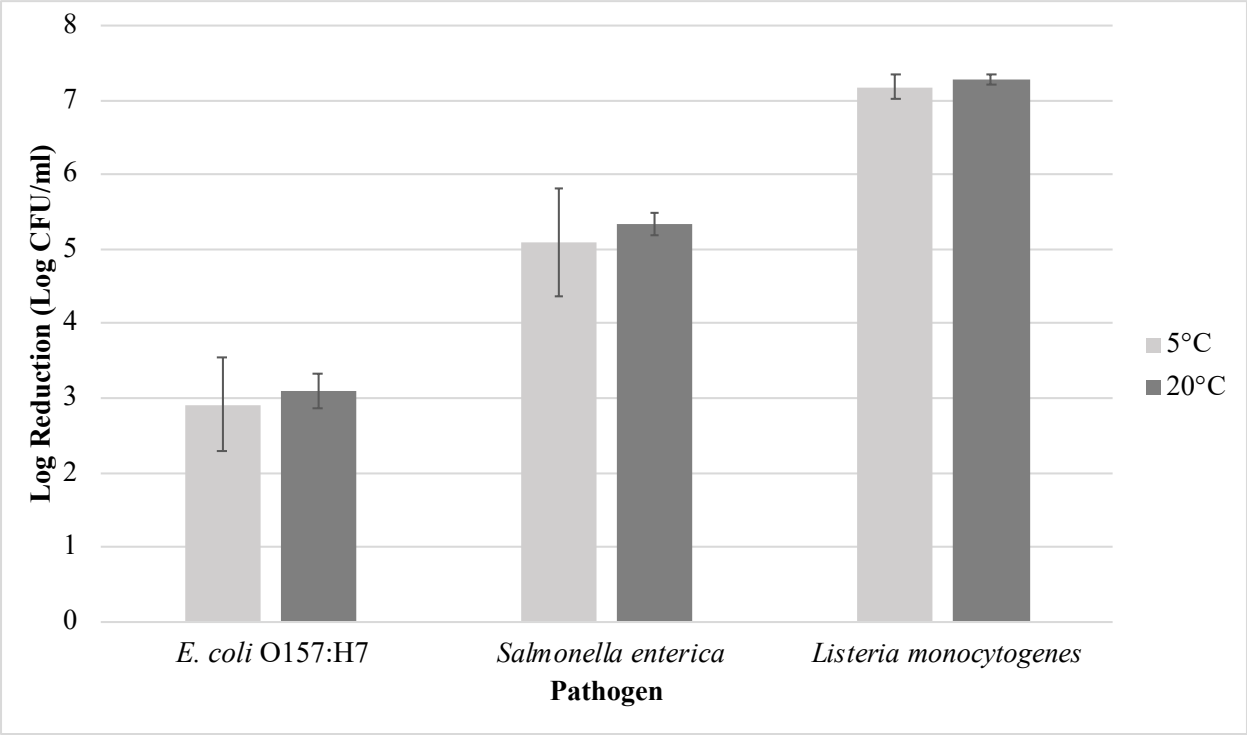

Figure 2.2. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* due to HPP at 550 MPa for 1 min, at 5°C (refrigeration temperature) and 20°C (ambient temperature) in orange juice adjusted to pH 4.5. Error bars represent standard deviation of the log reduction averages.

2.4.3 Reduction of pathogens in grape juice

In grape juice, the log reductions for *E. coli*, *Salmonella*, and *Listeria* were 1.15, 1.77, and 7.10 at 5°C and 4.65, 4.23, and 7.19 at 20°C (Figure 2.3). There were no significant

differences found between the two processing temperatures for *Listeria*. However, for both *E. coli* and *Salmonella*, the log reductions at 5°C were found to be significantly different ($p \leq 0.01$) from the log reductions achieved at 20°C.

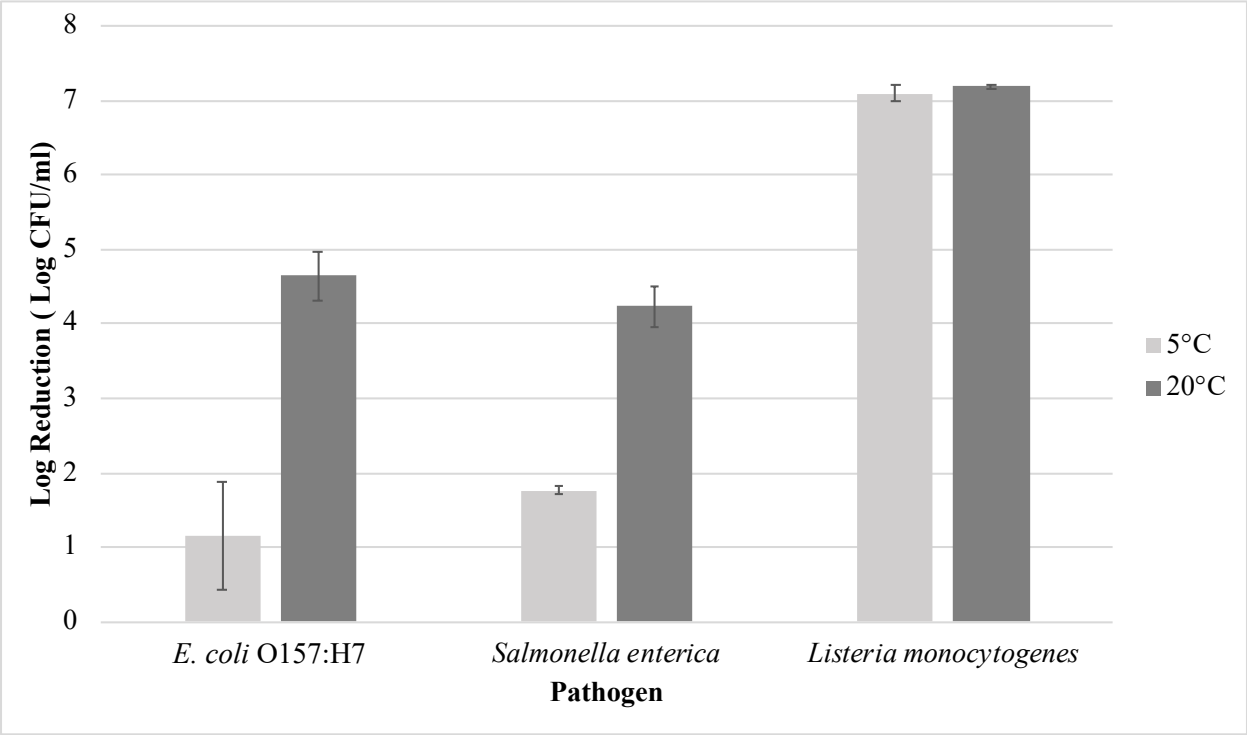

Figure 2.3. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* due to HPP at 550 MPa for 1 min, at 5°C (refrigeration temperature) and 20°C (ambient temperature) in white grape juice adjusted to pH 4.5. Error bars represent standard deviation of the log reduction averages.

2.5 Discussion

We conducted this study at both refrigerated (5°C) and ambient temperatures (20°C) to determine the overall effect of processing temperature on pathogen inactivation and to compare the pressure tolerances of the three pathogens tested. All three types of juice used were adjusted to pH 4.5 as a conservative measure to limit acid effects, as most bacteria have hindered growth below pH 4.6, and to stay within the typical pH range of acidic fruit juices (IFT/FDA, 2003). Current industrial HPP conditions utilize 600 MPa for 3 minutes to ensure a safe product.

Samples for this study were processed at a lower pressure and time of 550 MPa for 1 minute to study differences in pathogen response and inactivation. Using these processing conditions would also provide insight whether the combination of lower conditions would provide sufficient measures to ensure safety. Significant differences in log reductions were found in both apple juice and grape juice when processed at refrigerated temperatures and ambient temperatures. Under these processing conditions, a greater than 5-log reduction was achieved for *E. coli* and *Salmonella* at 20°C, and for *Listeria* at both temperatures in apple juice. In orange juice, temperature seemed to have less of an impact, as no significant differences were found between the log reductions at 5°C and 20°C. In terms of inactivation, *E. coli* was not able to achieve the 5-log reduction whereas *Salmonella* and *Listeria* could at both processing temperatures. The most pathogen resistance was found in grape juice, as the microbial counts of *E. coli* and *Salmonella* are barely reduced at 5°C. Only *Listeria* is able to achieve a greater than 5-log reduction at both temperatures. In all cases where significance was determined, the log reductions of pathogens at ambient temperatures were higher than at refrigerated temperatures.

These results clearly suggest that the HPP treatment is more effective at inactivating pathogens at ambient temperatures. However, as seen with the data, inactivation is also largely dependent on the product composition and the pathogen. In this study, more conservative conditions were used with an adjusted pH of 4.5 and processing time of 1 minute to elucidate and establish differences between temperature treatments. The resulting data could assist in determining the processing minimums needed to establish a 5-log reduction of certain pathogens for juice producers.

This study has also revealed the pressure tolerance differences among the three pathogens tested. *E. coli*, as seen in all three juices, appeared to be the most resistant pathogen to pressure at

refrigeration temperatures. Low inactivation rates observed at 5°C suggests that at low temperatures, *E. coli* has a more noticeable tolerance to high processing pressures. It has been reported that gram positive bacteria tend to be more pressure resistant due to their thicker peptidoglycan layer (Considine et al., 2008; Georget et al., 2015; Smelt, 1998). However, at pH 4.5 in all three fruit juices tested, *Listeria* was found to be completely inactivated under all experimental conditions, suggesting pressure tolerances depend on the product type and composition as well.

HPP products currently have to be validated by a process authority to achieve a 5-log reduction of the pertinent pathogens. For juice, this includes *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* (USFDA, 2004). The results have shown that pathogen inactivation is less effective at refrigeration temperatures, which could be attributed to pathogen pressure tolerances at lower temperatures. Since HPP is a cold temperature process, to safeguard a successful process validation, juice producers should consider employing hurdle technology to ensure food safety. These would include increased acidity (low pH), high water activity, changes in processing conditions (time and pressure), and other preservation methods. Government agencies must also be strict and cautious regarding validation parameters. A successful process validation completed at 20°C cannot be authorized for processing the same product at 5°C, as higher processing temperatures can possibly result in inflated inactivation rates. Temperature is only one of the many parameters involved in high pressure processing, and with more comprehensive studies and data, general safety guidelines for HPP could be developed and processing minimums and maximums established.

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and Inactivation in: Fish and Fishery Products Hazards and Controls Guidance Fourth Edition.

CHAPTER 3

EVALUATING THE EFFECTS OF PH AND ACID TYPE ON FOODBORNE PATHOGEN INACTIVATION IN FRUIT JUICES UTILIZING COMMERCIAL SCALE HIGH PRESSURE PROCESSING (HPP)

3.1 Abstract

Acid and low pH are commonly used as methods for food preservation due to antimicrobial effects and growth inhibition. Most fruit juices are considered to be acidic, with pH lower than 4.6. However, many foodborne outbreaks from unpasteurized fruit juices have been reported. High pressure processing (HPP) is a nonthermal processing method that utilizes high pressure to inactivate pathogens while maintaining fresh qualities of the product. Studies have indicated that the efficacy of HPP depends on several parameters, including the factors of the process that can be controlled (time, temperatures, pressure) and the product composition, such as pH. The effects of pH in fruit juices in combination with HPP on pathogen inactivation have yet to be fully characterized. This study was carried out to provide a comprehensive analysis of the acid effects of both pH and acid type in fruit juice products. The study was conducted in two parts: evaluating the effects of pH in fruit juices (apple, orange, grape) at a pH range from the initial pH of the product, 4.0, 4.5, and 5.0, and evaluating the effects of weak organic acids (acetic, citric, malic, tartaric) in a broth model. Five strains of each pathogen (*E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*) were grown and separately inoculated into juice and broth models. Juices were HPP treated at 550 MPa and the broth solutions at 400 MPa for 1 min at 5°C. All HPP treated samples were also held for 24 hr at 5°C to determine synergistic effects of pH post-HPP treatment. A greater than 5-log reduction of *E. coli* was

achieved at the initial pH (3.7) in apple, orange, and grape juice after HPP treatment, and in apple juice at pH 4 after 24 hr storage. In both apple and orange juices, *Salmonella* yielded a greater than 5-log reduction at the initial pH, 4.0, and 4.5. However, in grape juice, only a 5-log reduction at the initial pH and at pH 4.0 after 24 hr storage was obtained. *Listeria* achieved a greater than 5-log reduction under all experimental conditions. The weak acids showed no significant differences with the inactivation of *E. coli* or *Salmonella*, with few significant differences in *Listeria* inactivation. The data has indicated that pH plays a significant role in pathogen inactivation using HPP. The rate of inactivation appears to depend largely on the product composition and the pathogen species. The results have provided a pH range between which certain fruit juices should fall to ensure food safety, suggesting the needs for additional precautions for higher pH products when treated by HPP.

3.2 Introduction

Acidity in foods has long been a preventative measure to control for food safety. Acids lower the pH of a food, which creates unfavorable environments for non-acidophilic microorganisms, which includes foodborne pathogens, effectively limiting their growth (Doores, 2005). Many fruit and vegetable products have naturally occurring organic acids that could contribute antimicrobial effects (In et al., 2013). However, these effects are largely pertinent to the type and concentration of the acid, the genera and species of the microorganism, and food composition (Doores, 2005).

All microorganisms have defined optimum pH levels required for their growth and survival, whereas most bacteria prefer a pH near neutrality (pH 6.5 to 7.5) but can tolerate a pH range of pH 4 to 9 (Beales, 2004; Doores, 2005). Most commercial fruit juices are considered to

be acidic, which is defined by the U.S. Food and Drug Administration of a fruit juice having a pH of 4.6 and lower (USFDA, 2004). At this pH, growth of most bacteria is inhibited. However, pH alone cannot inactivate microorganisms. Bacterial cells may survive at low pH and thus still be metabolically active, which necessitates food safety processing measures (Beales, 2004; Mazzotta, 2001).

