HIGH PRESSURE PROCESSING, pH, AND THE EFFECT OF PRESERVATIVES ON THE SURVIVAL OF SPOILAGE FUNGI AND *ALICYCLOBACILLUS* SPP. IN A DILUTED APPLE JUICE CONCENTRATE AT VARYING WATER ACTIVITIES

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Elizabeth Claire Buerman

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Elizabeth Claire Buerman, Ph. D.

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ABSTRACT

Spoilage fungi and bacteria are capable of surviving typical juice pasteurization methods and growth during shelf life. High Pressure Processing (HPP) is a method used to extend the shelf life of foods by subjecting products in their final packaging to extreme pressure. This process leads to protein degradation in microbial cells, membrane degradation, and eventual cell death. Survival of spoilage organisms, especially spore formers, has not been closely considered in regard to the effect of water activity (a_w). HPP causes less nutrient and color degradation than traditional thermal processing which results in improved consumer acceptance. The purpose of this study was to determine the effect of pH, sulfites, dimethyl dicarbonate (DMDC) and a_w on HPP apple juice to prevent fungal and bacterial spoilage during storage.

Spoilage fungi, *Aspergillus pseudoglaucus, Aspergillus fischeri, Paecilomyces niveus* (*Byssochlamys nivea*)*, Penicillium* spp., *Paecilomyces variotii* (asexual form of *Byssochlamys spectabilis), Aspergillus niger, Candida parapsilosis, Rhodotorula mucilaginosa,* and *Torulaspora delbrueckii* as well as three strains of spoilage bacterium *Alicyclobacillus* spp. were inoculated into diluted apple juice concentration adjusted to 0.94, 0.96, 0.98 and 1.0 a_w at pH 3.5, 4.6 or 7.0 with either no preservative, 8 ppm sulfite or 0.025% DMDC. These products were

processed at 5°C in a commercial HPP unit at 300 MPa for 1.5 min, 450 MPa for 1.5 min, or 600 MPa for 1.5 - 15 min. These products were stored at 5 or 23°C for six months. Results display that processing pressure sensitive fungi at 450 MPa or higher and storing at refrigeration could extend storage up to 26 weeks. Pressure resistant organisms, *A. fischeri, A. pseudoglaucus, P. niveus, C. parapsilosis* and *Alicyclobacillus* spp. were capable of surviving the conditions tested. Additionally, using sulfites and DMDC did not significantly improve reductions of spoilage organisms, and storing HPP juices at refrigeration is necessary for extended shelf life.

BIOGRAPHICAL SKETCH

Elizabeth Claire Buerman, daughter of Karen and Gary Buerman, was raised in Newark, New York. She attended Cornell University for a degree in Interdisciplinary Studies with a concentration in Food Microbiology. After receiving her Bachelor of Science in 2012, Elizabeth moved to Englewood Cliffs, NJ. She worked as a Category Microbiologist with Unilever in the Research and Development Department. She was a member of a cross-functional team that produced many new, preservative free spreads under “I Can’t Believe It’s Not Butter” and “Country Crock” brand names. In 2014 Elizabeth returned to Cornell University to work under Dr. Olga Padilla-Zakour; studying osmophilic molds in low water activity foods. She earned her Master of Science degree in 2016 and chose to continue working with molds for her Ph.D.

To Grigor Robert Lynch and Winston Woolly Mammoth Buerman-Lynch:

For always being #1

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CHAPTER 1

NONTHERMAL FOOD PRESERVATION METHODS: HIGH PRESSURE PROCESSING, ULTRAVIOLET IRRADIATION AND PULSED ELECTRIC FIELD: A REVIEW

ABSTRACT

Consumers expect whole fruit and vegetables to be visually appealing. Fruits and vegetables that are not considered fit for grocery stores typically are processed into other products such as juice or sauce. “Ugly” fruits and vegetables can be processed into many types of products such as juices, fermented products, sauces, concentrates, and ciders. Non-thermal methods of food preservation have become more popular because the resulting product is generally considered “fresher” and minimally processed than traditionally processed foods. The public is increasingly searching for high quality, non-thermally treated, preservative free foods. High Pressure Processing (HPP), UV Light and Pulsed Electric Field are all methods that not only extend shelf life, but also meet consumer demands and provide safe, convenient foods.