There has been a recent trend with consumers gravitating toward products with functional benefits and improved nutrition and taste (Hogan et al., 2005). For instance, there is a high demand seeking fresh fruit juice products, such as fresh pressed or cold pressed juice, instead of traditionally pasteurized ones. However, though most fruit juices are acidic, unpasteurized fruit juices are still prone to bacterial contamination, leading to foodborne illness and outbreaks (Besser et al., 1993; CDC, 1995, 1996, 1997; Jain et al., 2009; Krause et al., 2001). This has prompted a need for alternative technologies to safely process juice products to retain fresh qualities and comply with the 5-log performance standard dictated by the Juice HACCP regulation (USFDA, 2004).

High pressure processing (HPP) is a nonthermal or cold process technology used for processing fruit juices and beverages. It utilizes water pressure to inactivate microorganisms with minimal damage to sensory properties and nutritional content, such as flavor, color, and vitamins (Georget et al., 2015; Oey et al., 2008). Many food products processed with HPP are acidic products, such as fruit juices and beverages, salsas and jams (Hogan et al., 2005). A comprehensive study to assess a range of pH effectiveness in unison with high pressure, as well as the acid type, has yet to be explored.

The purpose of this study is to evaluate the effects of pH and acid type on pathogen inactivation in fruit juices using HPP. Additionally, the study is aimed to provide insight on any

pressure tolerance differences among the pathogens tested and determine if there are any synergistic effects between pH and pressure. Three juices were utilized and inoculated with *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* and processed at 5°C. The acids tested were organic acids commonly found in food and fruit products, including malic, citric, tartaric, and acetic acid.

3.3 Materials and methods

Table 3.1. Concentrations and volume of acids used to adjust 1.0 L TSB to pH 5.0.

Acid Type (20% w/v)	**Average Concentration (M)**
Malic	0.015
Citric	0.013
Acetic	0.033
Tartaric	0.013

3.3.1 Juice, acids, and bacterial strains

Three types of juice were used for the pH effect study. Shelf stable apple (Wegmans, Geneva, NY), orange (Walmart, Geneva, NY), and white grape juice (Wegmans, Geneva, NY) were purchased from local supermarkets. Juices were stored at ambient temperatures before brought to 5°C before use. All juices were evaluated for °Brix and water activity values.

The second part of this study was to evaluate the effects of different acid types. 20% w/v solutions of malic, citric, acetic, and tartaric acid were prepared. Tryptic Soy Broth (TSB) (Alpha Biosciences, Baltimore, MD) was adjusted to pH 5 ± 0.05 using each acid solution and then autoclaved. The amount of each acid needed to adjust TSB are listed in Table 3.1. Broth solutions were stored at 5°C.

Five strains or serotypes of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* were used for this work. The strains and serotypes and origins are shown in

Table 3.2. Strains and serotypes were streaked out on Tryptic Soy Agar (TSA) (Alpha Biosciences, Baltimore, MD) from frozen cultures maintained at -80°C and re-streaked on fresh agar monthly.

Table 3.2. List of strain or serotype of each pathogen with origin.

Pathogen	Strain or serotype	Origin	Lab code
E. coli O157:H7	C7927	Human isolate, apple cider linked to an outbreak (date of outbreak unavailable)	E1
	ATCC 43890	Human isolate, date of outbreak unavailable	E2
	ATCC 43894	Human isolate, date of outbreak unavailable	E3
	ATCC 43889	Human isolate, date of outbreak unavailable	E4
	ATCC 35150	Human isolate, date of outbreak unavailable	E5
Salmonella enterica	Hartford H0778	Orange juice, US outbreak in 1995	S1
	Typhimurium FSL R9-5494	Orange juice, multistate US outbreak in 2005	S2
	Muenchen FSL R9-5498	Alfalfa sprouts, multistate US outbreak in 2016	S3
	Javiana FSL R9-5273	Tomatoes, multistate US outbreak in 2002	S4
	Enteriditis FSL-R9-5505	Beans sprouts, multistate US outbreak in 2014	S5
Listeria monocytogenes	Lineage I, serotype 4b FSL J1-108	Coleslaw, US outbreak in 1981	L1
	Lineage I, serotype 4d FSL J1-107	Coleslaw, US outbreak in 1981	L2
	Lineage II, serotype 1/2a FSL R9-0506	Cantaloupe, US outbreak in 2011	L3
	FSL R9-5411	Caramel Apple, multistate US outbreak 2014-2015	L4
	FSL R9-5506	Packaged Salad, multistate US outbreak in 2016	L5

3.3.2 High pressure processing specifications

A 55L Hiberbaric commercial scale High Pressure Processing machine was used to conduct all the processing experiments (Hiperbaric, Burgos, Spain). Water processing temperature was adjusted to 5 ± 3°C. The pH-adjusted juice samples were processed at 550 MPa (79,771 psi) for 1 minute. Acidified TSB samples were processed at 400 MPa (58,015 psi) for 1 minute.

3.3.3 Juice and acidified broth preparation and pathogen inoculation

Pathogen strains and serotypes were streaked out and grown on TSA plates. Single colonies were used to inoculate 5 ml TSB and were grown overnight (20 ± 2 hr) at 35°C, tilted

and shaking at 175 RPM. Equal amounts of each serotype were combined to form a cocktail mixture for each pathogen.

Juices were processed at the initial pH and at the adjusted pH. Each juice was adjusted to pHs of 4.00, 4.50, and 5.00 ± 0.05 using 50% w/w sodium hydroxide and aliquoted into 2 oz. bottles. The bottles were then adjusted to the targeted processing temperature of 5°C before inoculated with 0.5 ml of the desired pathogen cocktail to achieve a starting bacterial concentration of 10^7 CFU/ml. The same process was applied to the acidified TSB solutions. Bottles were then capped and sealed.

The samples to be processed were placed in a plastic bag filled with calcium hypochlorite solution (300 ppm) as a means to protect the contamination of the HPP unit in the event of container breach with the inoculated samples. The bags were then sealed using a vacuum sealer and then sealed again in an additional bag.

Four bottles per pathogen per experiment condition were prepared – one control sample, one unprocessed sample, and two samples to be HPP treated. The control sample was plated immediately after inoculation to obtain the initial microbial population counts and one HPP treated sample was plated immediately after processing. The unprocessed sample and additional HPP treated sample were stored at 5°C for 24 hr before plating. Samples were serial diluted in Bacto™ Peptone (Becton Dickinson, Sparks, MD). 1 ml of serial dilutions was plated using the pour plate method with the corresponding selective media. Violet Red Bile Agar (Alpha Biosciences, Baltimore, MD) was used for *E. coli* detection, Bismuth Sulfite Agar (Becton Dickinson, Sparks, MD) for *Salmonella*, and Oxford Listeria Agar (Alpha Biosciences, Baltimore, MD) with Modified Oxford Antimicrobic Supplement (Becton Dickinson, Sparks, MD) for *Listeria* enumeration. Poured plates were incubated at 35°C for 48 hr and then

enumerated to determine the CFU/mL, averaged, and converted into log numbers. Three biological replicates were conducted per juice/acidified TSB solution, pH, and pathogen.

3.3.4 Statistical analysis

The statistical software R (R Core Team, Vienna, Austria) and package lme4 were used to fit linear mixed effects regression models. Means and post-hoc comparisons were estimated from the model using the lsmeans package. Significant relationships were determined based on the log reductions derived from initial populations and after processing, comparing log reductions at each pH within each juice and pathogen grouping and against the same conditions after 24 hr storage. CFU/ml were converted to log CFU/ml and averaged with the standard deviation. Due to the method of plating, the lowest observable counts are recorded at 1 CFU/ml.

3.4 Results

Table 3.3. Initial microbial populations and standard deviation of each pathogen and populations after 24 hr storage at 5°C.

Apple Juice	*Average °Brix*		*Average a_w*	
	12.5		0.9805	
Pathogen Counts (log CFU/mL)	**Initial pH (3.7)**	**pH 4.0**	**pH 4.5**	**pH 5.0**
E. coli O157:H7	7.43 ± 0.22	7.31 ± 0.09	7.34 ± 0.08	7.25 ± 0.11
	7.21 ± 0.11	*7.20 ± 0.25*	*7.20 ± 0.22*	*7.21 ± 0.02*
Salmonella enterica	7.62 ± 0.07	7.50 ± 0.12	7.54 ± 0.05	7.56 ± 0.05
	7.53 ± 0.05	*7.56 ± 0.07*	*7.52 ± 0.06*	*7.51 ± 0.04*
Listeria monocytogenes	7.23 ± 0.12	7.10 ± 0.13	7.13 ± 0.11	7.18 ± 0.14
	7.07 ± 0.18	*7.13 ± 0.06*	*7.17 ± 0.06*	*7.12 ± 0.09*
Orange Juice	*Average °Brix*		*Average a_w*	

	11.8		0.9852	
Pathogen Counts (log CFU/mL)	**Initial pH (3.7)**	**pH 4.0**	**pH 4.5**	**pH 5.0**
E. coli O157:H7	7.21 ± 0.20	7.33 ± 0.07	7.23 ± 0.10	7.23 ± 0.09
	7.25 ± 0.10	*7.30 ± 0.05*	*7.11 ± 0.16*	*7.16 ± 0.07*
Salmonella enterica	7.49 ± 0.10	7.51 ± 0.06	7.51 ± 0.11	7.53 ± 0.09
	7.46 ± 0.14	*7.56 ± 0.05*	*7.44 ± 0.22*	*7.18 ± 0.24*
Listeria monocytogenes	7.09 ± 0.05	7.16 ± 0.06	7.10 ± 0.08	7.08 ± 0.02
	6.76 ± 0.46	*7.09 ± 0.10*	*7.13 ± 0.13*	*7.17 ± 0.12*
Grape Juice	*Average °Brix*		*Average a_w*	
	16.5		0.9739	
Pathogen Counts (log CFU/mL)	**Initial pH (3.4)**	**pH 4.0**	**pH 4.5**	**pH 5.0**
E. coli O157:H7	7.41 ± 0.10	7.35 ± 0.07	7.28 ± 0.12	7.35 ± 0.07
	7.16 ± 0.06	*7.10 ± 0.28*	*7.18 ± 0.12*	*7.21 ± 0.06*
Salmonella enterica	7.48 ± 0.18	7.59 ± 0.12	7.58 ± 0.03	7.56 ± 0.05
	6.99 ± 0.17	*7.50 ± 0.07*	*7.50 ± 0.03*	*7.53 ± 0.02*
Listeria monocytogenes	7.10 ± 0.05	7.18 ± 0.15	7.18 ± 0.24	7.13 ± 0.16
	7.03 ± 0.23	*7.06 ± 0.19*	*7.00 ± 0.10*	*7.09 ± 0.05*

Normal: control samples. *Italic*: unprocessed samples after 24 hr storage at 5°C. **Bold**: significantly different compared to control samples under the same experimental conditions ($p \leq 0.01$)

3.4.1 Comparing initial populations

One control sample and one unprocessed sample were prepared for each experimental condition. The control samples were used to determine initial microbial populations and evaluated immediately after inoculation. The unprocessed samples were inoculated with the same concentration of pathogens and stored at 5°C for 24 hr before evaluated for microbial counts. All microbial counts were recorded as CFU/ml and then converted into log numbers

(Table 3.3). It was found that there was only one incidence of significant difference between the control sample of *Salmonella* in grape juice and the unprocessed sample held at 5°C. All other pathogens in each juice were not found to significantly different between the two sample types.

Table 3.4. Effect of pH on pathogen inactivation in juice immediately after HPP and 24 hr storage at 5°C after high pressure processing at 550 MPa for 1 minute. Average pathogen reduction with standard deviation is presented for each juice and pathogen.

Apple Juice	*Average °Brix* 12.5		*Average a_w* 0.9805	
Pathogen Reduction (log CFU/mL)	**Initial pH (3.7)**	**pH 4**	**pH 4.5**	**pH 5**
E. coli O157:H7	6.43 ± 1.15^{A}	4.08 ± 0.61^{B}	3.23 ± 1.37^{BC}	1.78 ± 0.29^{C}
	7.43 ±0.22^{A}	***6.50*** *±0.56^{A}*	*4.44 ±1.48^{B}*	*2.41 ±0.26^{C}*
Salmonella enterica	7.62 ± 0.07^{A}	7.50 ± 0.12^{A}	5.78 ± 0.33^{A}	2.97 ± 1.03^{B}
	7.62 ±0.07^{A}	*7.50 ±0.12^{A}*	*7.14 ±0.47^{A}*	*4.06 ±0.89^{B}*
Listeria monocytogenes	7.23 ± 0.18^{A}	7.10 ± 0.13^{A}	7.13 ± 0.11^{A}	7.18 ± 0.14^{A}
	7.23 ±0.18^{A}	*7.10 ±0.13^{A}*	*7.13 ±0.11^{A}*	*7.18 ±0.14^{A}*
Orange Juice	*Average °Brix* 11.8		*Average a_w* 0.9852	
Pathogen Reduction (log CFU/mL)	**Initial pH (3.7)**	**pH 4**	**pH 4.5**	**pH 5**
E. coli O157:H7	6.66 ± 0.98^{A}	3.92 ± 0.57^{B}	2.13 ± 0.25^{C}	1.30 ± 0.18^{C}
	7.13 ±0.32^{A}	*4.29 ±0.10^{B}*	*1.26 ±0.27^{C}*	*1.00 ±0.18^{C}*
Salmonella enterica	7.49 ± 0.10^{A}	7.51 ± 0.06^{A}	5.90 ± 1.07^{B}	3.57 ± 0.35^{C}
	7.49 ±0.10^{A}	*7.51 ±0.06^{A}*	*6.02 ±1.43^{B}*	*2.49 ±0.41^{C}*
Listeria monocytogenes	7.09 ± 0.05^{A}	7.16 ± 0.06^{A}	7.10 ± 0.08^{A}	7.08 ± 0.02^{A}
	7.09 ±0.05^{A}	*7.16 ±0.06^{A}*	*7.10 ±0.08^{A}*	*7.08 ±0.02^{A}*

Grape Juice	*Average °Brix* 16.5		*Average a_w* 0.9739	
Pathogen Reduction (log CFU/mL)	**Initial pH (3.4)**	**pH 4**	**pH 4.5**	**pH 5**
E. coli O157:H7	7.31 ± 0.27^{A}	2.34 ± 0.59^{B}	0.82 ± 0.52^{B}	0.83 ± 0.42^{B}
	7.41 ± 0.10^{A}	***4.38 ± 1.02^{B}***	***2.76 ± 1.01^{BC}***	*1.76 ± 1.05^{C}*
Salmonella enterica	7.16 ± 0.52^{A}	4.72 ± 0.42^{B}	2.02 ± 1.08^{C}	1.57 ± 0.12^{C}
	7.48 ± 0.18^{A}	*5.69 ± 0.28^{B}*	***3.89 ± 1.00^{C}***	*2.52 ± 0.37^{C}*
Listeria monocytogenes	7.10 ± 0.05^{A}	7.18 ± 0.15^{A}	7.18 ± 0.24^{A}	5.51 ± 0.58^{A}
	7.10 ± 0.05^{A}	*7.18 ± 0.15^{A}*	*7.18 ± 0.24^{A}*	*6.67 ± 0.67^{A}*

Normal: HPP treated samples. *Italic*: HPP treated samples after 24 hr storage at 5°C. **Bold**: Significantly different compared to HPP treated samples under the same experimental conditions ($p \leq 0.01$). *: Different superscripts indicate significant differences ($p \leq 0.01$) within a row

3.4.2 Reduction of pathogens in apple juice

Juices were processed at the initial pH, and at the adjusted pH of 4.0, 4.5, and 5.0. Log reductions were calculated for each juice, pH, and pathogen (Table 3.4). Two processed samples were prepared for each experimental condition. One sample was high pressure processed and plated immediately after processing, and the other was high pressure processed and stored for 24 hr at 5°C before plating. In apple juice, a greater than 5-log reduction was achieved in *E. coli* at the initial pH right after processing, and the same was observed in *Salmonella* at initial pH, 4.0, and 4.5 (Figure 3.1). *Listeria* was completely inactivated under all processing and experimental conditions. There were significant differences ($p \leq 0.01$) between *E. coli* at the initial pH and the other adjusted pH of 4.0, 4.5, and 5.0. The inactivation at pH 4.0 was also found significantly different than that at pH 5.0. Regarding *Salmonella*, the log reductions at the initial pH, pH 4.0,

and pH 4.5 were not found to be significantly different from each other, though they were significantly different ($p \leq 0.01$) than the reduction at pH 5.0.

For samples that were HPP treated and held for 24 hr at 5°C, a greater than 5-log reduction of *E. coli* was achieved at initial pH and pH 4.0, and *Salmonella* at initial pH, pH 4.0, and pH 4.5 (Figure 3.1). Log reductions at initial pH and pH 4.0 of *E. coli* were found to be significantly different ($p \leq 0.01$) from the reductions at pH 4.5 and pH 5.0. At pH 4.0, *E. coli* reduction from HPP treated samples were found to be significantly lower ($p \leq 0.01$) from its 24 hr stored counterpart. The significance differences for HPP treated *Salmonella* samples were found to be the same for the 24 hr stored samples. At pH 4.0, *E. coli* reduction from samples immediately treated by HPP were found to be significantly different ($p \leq 0.01$) from its 24 hr stored counterpart.

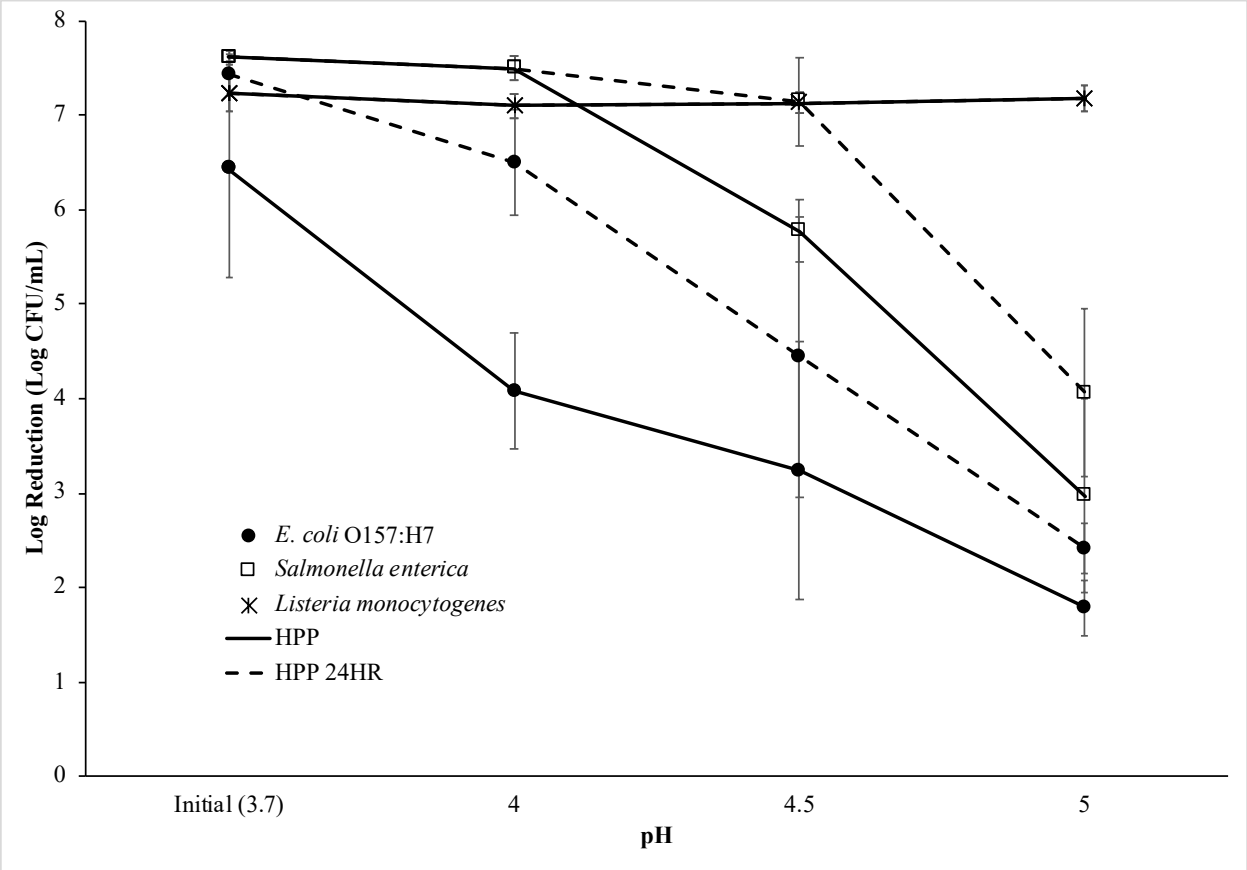

Figure 3.1. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in HPP treated samples (solid lines) and HPP treated samples stored for 24 hr at 550 MPa for 1 minute at 5°C (dashed lines) in apple juice. Error bars represent standard deviation of the log reduction averages.

3.4.3 Reduction of pathogens in orange juice

In orange juice, a greater than 5-log reduction of *E. coli* was achieved at initial pH, and at initial pH, pH 4.0, and pH 4.5 for *Salmonella* at both HPP and 24 hr stored HPP treated samples (Figure 3.2). *Listeria* was also completely inactivated in orange juice under all experimental conditions.

Significant differences were found when comparing the log reductions of *E. coli* at initial pH to pH 4.0, 4.5 and 5.0. We found pH 4.5 and 5.0 yielded low microbial reduction of *E. coli*, with no significant differences between the two treatments. *Salmonella* was completely inactivated at initial pH and pH 4.0. The resulting reductions at pH 4.5 and 5.0 were found to be

significantly different ($p \leq 0.01$) from each other and from the initial pH and pH 4.0. The same trends of significance were found in both HPP and 24 hr stored HPP samples.

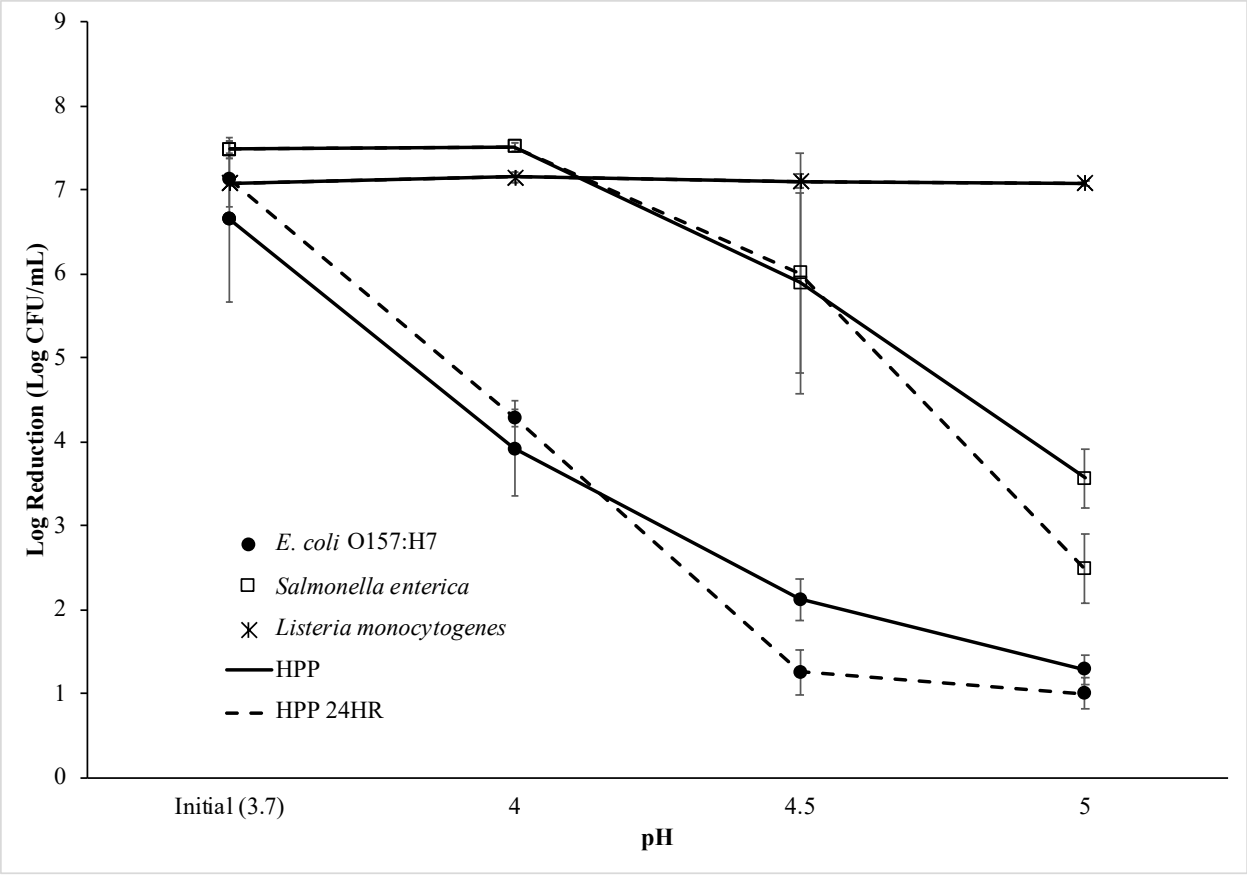

Figure 3.2. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in HPP treated samples (solid lines) and HPP treated samples stored for 24 hr at 550 MPa for 1 minute at 5°C (dashed lines) in orange juice. Error bars represent standard deviation of the log reduction averages.

3.4.4 Reduction of pathogens in grape juice

A greater than 5-log reduction of *E. coli* and *Salmonella* was achieved at initial pH in grape juice in both HPP treated and 24 hr stored samples. The results were found to be significantly different ($p \leq 0.01$) from the other pH values (Figure 3.3). *E. coli* had low inactivation at pH 4.0, 4.5, 5.0 with no significant differences found within these conditions. After 24 hr storage at 5°C, a greater than 5-log reduction of *Salmonella* was achieved at pH 4.0.

E. coli achieved significantly ($p \leq 0.01$) higher inactivation at pH 4.0 and 4.5 after 24 hr storage at 5°C. Similarly, *Salmonella* at pH 4.5 was also found to have significantly higher log reduction after 24 hr storage.

Listeria achieved a greater than 5-log reduction under all experimental conditions with no significant differences found within each condition.