HIGHLIGHTS

- The composition of the food has a direct relationship to the efficacy of the preservation method.
- HPP, UV and PEF are non-thermal methods of preservation that can be used to extend the shelf life of juices.
- Low oxygen concentration is less effective at preventing spoilage, even when combined with low water activity.

The popularity of high pressure processing (HPP) has exponentially increased in the past few years and has been commercially applied for the production of guacamole, deli meats, baby food, juice, soup, and shellfish products (Hiperbaric.com, 2018). Commonly referred to as "cold pasteurization," it subjects products in their final packaging to extreme pressure, typically at refrigeration (5°C) temperatures (Lado et al., 2002). This leads to protein degradation in microbial cells, cell membrane degradation, and eventual cell death (Mujica-Paz et al., 2011, Lado et al., 2002, Black et al., 2007). However, more resistant spores are not affected, and products must be refrigerated to prevent or slow growth of surviving spores. HPP products can have 50% or longer shelf lives than unprocessed or more minimally treated products (correspondence with Cornell HPP validation center). Because HPP does not use heat; vitamins, minerals, and pigments survive at improved rates when compared to thermally processed products (Mujica-Paz et al., 2011; Oey et al., 2008, Evelyn et al., 2016).

Uses of HPP are wide ranging from juices to meats. HPP can be used to reduce the incidence of food borne pathogens in raw meat, can result in more tender meat products compared to unprocessed meant products, and can reduce the risk of post-process *Listeria* contamination in cold cut deli meats (Souza et. al, 2011, Bajovic et al., 2012). HPP can reduce the incidence of *Vibrio parahaemolyticus* in raw oysters, that otherwise would have no preventative control (Ma and Su, 2011). Its effect on the reduction of norovirus in fresh produce has also been studied and found to cause more than 2-log CFU/mL reductions at 400 MPa for 2 min at 4°C. However, HPP can cause quality and textural issues with whole fruits and vegetables (Lou et al., 2011). As a result, it is commonly used with liquids such as juices and dressings.

These HPP products are perceived as “minimally processed” and better accepted by consumers than other processing methods (Wright et al., 2007; Black et al., 2007). Sensory panelists have been unable to detect differences in sensory tests between untreated juice and HPP juice. After a three-week refrigerated storage period, a panel of trained sensory judges could perceive fewer defects in the HPP product than the untreated control (Butz et al., 2002). HPP jabuticaba (a Brazilian fruit) juice scored the same as fresh, unprocessed jabuticaba juice in overall impression, taste, appearance, and intent to purchase but scored lower for aroma. However, other studies have found that other HPP juices, such as carrot, scored higher in the aroma category (Inada et al., 2018). For these reasons, HPP use has increased dramatically in the past decade (Hiperbaric).

HPP juice is a growing category. For example, Suja, Wegmans, Hain Celestial and PepsiCo all have HPP treated juice brands and smaller juice companies such as Evolution (found at Starbucks), and love grace produce HPP products too (Suja Juice, Wegmans, Naked Juice, BluePrint, Evolution Fresh, love grace foods). However, spoilage microorganisms found in traditionally processed juices can pose a risk to shelf life of HPP products as well. Thermally processed apple juice spoilage is commonly caused by yeast, filamentous fungi (mold), and spore forming bacteria that can either survive processing or are caused by post processing contamination (Hocking et al., 2006). Spoilage can cause unsightly colonies, bottle swelling, and off flavors. The juice category represents over a $6 billion industry and losses due to spoilage can be economically devastating (Mintel, 2018). Thermally treating juices until heat resistant organisms are undetectable can result in low quality juice (Lee et al., 2006). Juice is generally processed with hot fill methods by heating to 85 to 95°C for one to two min and then filled into its final packaging (Silva et al., 2012). The bottles are capped and then turned so that hot product

comes in contact with the cap. These packages are held for several minutes before cooling (Chang et al., 2004). HPP can be used as an alternative for safety and shelf life extension. Pathogen validations have been done studying the efficacy of HPP but have not closely studied the effect on spoilage organisms.