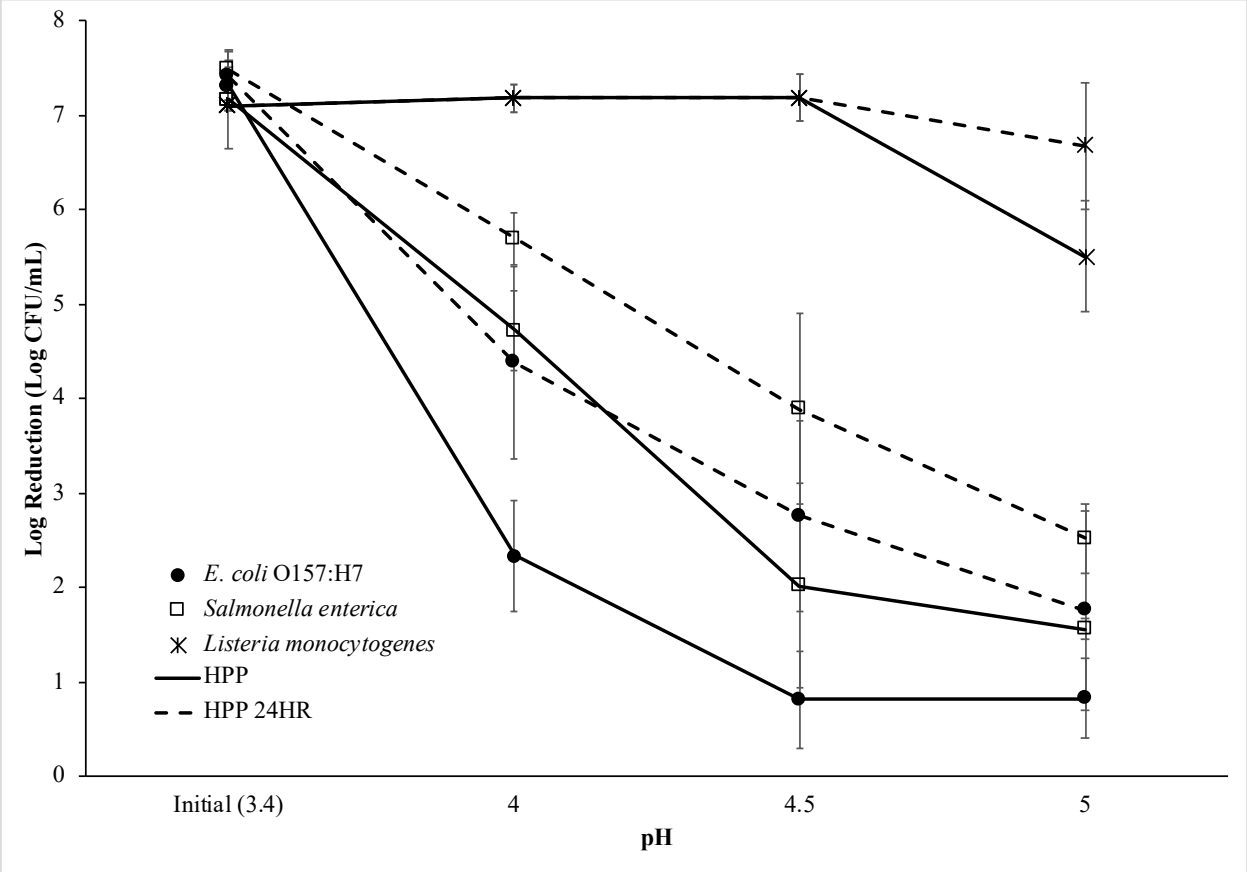

Figure 3.3. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in HPP treated samples (solid lines) and HPP treated samples stored for 24 hr at 550 MPa for 1 minute at 5°C (dashed lines) in white grape juice. Error bars represent standard deviation of the log reduction averages.

Table 3.5. Effect of acid type on pathogen inactivation in Tryptic Soy Broth (TSB) immediately after HPP and 24 hr storage at 5°C after high pressure processing at 400 MPa for 1 minute. Average pathogen reduction with standard deviation is presented for each acid type and pathogen.

TSB at pH 5.0	*Average °Brix*	*Average aw*

	3.9		0.9890	
Pathogen Reduction (log CFU/mL)	**Malic**	**Citric**	**Acetic**	**Tartaric**
E. coli O157:H7	0.58 ± 0.20^{A}	0.42 ± 0.35^{A}	0.43 ± 0.17^{A}	0.51 ± 0.04^{A}
	$\mathit{0.39} \pm 0.13^{\mathit{AB}}$	$\mathit{0.29} \pm 0.25^{\mathit{A}}$	$\mathit{1.91} \pm 1.53^{\mathit{B}}$	$\mathit{0.33} \pm 0.13^{\mathit{A}}$
Salmonella enterica	0.69 ± 0.14^{A}	0.91 ± 0.08^{A}	0.83 ± 0.07^{A}	0.69 ± 0.23^{A}
	$\mathit{0.77} \pm 0.23^{\mathit{A}}$	$\mathit{0.93} \pm 0.16^{\mathit{A}}$	$\mathit{1.19} \pm 0.20^{\mathit{A}}$	$\mathit{0.74} \pm 0.01^{\mathit{A}}$
Listeria monocytogenes	2.11 ± 0.44^{A}	4.04 ± 1.48^{B}	2.36 ± 0.71^{A}	2.74 ± 1.14^{AB}
	$\mathit{2.81} \pm 0.55^{\mathit{A}}$	$\mathit{3.99} \pm 0.30^{\mathit{A}}$	$\boldsymbol{\mathit{6.60}} \pm 0.57^{\mathit{B}}$	$\mathit{3.55} \pm 0.10^{\mathit{A}}$

Normal: HPP treated samples. *Italic*: HPP treated samples after 24 hr storage at 5°C. **Bold**: Significantly differences compared to HPP treated samples under the same experimental conditions ($p \leq 0.01$). *: Different superscripts indicate significant differences ($p \leq 0.01$) within a row

3.4.5 Reduction of pathogens in acid adjusted TSB

An acid adjusted study was completed to evaluate the effects of different weak acids on pathogen inactivation (Figure 3.4). TSB was adjusted to pH 5.0 using 20% w/v malic, citric, acetic, and tartaric acid and processed at 400 MPa for 1 min. *E. coli* and *Salmonella* reductions at each acid were not found to be significantly different after immediate HPP treatment. For experiments with 24 hr storage at 5°C, *E. coli* reduction in acetic acid was found higher and significantly different from inactivation in citric and tartaric acid. *Salmonella* reductions, on the other hand, were not found to be significantly different after 24 hr storage at 5°C. Log reduction of *Listeria* in citric acid was found to be significantly different ($p \leq 0.01$) than malic and acetic acid, but not tartaric acid. After storage at 5°C for 24 hr, *Listeria* reduction in acetic acid was found to be significantly higher ($p \leq 0.01$) than the other acids, and also from its HPP treated counterpart.

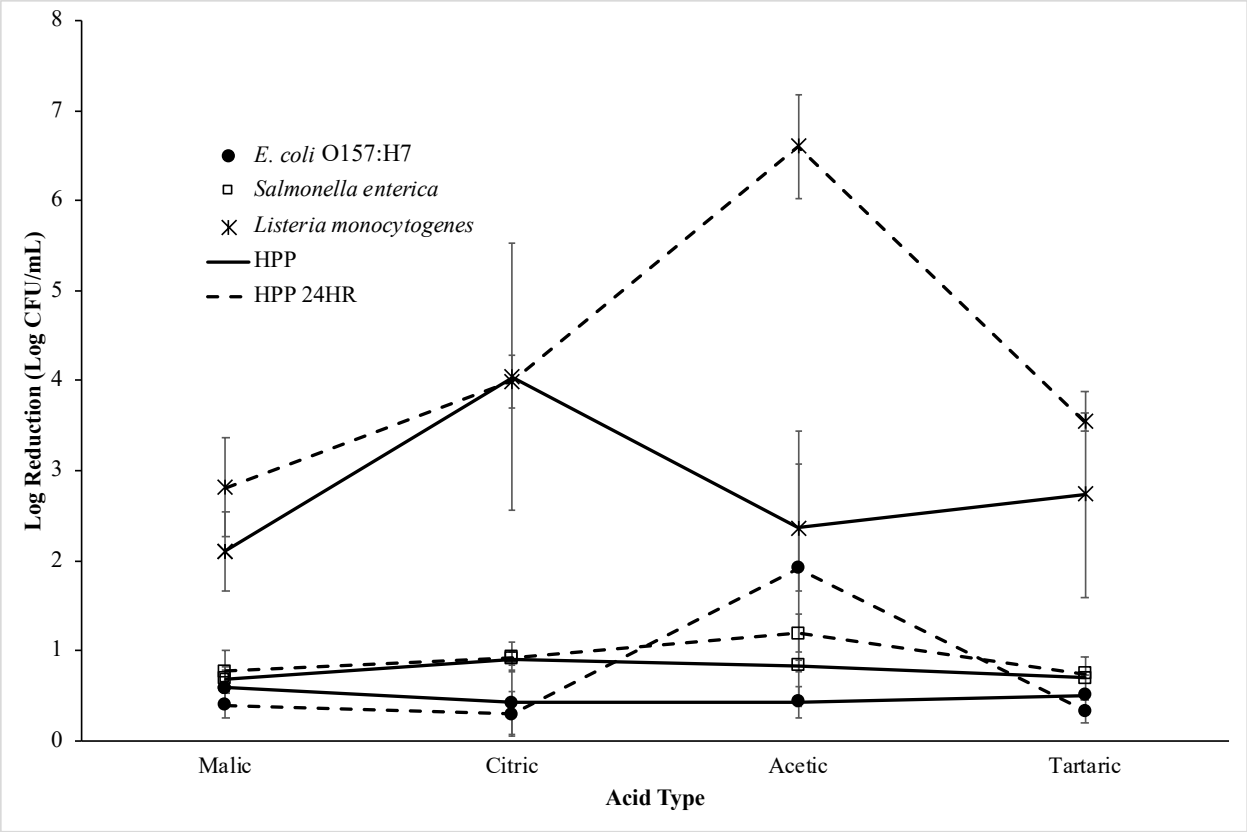

Figure 4. Microbial log reduction of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in HPP treated samples (solid lines) and HPP treated samples stored for 24 hr at 400 MPa for 1 minute at 5°C (dashed lines) in acidified TSB. Error bars represent standard deviation of the log reduction averages.

3.5 Discussion

Acidification of food products to reduce the pH has been used to control microbial populations for many years (Abee and Wouters, 1999). However, large quantities of acid can contribute negative sensory qualities, such as undesirable flavor or texture. More evidence suggests that acid alone is not feasible for microbial control (Beales, 2004). High pressure processing is a nonthermal method used to inactivate pathogens using water pressure. This method can preserve desirable qualities of food, making it an ideal process for treating raw juices to maintain freshness and food safety.

Several studies have noticed an increased pressure sensitivity of pathogens when treated at low pH (Bayındırlı et al., 2006; Garcia-Graells et al., 1998, Ritz et al., 1998, 2000). This study is aimed to provide more comprehensive data on this concept, evaluating the effects of pH, the pH range of common fruit juices, the type of acid, and possible synergistic effects. The pH range tested included the initial pH of the commercial juice used, and extended to pHs of 4.0, 4.5, and 5.0, accounting for pH variation that comes from fruit maturity and variety (Belitz et al., 2004; Kader and Barrett, 2004).

A greater than 5-log reduction of *E. coli* was achieved only at the initial pH in apple, orange, and grape juice, and after 24 hr storage at pH 4.0 in apple juice. *Salmonella* was reduced over 5-logs at the initial pH, pH 4.0 and 4.5 in apple and orange juice at both processing and storage conditions. In grape juice, a greater than 5-log reduction was achieved at initial pH and at pH 4.0 after 24 hr storage. *Listeria* was completely inactivated under all pH and storage conditions in apple and orange juice. In grape juice, *Listeria* was completely inactivated at initial pH, pH 4.0 and 4.5, and achieved a greater than 5-log reduction at pH 5.0. Under 24 hr storage conditions, *Listeria* was completely inactivated. The results show that HPP is more effective at pathogen inactivation at lower pH, supporting the previous studies that reported an increased pressure sensitivity at low pH. Regarding pressure tolerances, *E. coli* is the least affected by pH and HPP treatment, showing lower inactivation rate under all pH conditions compared to *Salmonella* and *Listeria*. In contrast, *Listeria* showed the least resistance among all the pathogens, with complete inactivation under almost all experimental conditions.

To determine the synergistic effects, control samples and HPP treated samples were compared to unprocessed samples and HPP treated samples held for 24 hr at 5°C. Comparing control samples and unprocessed samples, only one incidence of significance was found,

suggesting that after 24 hr storage at the tested pH values, there is little to no pH effect on pathogen inactivation. Most HPP treated samples that were held overnight had either no change or slightly increased log reductions. Four of the 24 hr stored HPP samples had significantly higher ($p \leq 0.01$) inactivation, suggesting mild synergistic effects between pH and pressure.

HPP has several mechanisms for pathogen inactivation, as high pressure can cause protein and enzyme unfolding, resulting in changes in cell membrane, enzyme or protein synthesis inhibition, and disrupting intracellular functions (Considine et al., 2008; Rastogi et al., 2007). Many of the acids found in fruit juices are weak organic acids, which are mainly undissociated in solution (IFT/FDA, 2003). Weak acids in their undissociated form can diffuse through the cell membrane and lower the intracellular pH, inhibiting essential metabolic pathways (Abee and Wouters, 1999). In most cases, this would inhibit growth, but not necessarily cause bacterial inactivation (Doores, 2005). This can be seen in the unprocessed samples held for 24 hr at 5°C, where no additional growth was recorded, nor were there significant differences compared to the control samples. However, HPP treated samples after 24 hr storage seemed to have increased inactivation after storage, which could be explained by the effect of high pressures altering cell membranes, allowing for greater diffusion of undissociated acids and a larger intracellular pH imbalance.

E. coli and *Salmonella* inactivation in grape juice seemed to be the least effected by HPP treatment, suggesting there is some protective effect despite having the same pH conditions as apple juice and orange juice. This suggests that product composition plays a sizeable role in pathogen inactivation, depending on the food constituents, including solute type, solids, and acids.

We carried out additional studies to investigate the effects of different types of weak organic acids. The acids tested were malic, citric, acetic, and tartaric acids, commonly found in fruit and food products, and representative of the main acids found in fruit juices tested (apple, orange, and grape) (Belitz et al., 2004). TSB was adjusted to pH 5.0 and processed at 400 MPa for 1 min. The higher pH and lower pressure processing conditions used here were based on preliminary studies, which showed *Listeria* completely inactivated at pH ≤4.5 and higher pressures. These conditions made it impossible to show differences between acid types concerning *Listeria* inactivation. The results show there were no significant differences found in *E. coli* and *Salmonella* inactivation rates among the acid types. *Listeria* inactivation in citric acid was found to be significantly different ($p \leq 0.01$) from malic and acetic, but after 24 hr storage, the log reductions were no longer significantly different. The 24 hr stored HPP samples of acetic acid showed a significant log reduction of *Listeria* ($p \leq 0.01$) compared to the other acid types and its immediately processed counterpart, from a 2-log reduction to over 6-log reduction overnight. This suggests there might be some acid differences in resulting pathogen inactivation. However, it seems to highly depend on the pathogen and product composition.