The composition of the food has a direct relationship to the efficacy of the preservation method (Georget et al., 2015). Nutrients, minerals, water activity (a_w), pH and fat content can all contribute to survival of organisms (Black et al., 2007). Higher survival rates of fungi have been observed in juices with higher sugar concentrations (Black et al., 2007). Differences in solutes such as NaCl, sugar and glycerol have also been observed to have an effect on survival (Goh et al., 2007). Butz and others (1996) saw varying survival results of heat resistant fungus *Byssochlamys nivea* in grape juice, bilberry (European blueberry) preserves, and salt water. Very little inactivation was achieved during processing at 700 MPa and 70°C in the preserves. However, inactivation rates achieved in juice were relatively similar to those achieved with salt as a solute. Initially, inactivation rates were faster in juice, but after 30 min, inactivation rates between salt water and juice were the same. Butz and others also found little difference in survival rates at differing pH values (Butz et al., 1996).

Oxen and others (1993) found that yeast *Rhodotorula rubra* populations could not be reduced by HPP when the water activity was at or below 0.91, regardless of whether the solute used was glucose, fructose, or sodium chloride (Oxen et al., 1993). The same researchers found that 0.94 a_w required long processing times, but significant reductions could occur at 0.96 a_w. Finally, Oxen and others found that the water activity better predicted survival than pH values between 3.0 and 8.0 (Oxen et al., 1993). Subsequent studies displayed similar results with other yeasts showing increased survival at 0.95 a_w as compared to 0.98 a_w. Sucrose has also been

shown to be more protective than salt in some instances, while salt was protective in others. Many hypotheses have been proposed regarding how solutes and water activity affect microorganisms' survival during HPP. It is believed that the saturation of the medium may have an effect on "displac[ing] … water from the core of the protein, and hence decreased the volume and compressibility of the protein interior and thus stabilized proteins against denaturation" (Georget et al., 2015). However, many of these studies were carried out at temperatures higher than typically used in processing or with bench top models of HPP units. High temperature as a critical control point may be risky as temperature throughout the pressurization chamber is not always uniform (Georget et al., 2015). Therefore, it is necessary to determine the effects using commercially available equipment and food systems.

Another non-thermal technology that can cause a 5-log reduction of pertinent pathogens as well as extend shelf-life is Ultraviolet Irradiation (UV). UV is an FDA approved method for achieving a 5-log reduction of pertinent pathogens in juices and cider (Usaga et al., 2015, Kaya et al., 2015). It is also commonly used to treat drinking water in both North America and Europe (Pereira and Vicente, 2010). UV can inactivate bacteria and fungi in the range of 200 - 280 nm (Kaya et al., 2015). The flow rate can be adjusted, or juice can be re-cycled to increase exposure to UV (Guidance for Industry: Juice HACCP Hazards and Controls Guidance First Edition). The FDA requires that the "UV radiation be provided by low pressure mercury lamps emitting 90 percent of the emission at a wavelength of 253.7 nanometers (2,537 Angstroms), and that during the treatment, the juice undergo turbulent flow through tubes with a minimum Reynolds number of 2,200." (FDA Guidance for Industry: Juice HACCP Hazards and Controls Guidance First Edition, FDA 21CFR179.39). UV works by damaging crosslinks between thymine and cytosine of microorganisms' DNA (Choi and Nielsen, 2004). UV has other benefits as well, including the

ability to reduce levels of patulin (a mycotoxin produced by fungi commonly found on apples and can result in chronic long-term health problems), and insect larvae. UV has also been used to decrease surface contamination of meat and water, both drinking and waste. UV units can be $5-$15,000 cheaper than more traditional thermal pasteurization systems (Choi and Nielsen, 2004). However, there are a few drawbacks to UV treatment. For example, as UV is absorbed by ascorbic acid, polyphenols, pectin, cellulose and other compounds its effectiveness in certain juices decreases. For these reasons, more UV must be applied to unfiltered apple cider than filtered apple juice (Assatarakul et al., 2012).