The results of this study provide a comprehensive understanding of the pH effect on pathogen inactivation and a pH range the juices tested should fall between to assure food safety. Mild synergistic effects of pH and pressure were observed at 5°C, and increased cold storage after HPP at low pH could result in further pathogen inactivation (Jordan et al., 2001). Although products below pH 4.6 are considered to be acidic, this study shows that at pH 4.5, not all pathogens tested can achieve a 5-log reduction under the experimental conditions. Current industrial HPP conditions have an increased pressure of 600 MPa and are processed for 3 minutes. However, not all products can be successfully processed by HPP, and additional

protections to ensure a 5-log reduction in juice products could include lowering the pH, adjusting product composition, and continued cold storage after HPP. All these factors affect high pressure processing, and additional studies of these parameters could assist juice producers to optimize their processes.

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CHAPTER 4

EFFECT AND ROLE OF WATER ACTIVITY ON PATHOGEN INACTIVATION USING HPP IN ACIDIC AND NEUTRAL SOLUTIONS

4.1 Abstract

Low water activity (a_w) has been used as a method of preservation and ensuring the safety of food products. High Pressure Processing (HPP) is a nonthermal processing technology utilizing high pressures to extend shelf life and food safety of certain food products. In HPP, pressure is transferred using water, and the efficacy is impacted by the available water present in a food product. This study was conducted to evaluate the effects of a range of water activity on pathogen inactivation, which compares pathogen reduction at two solutes under neutral (pH 7.0) and acidic (4.5) conditions. Tryptic soy broth (TSB) was adjusted to a_w of 0.95, 0.96, 0.97, 0.98 and 0.99 using sodium chloride (NaCl) or fructose, and these solutions were then stored at pH 7.0 or adjusted to pH 4.5, depending on experimental needs. Pathogens cocktails of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* were inoculated into the TSB solutions and HPP treatment was conducted at 550 MPa for 1 minute at 5°C. The treated samples were plated immediately after processing and plated again after 24 hr storage at 5°C. The results show that a 5-log reduction could not be achieved at any a_w values in NaCl and fructose solutions at pH 7.0. Complete inactivation of *E. coli* was found at a_w 0.98 and 0.99, and of *Salmonella* and *Listeria* at a_w 0.97, 0.98, and 0.99 in pH 4.5 NaCl solutions. At pH 4.5 in fructose, complete reduction of *Salmonella* was achieved at a_w 0.99 and of *Listeria* at a_w 0.98 and 0.99. Inactivation at pH 4.5 in both solutions were either the same or significantly higher ($p \leq 0.01$) than at pH 7.0. The results have also shown that there are significant inactivation differences ($p \leq 0.01$) between

NaCl and fructose solutions at both pH 7.0 and pH 4.5. Pathogen inactivation rate was found to be generally higher in NaCl solutions, suggesting that NaCl is less protective than fructose. This study revealed the baroprotective effects of NaCl and fructose under two pH conditions, where significant differences suggest that pathogen inactivation is more pronounced at higher a_w under acidic conditions. The results have also shown inactivation differences within a narrow range of activity (0.01 between a_w values). Water activity is influenced by a variety of factors, including food constituents, emphasizing the importance of available water and product composition for effective HPP treatment.

4.2 Introduction

High pressure processing (HPP) is a nonthermal processing technology used to preserve foods, maintaining fresh-like qualities. This technology has gained considerable interest in processing food products to retain flavor and nutritional content that might otherwise be damaged by heat (Hogan et al., 2005). Common HPP treated products include jams, guacamole, salsas, salad dressings, fruit and vegetable juices, and beverages. HPP is able to effectively extend the shelf life and food safety of these products through microbial inactivation under mild conditions (Rendueles et al., 2011). The U.S. Food and Drug Administration has required that products treated with HPP must have the process validated and be able to achieve a 5-log reduction of the most pertinent pathogens (USFDA, 2004). Like with all processing methods, there are certain factors and parameters that affect the effectiveness. With HPP, important product parameters that affect microbial inactivation include pH, composition, and water activity (Daryaei et al. 2016).

Low pH and water activity (a_w) have been methods used for food preservation and to inhibit growth of microorganisms in food products (IFT/FDA, 2003). Microorganisms have a minimum a_w level, below which, they will not reproduce or grow (Tapia et al., 2008). Most foodborne pathogenic bacteria require a minimum a_w of 0.90, and no microbial proliferation will occur below a_w 0.60 (Fontana, 2008). However, HPP utilizes water pressure to exert the effect, and therefore certain dry foods or foods with low water content or activity are not suitable for processing by HPP due to a lack of means to transfer pressure through the product (Huang et al., 2017). This makes it difficult to utilize low water activity as a preservation method to reduce microbial growth.

Some food additives, such as sugar and salt, are natural microbial inhibitors and commonly used in food preservation. An additional effect of these additives is that they can decrease the overall water activity and possibly lower HPP efficiency (Chirife and Favetto, 1992; Leistner, 1992). It has been reported that the type of solute (sugars or salt) in the food product can also have a significant effect on pathogen survival (Patterson et al., 1995; Patterson, 2005).

The purpose of this work is to evaluate the effect of a narrow range of water activity and its impact on pathogen inactivation using HPP. The study was conducted using a broth model inoculated with *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. We carried out the experiments using two types of solutes (sugar and salt) and at two different pH values to determine inactivation differences between and within these conditions.

4.3 Materials and methods

4.3.1 Broth model and bacterial strains

This study was conducted in a broth model, using Tryptic Soy Broth (TSB) (Alpha Biosciences, Baltimore, MD) as the sample media. Prepared broth solutions were stored at

ambient temperatures before brought to 5°C before use. All solutions were measured prior to bacterial inoculation to determine the correct pH and water activity value.

Five strains or serotypes of *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* were used for this work. The strains, serotypes, and origins are listed in Table 4.1. Strains and serotypes were streaked out on Tryptic Soy Agar (TSA) (Alpha Biosciences, Baltimore, MD) from frozen cultures maintained at -80°C and re-streaked on fresh agar monthly.

Table 4.1. List of strain or serotype of each pathogen with origin.

Pathogen	Strain or serotype	Origin	Lab code
E. coli O157:H7	C7927	Human isolate, apple cider linked to an outbreak (date of outbreak unavailable)	E1
	ATCC 43890	Human isolate, date of outbreak unavailable	E2
	ATCC 43894	Human isolate, date of outbreak unavailable	E3
	ATCC 43889	Human isolate, date of outbreak unavailable	E4
	ATCC 35150	Human isolate, date of outbreak unavailable	E5
Salmonella enterica	Hartford H0778	Orange juice, US outbreak in 1995	S1
	Typhimurium FSL R9-5494	Orange juice, multistate US outbreak in 2005	S2
	Muenchen FSL R9-5498	Alfalfa sprouts, multistate US outbreak in 2016	S3
	Javiana FSL R9-5273	Tomatoes, multistate US outbreak in 2002	S4
	Enteriditis FSL-R9-5505	Beans sprouts, multistate US outbreak in 2014	S5
Listeria monocytogenes	Lineage I, serotype 4b FSL J1-108	Coleslaw, US outbreak in 1981	L1
	Lineage I, serotype 4d FSL J1-107	Coleslaw, US outbreak in 1981	L2
	Lineage II, serotype 1/2a FSL R9-0506	Cantaloupe, US outbreak in 2011	L3
	FSL R9-5411	Caramel Apple, multistate US outbreak 2014-2015	L4
	FSL R9-5506	Packaged Salad, multistate US outbreak in 2016	L5

4.3.2 High pressure processing specifications

A 55L Hiberbaric High Pressure Processing machine was used to perform all the processing experiments (Hiperbaric, Burgos, Spain). All of the samples were processed at 550 MPa (79,771 psi) for 1 minute at 5 ± 3°C.

Table 4.2. Average concentration of solutes in solution needed to reach the targeted water activity.

Water Activity (a_w) ± 0.003	**NaCl (w/w%)**	**75% Fructose (w/w%)**
0.990	0.80%	4.75%

0.980	3.00%	13.0%
0.970	4.75%	26.5%
0.960	6.00%	34.0%
0.950	7.50%	40.5%

4.3.3 Broth preparation and pathogen inoculation

Pathogen strains and serotypes were streaked out and grown on TSA plates. Single colonies were used to inoculate 5 mL TSB and were grown overnight (20 ± 2 hr) at 35°C, tilted and shaken at 175 RPM. Equal amounts of each culture were combined to form a cocktail mixture for each pathogen.

The targeted water activity values for this experiment were 0.99, 0.98, 0.97, 0.96 and 0.95. Different concentrations of sodium chloride (NaCl) and fructose were added to TSB to reach the specific water activity value (± 0.003) (Table 4.2).

NaCl quantities were added directed into TSB and then autoclaved. A 75% w/w fructose solution was prepared and autoclaved before added to autoclaved TSB. Each solution was then either left at pH 7.0 (± 0.05) or adjusted to pH 4.5 (± 0.05) with 2.0 M HCl. Solutions were aliquoted into 2 oz. bottles and adjusted to the targeted processing temperature of 5°C before inoculated with 0.5 ml of the desired pathogen cocktail to achieve a starting bacterial concentration of 10^7 CFU/ml. Bottles were then capped and sealed. The samples to be processed were then placed in a plastic bag filled with calcium hypochlorite solution (300 ppm) at the required temperature. The hypochlorite solution was used as a means to prevent contamination of the HPP unit in the event of a container breach of the pathogen inoculated samples. The bags were sealed using a vacuum sealer and sealed again in an additional bag.

Three bottles per pathogen per experiment condition were prepared – one control sample and two samples to be HPP treated. The control sample was plated immediately after inoculation

to obtain the initial microbial population counts and one HPP treated sample was plated immediately after processing. The remaining HPP treated sample was stored for 24 hr at 5°C before plating. Samples were serial diluted in Bacto™ Peptone (Becton Dickinson, Sparks, MD). One milliliter of serial dilutions was plated using the pour plate method with the corresponding selective media. Violet Red Bile Agar (Alpha Biosciences, Baltimore, MD) was used for *E. coli* detection, Bismuth Sulfite Agar (Becton Dickinson, Sparks, MD) was used for *Salmonella*, and Oxford Listeria Agar (Alpha Biosciences, Baltimore, MD) with Modified Oxford Antimicrobic Supplement (Becton Dickinson, Sparks, MD) was used for *Listeria* enumeration. Poured plates were incubated at 35°C for 48 hr and then enumerated to determine the CFU/ml, averaged, and converted into log numbers. Three biological replicates were conducted per solute type, pH, and pathogen.

4.3.4 Statistical analysis

The statistical software R (R Core Team, Vienna, Austria) and package lme4 were used to fit linear mixed effects regression models. Means and post-hoc comparisons were estimated from the model using the lsmeans package. Significant relationships and analysis were determined based on the log reductions derived from initial populations and after processing, which compares log reductions at each water activity within each solute type and pathogen grouping and against the same conditions after 24 hr storage. Log reductions at each water activity were also compared depending on solute type at each pH level. CFU/ml were converted to log CFU/ml and averaged with the standard deviation. Due to the method of plating, the lowest observable counts are recorded at 1 CFU/ml.

4.4 Results

Table 4.3: *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* inactivation by HPP at 550 MPa for 1 minute at 5°C in NaCl adjusted TSB solutions (pH 4.5 and 7) at water activity values (a_w) 0.95 – 0.99. Average pathogen reduction with standard deviation is presented for each solution and pathogen.

***E. coli* O157:H7 Reduction (Log CFU/mL)**

	a_w				
Solute	**0.95**	**0.96**	**0.97**	**0.98**	**0.99**
NaCl (pH 7)	1.38 ± 0.07^{A}	$1.77 \pm 0.17^{*A}$	$2.76 \pm 1.05^{*AB}$	$3.93 \pm 0.73^{*BC}$	$4.63 \pm 0.92^{*C}$
	$2.18 \pm 0.47^{*A}$	*$2.43 \pm 0.22^{*AB}$*	*$2.84 \pm 0.42^{*AB}$*	*$4.05 \pm 0.72^{*B}$*	*$3.51 \pm 0.77^{*AB}$*
NaCl (pH 4.5)	2.43 ± 0.61^{A}	$4.40 \pm 0.90^{*B}$	$7.11 \pm 0.17^{*C}$	$7.05 \pm 0.13^{*C}$	$6.89 \pm 0.41^{*C}$
	$4.70 \pm 0.12^{*A}$	***$7.01 \pm 0.05^{*B}$***	*$7.06 \pm 0.10^{*B}$*	*$7.05 \pm 0.13^{*B}$*	*$6.89 \pm 0.41^{*B}$*

***Salmonella enterica* Reduction (Log CFU/mL)**

	a_w				
Solute	**0.95**	**0.96**	**0.97**	**0.98**	**0.99**
NaCl (pH 7)	1.45 ± 0.31^{A}	$2.00 \pm 0.72^{*AB}$	$3.70 \pm 0.54^{*B}$	$3.15 \pm 1.14^{*AB}$	$1.44 \pm 0.25^{*A}$
	$1.25 \pm 0.08^{*A}$	*$1.94 \pm 0.20^{*AB}$*	*$3.94 \pm 0.14^{*C}$*	*$3.31 \pm 1.30^{*BC}$*	*$1.48 \pm 0.05^{*A}$*
NaCl (pH 4.5)	3.05 ± 0.21^{A}	$4.68 \pm 0.57^{*A}$	$7.52 \pm 0.07^{*B}$	$7.52 \pm 0.07^{*B}$	$7.55 \pm 0.03^{*B}$
	$3.58 \pm 0.21^{*A}$	*$6.04 \pm 0.91^{*B}$*	*$7.52 \pm 0.07^{*B}$*	*$7.52 \pm 0.07^{*B}$*	*$7.55 \pm 0.03^{*B}$*

***Listeria monocytogenes* Reduction (Log CFU/mL)**

	aw				
Solute	**0.95**	**0.96**	**0.97**	**0.98**	**0.99**
NaCl (pH 7)	3.33 ± 0.18^{A}	$2.73 \pm 0.50^{*AB}$	$1.25 \pm 0.55^{*BC}$	$0.63 \pm 0.30^{*C}$	$1.38 \pm 0.37^{*BC}$
	3.41 ± 0.21^{A}	*$2.92 \pm 0.63^{*AB}$*	*$1.41 \pm 0.66^{*BC}$*	*$0.38 \pm 0.08^{*C}$*	*$0.33 \pm 0.18^{*C}$*
NaCl (pH 4.5)	2.72 ± 0.65^{A}	$5.18 \pm 1.71^{*B}$	$7.00 \pm 0.13^{*C}$	$7.00 \pm 0.14^{*C}$	$7.01 \pm 0.12^{*C}$
	3.82 ± 1.00^{A}	*$6.91 \pm 0.19^{*B}$*	*$7.00 \pm 0.13^{*B}$*	*$7.00 \pm 0.14^{*B}$*	*$7.01 \pm 0.12^{*B}$*

Normal: HPP treated samples. *Italic*: HPP treated samples after 24 hr storage at 5°C. **Bold**: Significant differences compared to HPP treated samples under the same experimental conditions ($p \leq 0.01$). (^{ABC}): Different alphabet superscripts indicate significant differences ($p \leq 0.01$) within a row. (*): Indicates significant differences ($p \leq 0.01$) between pH 4.5 and 7.0 within the same pathogen and under the same experimental conditions.

4.4.1 Pathogen inactivation in NaCl adjusted TSB

TSB was adjusted with two solutes, NaCl and fructose, to obtain water activity values of 0.99, 0.98, 0.97, 0.96 and 0.95. Solutions were then kept at neutral pH (pH 7.0) or adjusted to pH 4.5. Solutions were inoculated with *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* before processed at 550 MPa for 1 minute. As discussed above, two samples were HPP treated under each experimental condition, with one plated immediately after processing and the other stored for 24 hr at 5°C before plating. Log reductions at each water activity value, pH, and storage condition for NaCl solutions are shown in Table 4.3. From the results, *E. coli* was unable to achieve a 5-log reduction under any experimental condition at pH 7.0. At pH 4.5, however, a greater than 5-log reduction was achieved at a_w 0.97 without storage and at a_w 0.96 after 24 hr cold storage. *E. coli* inactivation was also found to be significantly higher ($p \leq 0.01$) at a_w 0.95 and 0.96 after 24 hr storage. Total inactivation was achieved at 0.98 and 0.99 for both conditions after HPP treatment.

Neither *Salmonella* nor *Listeria* was able to achieve a 5-log reduction at pH 7.0 under any experimental condition. At pH 4.5, *Salmonella* was completely inactivated at a_w 0.97, 0.98, and 0.99, and a greater than 5-log reduction was achieved after 24 hr storage at a_w 0.96. *Listeria* was also completely inactivated at a_w 0.97, 0.98, and 0.99, and a greater than 5-log reduction was achieved at a_w 0.96.

Though unable to achieve a 5-log reduction at pH 7.0, *E. coli* inactivation at a_w 0.98 and 0.99 were significantly higher ($p \leq 0.01$) than at a_w 0.95 – 0.97. However, *Listeria* inactivation was significantly higher ($p \leq 0.01$) at a_w 0.95 and 0.96 than at a_w 0.98 and 0.99. Taken all inactivation data at pH 7.0 and pH 4.5 into consideration, *E. coli*, *Salmonella*, and *Listeria* inactivation for both HPP treated and stored samples with a_w in the range of 0.96 – 0.99 were all significantly higher ($p \leq 0.01$) at pH 4.5. At a_w 0.95, inactivation of *E. coli* and *Salmonella* after 24 hr cold storage were found to be significantly higher ($p \leq 0.01$) at pH 4.5 than at pH 7.0.

Table 4.4: *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* inactivation by HPP at 550 MPa for 1 minute at 5°C in fructose adjusted TSB solutions (pH 4.5 and 7) at water activity values (a_w) 0.95 – 0.99. Average pathogen reduction with standard deviation is presented for each solution and pathogen.

***E. coli O157:H7* Reduction (Log CFU/mL)**

	a_w				
Solute	**0.95**	**0.96**	**0.97**	**0.98**	**0.99**
Fructose (pH 7)	0.11 ± 0.15^{A}	NA	0.44 ± 0.09^{A}	$1.15 \pm 0.61^{*A}$	$1.43 \pm 0.61^{*A}$
	0.77 ± 0.28^{A}	*0.79 ± 0.27^{A}*	*$1.33 \pm 0.15^{*A}$*	*$1.86 \pm 0.06^{*A}$*	*$1.87 \pm 0.30^{*A}$*
Fructose (pH 4.5)	0.75 ± 0.67^{A}	0.32 ± 0.13^{A}	1.46 ± 0.25^{A}	$5.12 \pm 1.32^{*B}$	$4.61 \pm 0.57^{*B}$
	0.84 ± 0.42^{A}	*0.94 ± 0.16^{A}*	***$3.54 \pm 0.62^{*B}$***	***$6.91 \pm 0.16^{*C}$***	***$7.06 \pm 0.09^{*C}$***

***Salmonella enterica* Reduction (Log CFU/mL)**

	a_w				
Solute	**0.95**	**0.96**	**0.97**	**0.98**	**0.99**
Fructose (pH 7)	0.26 ± 0.05^{A}	0.31 ± 0.04^{AB}	$0.63 \pm 0.22^{*AB}$	$1.83 \pm 0.39^{*B}$	$1.53 \pm 0.14^{*AB}$
	0.65 ± 0.02^{A}	*$0.64 \pm 0.06^{*A}$*	*$1.69 \pm 0.15^{*AB}$*	*$3.18 \pm 0.18^{*B}$*	*$1.82 \pm 0.12^{*A}$*
Fructose (pH 4.5)	0.84 ± 0.19^{A}	0.87 ± 0.15^{A}	$2.46 \pm 0.23^{*B}$	$7.40 \pm 0.08^{*C}$	$7.57 \pm 0.06^{*C}$
	1.07 ± 0.15^{A}	*$2.24 \pm 0.37^{*A}$*	***$6.26 \pm 0.95^{*B}$***	*$7.45 \pm 0.11^{*B}$*	*$7.57 \pm 0.06^{*B}$*

***Listeria monocytogenes* Reduction (Log CFU/mL)**

	a_w				
Solute	**0.95**	**0.96**	**0.97**	**0.98**	**0.99**
Fructose (pH 7)	0.28 ± 0.09^{A}	$0.31 \pm 0.09^{*A}$	$1.39 \pm 0.01^{*A}$	$1.78 \pm 1.01^{*A}$	$1.63 \pm 0.02^{*A}$
	0.53 ± 0.05^{A}	*$1.48 \pm 0.13^{*A}$*	***$3.63 \pm 0.97^{*B}$***	***$3.51 \pm 1.21^{*B}$***	*$1.01 \pm 0.68^{*A}$*
Fructose (pH 4.5)	0.50 ± 0.38^{A}	$1.88 \pm 0.49^{*A}$	$6.69 \pm 0.49^{*B}$	$6.99 \pm 0.16^{*B}$	$6.99 \pm 0.02^{*B}$
	1.41 ± 0.95^{A}	***$5.63 \pm 1.20^{*B}$***	*$6.96 \pm 0.13^{*B}$*	*$6.99 \pm 0.16^{*B}$*	*$6.99 \pm 0.02^{*B}$*

Normal: HPP treated samples. NA: no inactivation or HPP effect observed. *Italic*: HPP treated samples after 24 hr storage at 5°C. **Bold**: Significant differences compared to HPP treated samples under the same experimental conditions ($p \leq 0.01$). (^{ABC}): Different alphabet superscripts indicate significant differences ($p \leq 0.01$) within a row. (*): Indicates significant differences ($p \leq 0.01$) between pH 4.5 and 7.0 within the same pathogen and under the same experimental conditions.

4.4.2 Pathogen inactivation in fructose adjusted TSB

Fructose solutions of a_w values of 0.95 – 0.99 were also adjusted to pH 4.5 and 7.0 and HPP treated, with inactivation and significance recorded and summarized in Table 4.4. At pH 7.0, *E. coli*, *Salmonella*, and *Listeria* were unable to achieve a 5-log reduction under any experimental condition, similar to NaCl. At pH 4.5, *E. coli* was able to achieve a greater than 5-log reduction at a_w 0.98 and at a_w 0.99 after 24 hr storage. *E. coli* inactivation was found to be significantly higher ($p \leq 0.01$) after 24 hr storage at a_w 0.97, 0.98, and 0.99. *Salmonella* achieved complete inactivation at a_w 0.99, and a greater than 5-log reduction at a_w 0.98 and at a_w 0.97 after 24 hr storage. Inactivation at a_w 0.97 after HPP treatment was significantly lower than ($p \leq 0.01$) its 24 hr stored counterpart. *Listeria* was completely inactivated at a_w 0.98 and 0.99 and achieved a greater than 5-log reduction at a_w 0.97 and at a_w 0.96 after 24 hr storage. There was a

significant increase ($p \leq 0.01$) in inactivation at a_w 0.96 from immediate HPP treatment to overnight storage.

At pH 7.0, little significance was found between samples of different water activity values. At pH 4.5, inactivation of *E. coli* at a_w 0.98 and 0.99, *Salmonella* at a_w 0.97 – 0.99, and *Listeria* at a_w 0.96 – 0.99 were all found to be significantly higher ($p \leq 0.01$) than at the same a_w values at pH 7.0. Inactivation of *E. coli* and *Salmonella* in 24 hr stored samples were also found to be significantly higher ($p \leq 0.01$) at a_w 0.97 and 0.96, respectively, at pH 4.5.

4.4.3 Comparison of NaCl and fructose solutions

NaCl and fructose solutions were also compared for significance at pH 4.5 (Figure 4.1) and pH 7.0 (Figure 4.2). At pH 4.5, *E. coli* inactivation in NaCl was significantly higher ($p \leq 0.01$) at all water activity values tested. *Salmonella* inactivation was also significantly higher ($p \leq 0.01$) in NaCl at a_w 0.95 - 0.97, and *Listeria* at a_w 0.95 and 0.96.

At pH 7.0, *E. coli* inactivation was found to be significantly higher in NaCl at a_w 0.96 – 0.99, *Salmonella* at a_w 0.96 and 0.97, and *Listeria* at a_w 0.95 and 0.96, respectively.

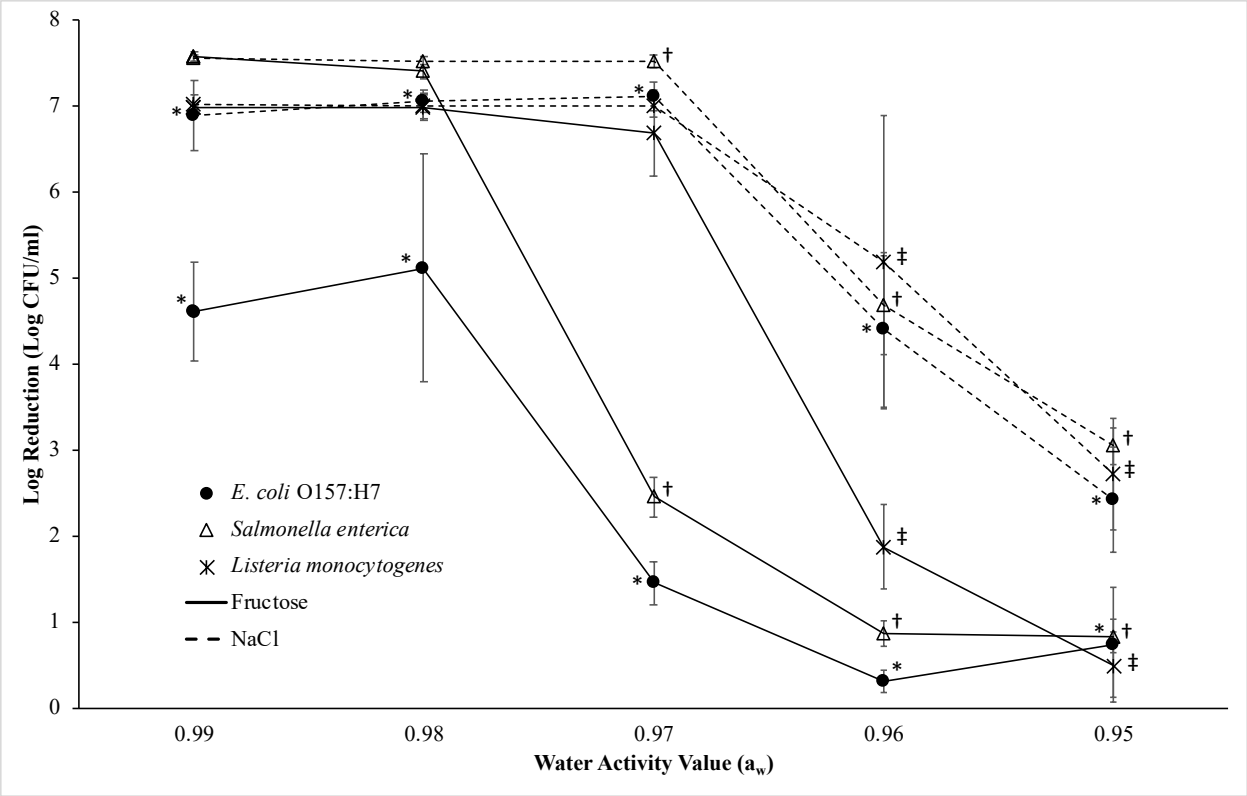

Figure 4.1. Effect of water activity on *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in fructose (solid lines) and NaCl (dashed lines) solutions high pressure processed at 550 MPa for 1 minute at 5°C at pH 4.5. (*) represents significant differences between fructose and sucrose *E. coli* inactivation at each water activity value, (†) for *Salmonella* inactivation, (‡) for *Listeria* inactivation. Error bars represent standard deviation of the log reduction averages.

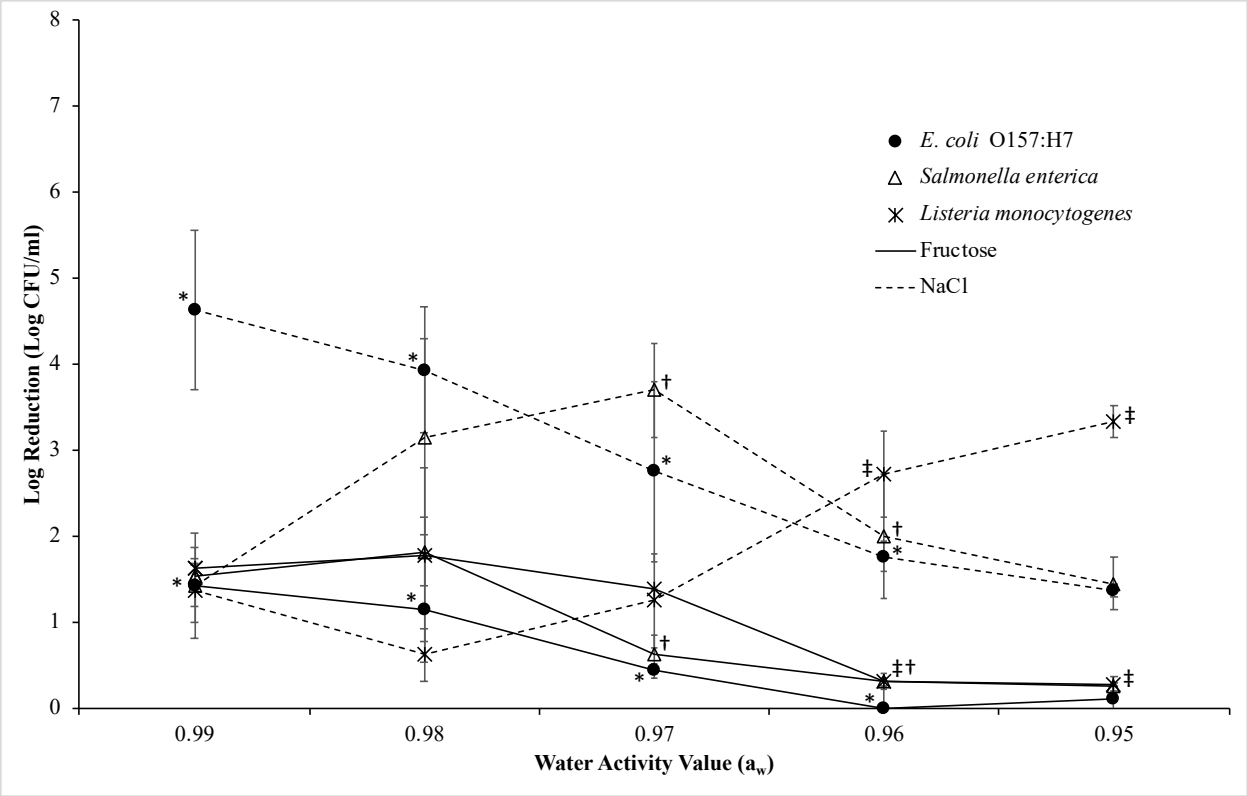

Figure 4.2. Effect of water activity on *E. coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in fructose (solid lines) and NaCl (dashed lines) solutions high pressure processed at 550 MPa for 1 minute at 5°C at pH 7.0. (*) represents significant differences between fructose and sucrose *E. coli* inactivation at each water activity value, (†) for *Salmonella* inactivation, (‡) for *Listeria* inactivation. Error bars represent standard deviation of the log reduction averages.

4.5 Discussion

The water activity of a food product has been shown to have significant effects on microbial growth, with low water activity (a_w <0.60) effectively inhibiting all microbial growth. However, it has been reported that low water activity with certain solutes (< a_w 0.95) can exhibit baroprotective effects on pathogens, leading to decreased sensitivity and increased resistance to high pressures (Hayman, et al., 2008; Oxen and Knorr, 1993; Palou et al., 1997).

HPP utilizes water pressure to transfer uniform pressure on the food product. Several mechanisms have been proposed for microbial inactivation by high pressure. HPP does not break covalent bonds, instead relying on protein and enzyme denaturation and cell membrane changes

to disrupt essential enzymatic functions. (Considine et al., 2008; Rastogi et al., 2007). As previously mentioned, low water activity can be protective of pathogens during HPP treatment. However, how narrow the range of water activity precisely influences bacterial inactivation in HPP remains elusive. We investigated the effects of water activity in a range from a_w 0.95 to 0.99 at both neutral (pH 7.0) and acidic (pH 4.5) conditions. We also studied the effects of two solutes that were used to adjust water activity, as it has been reported that solute type may confer different protections. Solutions were made using TSB to provide a few growth nutrients to facilitate more ideal growing conditions and to minimize interactions of other food constituents, such as solids, natural chemical compounds, and active enzymes. Fructose, or fruit sugar, was utilized to simulate the main sugar found in fruit juices and beverages. Samples were processed at 550 MPa for 1 min at 5°C (refrigeration temperatures) to mimic commercial processing conditions.

Inactivation comparisons were completed between solute types and pH at each water activity value for each pathogen. In NaCl solutions, no pathogen was able to achieve a 5-log reduction at pH 7.0. Most log reductions of *E. coli*, *Salmonella*, and *Listeria* were significantly higher at pH 4.5, with complete inactivation of *E. coli* at a_w 0.98 and 0.99 and *Salmonella* and *Listeria* at a_w 0.97, 0.98, and 0.99. Similarly, in fructose solutions, a 5-log reduction could not be achieved for any pathogen at pH 7.0. A greater than 5-log reduction of *E. coli* was achieved at a_w 0.98 and at a_w 0.99 after 24 hr storage at pH 4.5. *Salmonella* yielded a greater than 5-log reduction at a_w 0.98 and at a_w 0.97 after 24 hr storage. A complete reduction of *Salmonella* was achieved at a_w 0.99 and of *Listeria* at a_w 0.98 and 0.99. A greater than 5-log reduction of *Listeria* was achieved at a_w 0.97 and at 0.96 after 24 hr storage.

Comparing inactivation rates at different pHs indicated that inactivation at pH 4.5 was significantly higher ($p \leq 0.01$) than at pH 7.0. At pH 7.0, NaCl and fructose solutions were unable to inactivate any of the pathogens to a 5-log reduction, with a few significant differences in certain water activity values in NaCl solutions and almost no significant differences in fructose solutions. Low pH has been known to slow and hinder microbial growth. However, the effect of pH alone is insufficient to inactivate microorganisms (Beales, 2004; Mazzotta, 2001). One possible mechanism for pH inhibited growth involves undissociated weak acids, which diffuse through the cell membrane and cause intracellular imbalance, leading to inhibition of metabolic functions (Abee and Wouters, 1999). Based on this mechanism, the increased inactivation at pH 4.5 could be due to high pressure that causes cell membrane damage, allowing for increased diffusion of weak acids and further decreasing the intracellular pH.

The results have also shown that there are significant inactivation differences ($p \leq 0.01$) between a_w 0.95 and 0.99 at pH 4.5, with much higher inactivation rate at higher water activity values. The effect of solute type on inactivation of pathogens was also compared. It was found that significantly higher inactivation was generally observed in NaCl solutions. Some studies suggested that with HPP treatment, NaCl is less protective as a solute, which is further confirmed by our study (Setikaite et al., 2009). This could be due to the fact that less NaCl (g) is needed to lower the water activity as compared to fructose (g), which requires a large quantity to reach the same a_w level. These findings support the previous work that determined the protective effects of low a_w and increased concentration of solutes (Setikaite et al., 2009). It also demonstrates the crucial role the availability of water plays in pathogen inactivation (Hayman et al., 2008).

In this study, differences in inactivation were found at different a_w values adjusted with either NaCl and fructose at pH 7.0 and pH 4.5. The results have provided key parameters for

minimum a_w values a solution must be at to achieve a 5-log reduction. It is acknowledged that the study was completed in a broth-based model and is not representative of all beverage products. However, the broth model does not contain many other factors that could influence pathogen inactivation, with either protective or antimicrobial constituents, providing a solid foundation to determine the effect of water activity. In addition, the media provided conditions that simulated the "worst case" scenario for pathogen growth in food matrices. In general, the higher the a_w value, the greater the microbial inactivation. However, this work also suggests that pH plays a critical role in pathogen inactivation, and the effect of water activity appeared to be stronger at acidic pH when compared to neutral conditions. From this study, these factors suggest that HPP effectiveness depends largely on product composition, and additional research is necessary to determine the interactions between complex food constituents to provide a more in-depth understanding on high pressure induced pathogen inactivation.

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CHAPTER 5

HIGH PRESSURE PROCESSING – ECONOMIC ANALYSIS

5.1 Introduction

High pressure processing (HPP), also known as ultra-high pressure (UHP) or high hydrostatic pressure (HHP) processing, is a non-thermal processing method used to inactivate pathogens and preserve fresh qualities in certain food products. The juice industry has seen a strong shift towards functional and nutritional juices, which has led to an exploration of alternative non-thermal processes that ensure desirable qualities as well as food safety. This is in response to markets where consumers are becoming increasingly health conscious and demanding food products with functional benefits, such as increased nutritional value, freshness, and flavors (Hogan et al., 2005, Huang et al., 2017).

Currently, most of the 100% juice and juice products are heat pasteurized. However, thermal processes can negatively affect certain qualities, such as sensory characteristics, loss of color, flavors, and nutritional contents of food (Considine et al., 2008). HPP utilizes water pressure to process products and the high-pressure treatment has minimal effect on flavor and nutrients, such as vitamins (Georget et al., 2015; Oey et al., 2008b).

At present, juice products treated with HPP represent a small but innovative and fast-growing segment of the juice market (Mintel, 2019). One major barrier to growth is the high investment costs associated with high pressure processing (Elamin et al., 2015). The objective of this study was to investigate and compare capital and operational costs of an HPP system to a thermal processing equivalent. This study will also analyze the feasibility for small producers to enter the HPP product market by investing in HPP technology or exploring alternative high-pressure processing avenues, such as toll processing or contract manufacturing.

5.2 High pressure processing

High pressure processing (HPP) is primarily carried out as a batch process but can be semi-continuous for liquid products under the right conditions (Considine et al., 2008; Hogan et al., 2005). Untreated food products are packaged in flexible containers and loaded into a high-pressure chamber (Balasubramaniam and Farkas, 2008). Desired settings, including pressure, dwell time, and temperature can be adjusted for the treatment. The chamber is then filled with water, sealed, and filled with more water to reach the set pressure. The chamber holds the pressure for a specified period of time before discharging the pressure and removing the products. Currently, commercial HPP treatments are processed between 400 – 600 MPa for three minutes at refrigeration temperatures. Unlike most processing methods, including HTST, HPP has the advantage of processing food products in its final packaging, reducing the risk of additional contamination.

Industrial HPP machines are currently installed and operational in North America (United States, Mexico, and Canada), South America (Peru and Chile), Europe, and Asia (Japan, China, and South Korea) (Tonello, 2011). The main application of HPP is to produce fresh-like products without compromising food safety, such as ready-to-eat (RTE) vegetables, juices, and beverages (Tonello, 2011). Compared to other nonthermal processes, such as irradiation and pulsed electric fields, studies have shown that consumers have a more positive response and higher acceptance of HPP as a newer and emerging processing technology (Cardello et al., 2007; Wright et al., 2008). Over the last several years, the number of HPP equipment installations has been rising, with a five-fold increase between 2004 and 2014 (Elamin et al., 2015). Despite the rising number of installed units, HPP is still not commercially widespread due to higher capital and

maintenance costs compared to other processing methods, including thermal pasteurization (Toepfl et al., 2006).

5.3 Thermal processing

Thermal processing has long been documented and utilized as an effective method to ensure food safety by inactivating pathogens through high heat. As a comparable thermal equivalent to HPP, a high-temperature short-time (HTST) pasteurizer will be analyzed as HTST and HPP treated products must be refrigerated post-processing and share many of the same processing parameters and factors. Products that are treated with HTST include dairy, such as fluid milk, and some fruit juices, with treatment conditions that can range from 70°C - 90°C (158°F – 194°F) for six to fifteen seconds, depending on the type of product (ICMSF, 2005). Not all spoilage bacteria will be inactivated under these processing conditions, necessitating refrigeration to keep them under control (Meer et al., 1991; Schröder et al., 1982). Similarly, HPP products must also be refrigerated due to remaining enzymatic activity, which can cause detrimental sensory changes, such as product browning (Oey et al., 2008b). In the process, HTST pasteurized products are heated and then cooled down to refrigeration temperatures before bottling and storage, and HTST pasteurizers are typically continuous systems fitted with either plate or tubular heat exchangers. Attachment options include electric, natural gas, propane, or oil boilers and chiller systems. Water and compressed air are also necessary for operation start-up and sanitization. However, the costs associated with these are minimal.

5.4 Costs for high pressure processing

For the purpose of this study, we will primarily evaluate operational costs that are different across HPP and HTST systems, including the main processing units and energy requirements. Other factors associated with manufacturing, including processes before and after treatment, storage, transport, charges for facility development, and other ancillary or supplementary materials and equipment (filling, labeling, packing), are not included in the estimates. Processing units were chosen based on similar output flow rates, which is shown in Table 5.1 along with detailed main capital costs for HPP and thermal processing.

- Capital Investment: High pressure processing units can range from $770,000 to $3,150,000, depending on size, capacity, and manufacturer (Tonello, 2011). For this analysis, a Hiperbaric 135L was considered, with an estimated cost of $1,140,000 per unit (Hiperbaric, Burges, Spain). Shipping estimate for this unit is $36,000. For most commercial HPP processes, a chiller is required and estimated to be $15,000 - $20,000.
- Utilities: Energy consumption depends on equipment model and size. For the Hiperbaric 135L, electricity usage is estimated to be 5.7 kWh per cycle with 9.1 cycles per hour. Based on an average industrial electricity rate in the Middle Atlantic geographic area of $0.0696 per kWh (EIA, 2018) this amounts to $3.61 per hour. Chiller electricity usage is estimated to be 11.9 kW per hour. Assuming an eight hour processing period, electricity usage is estimated to be 95.2 kWh per day, resulting in charges of $28.88 per day for the HPP unit and $6.63 per day for the chiller.
- Labor: HPP treatment is mainly a batch process and necessitates operators for the equipment programming and for loading and unloading of product. We assume three full-time equivalent (FTE) operators at an average labor rate of $40 per hour (Sampedro et al., 2014).

- Packaging: HPP compliant bottles generally have a heavier weight of 33 grams. A 33 grams, 16 oz. unit would cost an estimated $0.21 per bottle (Crompton, 2019).
- Depreciation: Assuming a straight line depreciation method, annual depreciation (a non-cash cost), is estimated at 10% of capital cost, based on HPP equipment useful life of 10 years (Sampedro et al., 2014).
- Maintenance: Annual maintenance and repair costs are estimated at 8.0% of capital cost (for every 200,000 – 500,000 cycles) (Sampedro et al., 2014).

Table 5.1. Capital cost of thermal and high-pressure processes

Process parameters	Unit of measure	Thermal	High pressure
Process flow*	gal/yr	624,000	521,000†
	l/yr	2,362,000	1,972,000
Pasteurizer with heater	$	110,000	–
High pressure equipment	$	–	1,140,000
Process chillers	$	8,000	15,000
Total equipment cost	$	118,000	1,155,000
Shipping costs	$	1,000	36,000
Total capital cost	$	119,000	1,191,000
Capital cost	$/l	0.05	0.60

*: Based on 8 hr/day manufacturing period, 5 days/week, 52 weeks/year.
†: Assuming 75% filling ratio (Tonello, 2011).

5.5 Costs for HTST pasteurization

- Capital Investment: HTST pasteurizer costs also depend on size and output rates. For this study, a Goodnature XT Series Pasteurizer with a 300 gal/hr flowrate was considered (Goodnature, Buffalo, NY). The estimated equipment cost is $110,000 for the pasteurizer with attached heater and $8,000 for the attached chiller (Whitehead, 2019). Total shipping estimate for these units is around $1,000.

- Utilities: The HTST pasteurizer with an electric boiler and chiller are estimated to use 18.4 kW per hour and 7.8 kW per hour, respectively. Assuming an 8 hour manufacturing period at $0.0696/kWh, both units would require a total of 209.6 kWh per day, or $14.58.
- Labor: The HTST pasteurizer is a continuous flow process and advertised to have little to no operator attention needed. We assume one FTE operator at an average labor rate of $40 per hour (Sampedro et al., 2014).
- Packaging: Many juice producers opt for 33 grams plastic bottles. However, a lower weight 23 grams, 16 oz. bottle would be sufficient for packaging at $0.18/bottle (Crompton, 2019).
- Depreciation: As above, 10% of capital cost based on a life of 10 years (Sampedro et al., 2014).
- Maintenance: Annually at 2.0% of capital cost (Sampedro et al., 2014).

5.6 Process cost comparison

We analyze and compare the costs discussed above and related solely to the main processing component. Total annual production costs are listed in Table 5.2. We assume the processing equipment is paid in cash (i.e., no loan interest charges), while depreciation represents a noncash cost. Accordingly, the total costs included in Table 5.2 represent an annualized cost to purchase and operate the equipment.

Table 5.2. Annualized costs of thermal (HTST) and high-pressure (HPP) processes per year

Process parameters	Unit of measure	Thermal	High pressure
Electricity			
Electric boiler	kWh/yr	38,000	–
Refrigeration units	kWh/yr	16,000	25,000
High pressure unit	kWh/yr	–	108,000

Total Electricity	kWh/yr	55,000	133,000
Electrical Costs	$/yr	4,000	9,000
Labor			
Plant operators per shift	Number	1	3
Labor costs	$/hr	40	40
Total labor costs	$/yr	83,000	250,000
Unit-related costs			
Estimated unit life	year	10	10
Maintenance charges*	**%**	2	8
	$/yr	2,000	95,000
Total production costs	$/yr	89,000	354,000
Depreciation*†	%	10	10
	$/yr	12,000	119,000
Total annualized costs	$	101,000	473,000

*: Applied to capital investment cost
†: Noncash cost

Equipment for both process technologies was chosen based on similar flow output capabilities. This study has shown that capital and production costs of HPP are much higher than HTST pasteurization, with HPP capital costs, production cost, and total annualized costs 10.0, 4.0, and 4.7 times, respectfully, the costs associated with HTST pasteurization. The capital cost was calculated to be $0.05/l and $0.60/l for HTST and HPP, respectively.

HPP is mainly a batch process, which requires multiple plant operators, and of the total production costs, labor costs covered the majority at 70.6%. These costs could be lowered if fewer operators are needed and if the operation were to become continuous. However, Sampedro et al. (2014) estimated that reconfiguring the system to a continuous or automatic process would increase equipment costs by 10 - 35%, or an additional 1.0 – 3.5% increase to annualized capital cost. Regarding energy usage of both the unit and chiller, the electricity costs only accounted for 2.54% of the total annual production cost. Other main costs include maintenance and

depreciation, which is estimated to be 20.1% and 25.2%, respectively, of the total annualized costs.

The HTST pasteurizer was estimated to have a much lower capital and production cost. Maintenance (2.0%) and depreciation (11.9%) estimates accounted for much less of the total annualized costs as compared to HPP. The majority of the production cost, similar to HPP, was labor, taking up 93.3%. As a continuous process, the HTST pasteurizer requires a maximum of 1 operator to monitor the process. However, labor estimates in this study were on a conservative side as most HTST monitoring would not be continuous nor the sole duty of a plant operator.

5.7 Alternative processing costs

From this study, it has been shown that startup costs for HPP manufacturing are incredibly high and most likely infeasible for small producers and companies. An alternative to directly investing in processing technology would be the use of contract manufacturers and/or toll processors. A toll processor would be able to supply the manufacturing processing, while a contract packer or manufacturer would supply the manufacturing process with the capability for additional arrangements, such as sourcing raw materials, treating and packaging the product, labeling, storage, and so on (Kim, 2003).

Costs for contract manufacturing depend on a variety of factors, including product type, product development, volume of product, bottle type and size, processing parameters, packaging, number of products, etc. (Baudier, 2019). Based on current industry rates of select companies, tolling or manufacturing fees can be in the range of $0.15 – $0.25 for HTST pasteurization and $0.25 – $0.33 for HPP treatment per 16 oz. unit, or $0.33 - $0.43 and $0.46 - $0.54, respectively, including the price of bottles (Baudier, 2019; Brown, 2019). Based on the estimated process flow

in Table 5.1 and annualized costs in Table 5.2, the tolling costs are calculated to be $0.04/l or $0.02 per 16 oz. for HTST pasteurization and $0.24/l or $0.11 per 16 oz. for HPP treatment. While our computed tolling rates are well below those suggested in the industry today, they include only cost recovery (including capital) with no processor margin (i.e., the returns above costs to owners of processing firms). In addition, related overhead costs for infrastructure and other equipment is excluded from our estimates. That said, compared to the industry rates mentioned above, implied gross processor margins (i.e., for overhead and residual profits to owners) would amount to 86.7% - 92.0% for HTST pasteurization and 56.0% - 66.7% for HPP treatment. Average processing margins in the neighborhood of 50% - 60% is not uncommon based on aggregated industry data, thus the cost-only toll rates for HPP processing appear reasonable to industry standards. The further discrepancy for HTST processing is likely due to a wider array of HTST toll processing equipment than our comparison HTST model dictates.

5.8 Conclusion

Many studies have reported the high HPP investment costs (Sampedro et al., 2014; Toepfl et al., 2006; Tonello, 2001), which was further supported by our study. However, in certain sectors, such as the juice industry, HPP products are a small but rapidly growing market. Consumers are gravitating towards products with more functionality and nutritious quality, without compromising on taste and safety. Compared to other nonthermal processing technology, HPP products have higher consumer acceptance due to improved quality compared to their thermally processed counterparts and the process perceived as a natural technology (Mújica-Paz et al., 2011).

Both HPP and HTST treatment would extend the shelf life of food products and ensure food safety. However, studies between HPP and HTST processed juices have shown that the quality of HPP treated products are much more comparable to fresh juices, as HTST treatment caused detrimental aroma and color changes, as well as vitamin loss (Huang et al., 2018; Liu et al., 2016; Yi et al., 2017). Sensory tests conducted have also shown panelists scoring HPP juices higher in sensory properties (flavor, mouthfeel, freshness) and overall higher acceptability compared to thermally treated juices (Laboissière et al., 2007; Liu et al., 2016). Consumers that value fresh-like products would likely have a higher willingness to pay the higher price for HPP products due to the similarity in taste with improved shelf life and safety. Currently, entering the market for most startup and smaller companies is expensive and difficult, with toll processing or contract manufacturing services a more feasible option than investing in HPP equipment. Nevertheless, with a higher demand for more functional and beneficial products, the increase in demand for HPP products and units would require consumers to be willing to pay an estimated $0.09 more per 16 oz bottle for HPP over HTST products, a reasonable difference to be sure. Future HPP developments include more effective options to upgrade the functionality and design of the unit to lower costs while maintaining the integrity and effectiveness of high-pressure processing.

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CHAPTER 6

IMPLICATIONS AND FUTURE WORK

Nonthermal processing has been growing in popularity due to its ability to process certain products and ensure food safety without the use of heat. The work and research that has been detailed in the previous chapters focus on nonthermal technologies and how they impact pathogen survival in fruit juices or aqueous solutions, specifically focusing on a chemical method (dimethyl dicarbonate) and use of high pressure (high pressure processing).

Dimethyl dicarbonate (DMDC), a microbial control agent, was used on *Salmonella enterica* and natural yeast and mold to determine the efficacy of inactivation in orange juice. The usage of DMDC is effective under certain conditions, and yet for a small business or company, its usage might be unfeasible or impractical due to the high costs associated with the dosing mechanism and instrument. However, the use of DMDC results in a final product with minimal changes to quality and sensory, as the DMDC reaction only produces small amounts of carbon dioxide and methanol. Some studies have already explored the combination effects of DMDC with other types of nonthermal processing methods. This research could be expanded in terms of the type of processing methods and products, looking at a wider range of dosing concentrations, shelf life, and overall sensory quality.

A major portion of the research in this thesis focuses on high pressure processing and how certain parameters associated with the process affect pathogen inactivation in juices. As a relatively new technology, the parameters associated with HPP have been understudied. Much of the research on HPP has been conducted using a small, benchtop machine instead of an industrial or commercial processing unit. The results from small scale HPP machines, however, might not accurately reflect the processes that are occurring during commercial production, emphasizing

the importance of conducting research using commercial HPP units for results that can be directly related and associated with the industrial process and final product.

There are several parameters associated with the high pressure process, with the majority falling under machinery and process factors or associated with the product itself. Process parameters include the processing pressure, dwell time, water processing temperature, while product factors are associated with the product composition, such as the pH, soluble solids, °Brix, and water activity of the product. The research detailed in the previous chapters focused on three main parameters (water processing temperature, pH, and water activity) to determine some of the limitations and restrictions associated with using HPP on fruit juice, beverages, and aqueous solutions.

A study was conducted on HPP water processing temperatures and found that there were pathogen inactivation differences between the temperatures tested (refrigeration against ambient). Future study could be conducted to monitor actual temperature changes that happen during the process, or in the HPP machine as some studies have indicated that the temperature changes for each increment of pressure used. Examining a wider range of water processing temperatures could also lead to a better understanding of the minimum temperatures needed to achieve food safety. More experiments could be planned using different product mediums to determine if processing temperature effects are universal for pathogen inactivation.

In this thesis, research was also conducted regarding the effects of pH and acid on pathogen inactivation, with results indicating mild synergistic effects between low pH and pressure. Three different fruit juices were tested, and distinct differences in pathogen reduction were found between the lowest pH tested (around pH 3.4) and pH 4.0. Future work could be conducted to examine the pH changes with a narrow pH range of 0.1 increments, as compared to

the 0.5 increments tested in the study mentioned. The results of this study also indicated that pathogen inactivation did depend on the pH of the product, but a more comprehensive understanding could be constructed when a variety of other product composition factors are taken into consideration, including solute content and type.

The last HPP study focused on the water activity of aqueous solutions. Two main solutes, fructose and sodium chloride, were used to adjust the water activities of broth solutions to look at the combination effects of water activity and pressure on pathogen inactivation. The study was also conducted at two different pH (7.0 and 4.5), which showed that pH played a large role in pathogen inactivation, despite the water activity level. It would be interesting to assess the long-term effects of low water activity and pressure and determine the pathogen inactivation trend during storage after HPP. The broth model used is simple and effective, but may not represent all beverages, and an adapted version of this study in a juice model would likely produce more information on the log reduction of pathogens.

Currently, all products processed by HPP must have the process validated, according to the U.S. Food and Drug Administration (USFDA). Thermal processing has long been evaluated, and several studies have validated the methodology for fruit juice processing. To achieve standardized processing conditions with HPP, additional research must be conducted to determine at the combinations of pressure and time and the limitations of product composition to achieve food safety. The current industry standard for processing fruit juice and beverage products is 600 MPa for 3 minutes. However, even at this standard, not all products are able to achieve a 5-log reduction due to the product composition. Each HPP experiment mentioned previously in this work was conducted at 550 MPa for 1 minute, which is less than the commercial standard. This pressure and processing time were initially chosen to determine

differences between pathogen responses. However, if the same pathogen inactivation levels can be achieved at a lower pressure and time, such as a 5-log reduction necessitated by the USFDA to complete a successful process validation, this implicates that safe products can be produced under these lower processing conditions. Lower processing times would increase the efficiency, allowing more units to be processed per hour. Lower pressures would also result in less wear and tear of the machine, as achieving such high pressures can cause strain on the equipment.

The research completed in this work has addressed a number of important parameters associated with HPP, in terms of both the process factors and product composition. Future works regarding HPP could expand to individual testing of each essential parameter in both parameter categories, such as the pressure holding or dwell time, which have yet to be fully explored. Additional research could be carried out on testing a wide range of pressures against processing times to determine the decimal reduction time (D-value) and the pressure equivalent of a Z-value. These experiments, if extended to other types of food products, would also provide information for the lowest combinations of pressure and time necessary to achieve food safety.

HPP has received increasing interest as a nonthermal processing method, due to the current trend for fresh-like products with increased nutrition and benefits. As the interest in HPP grows, additional research and study is necessary to determine the limitations of processing and product factors as well as the possibilities for innovation in new products and increased nutrition. This would greatly help establish the standard conditions necessary to ensure food safety and deliver a safe, quality product.