Pulsed Electric Field or PEF is another non-thermal technology used to treat juices. Pulses (static or continuous) of high voltage electric current are sent between two electrodes in a chamber holding the food resulting in damage to the cell membrane and microbial inactivation up to 9-logs (Cserhalmi et al., 2006). The length of pulse, number of pulses and field intensity can also be adjusted. A small amount of heat may be generated, depending on the processing parameters, with higher temperatures shown to be more effective. Preservatives may act synergistically with PEF and permeate the cell through membrane damage. pH of the product tested can have an effect as well; the further the product is from the target organism's optimum, the better it will work synergistically with PEF. Like HPP, PEF is less effective at lower water activities, most likely due to shrinkage of the cell from reduced available water and subsequent cell membrane thickening (Aronsson and Rönner, 2001, Pereira and Vicente, 2010). Also, like HPP products, PEF juices fare better nutritionally and sensorially as compared to heat treated juices (Cserhalmi et al., 2006). HPP, PEF, and low pasteurization (70°C for 30s) of orange juice were compared in regard to the Vitamin C concentration. HPP had the highest amount of Vitamin C retained in comparison to the other treatments while PEF retained the lowest amount.

However, all treatments were fairly similar and met the daily recommended amount of Vitamin C for an adult even at refrigerated storage for 40 days (Plaza et al., 2006).

Similar to results by HPP, sexual spore forming fungi have been shown to be PEF resistant. Raso and others (1998) found that *Byssochlamys fulva* survived best in tomato juice that was treated with PEF and worst in cranberry juice. The voltage administered to the fungi was juice conductivity dependent, even though the pulse strength and length were kept consistent. Raso and others also tested the survival of *Neosartorya fischeri;* which was resistant to PEF in both cranberry and tomato juice. Ascospore structure differences may have contributed to the differences in reduction between species treated by PEF (Raso et al., 1998). Evrendilek and others (2008) tested the germination of *Penicillium expansum* in cherry juice (13.5 °Brix), apricot nectar (11 °Brix) and peach nectar (10 °Brix) which was significantly slowed by PEF in all juices. Inactivation was highest in the cherry juice which also had the lowest pH (Evrendilek et al., 2008). Fungi that produce ascospores have been shown to survive PEF treatments of juice and later germinate during shelf life (Min et al., 2003).

Reducing the environmental oxygen has also been proposed as a hurdle to reduce spoilage when combined with other hurdles such as lower thermal treatment and low water activity. Filamentous fungi in particular have the ability to survive the heat processing that is regularly used in the food industry. Xerophilic molds can produce dormant, heat resistant ascospores through sexual recombination. Heat processing temperatures can act as a heat shock, which activate the spores and can initiate germination. Less resistant molds that may not survive thermal processing can still cause contamination post-process through improper cooling which causes the formation of condensation or exposure to the environment. The large number of foods that are affected by mold spoilage and the current clean label trend demanding the removal of

preservatives demonstrates that it is imperative to find solutions that can be implemented by the food industry that will also be accepted by consumers. Hot filling products uses thermal activation and reduces oxygen within the package. Hot filling is a process in which a product is heated and then added to a package. The heating forms steam in the headspace, from the available free water. A cap is added to the package. The steam condenses and a vacuum is formed as the product is cooled thus sealing it for an extended shelf life. The heated product also serves to sterilize the container. Hot fill products are expected to have long shelf lives at ambient temperatures. However, fruit products are typically only hot filled at 70-75°C to preserve quality (Pitt, and Hocking, 2009, Shearer et al., 2002). The survival of fungi and their ability to germinate after lesser thermal processing can vary but fungal spoilage has been known to occur (Lawlor et al., 2009). Hurdle methods, such as thermal or non-thermal processing, lower water activity and oxygen concentration reduction combined synergistically to inhibit spoilage, must be put into place to extend shelf life and reduce economic losses in the food industry. The survival of fungal ascospores and their ability to germinate after processing can vary based on the strain, water activity, soluble sugar content, and acid presence in the product.

We set up an experiment to find which combinations of water activity reductions and oxygen concentrations work best to limit fungal spoilage. This research was meant provide valuable resources for the food industry to fill current gaps in knowledge. We started by calculating the headspace in hot filled products and used that information to dictate oxygen levels tested with inoculated samples. These results were used to determine the ability of *Talaromyces, Cladosporium, Penicillium, Aspergillus* and *Byssochlamys* to grow in 0.85, 0.90 and 0.93 a_w apple juice concentrate and malt extract broth models at 0.5% (0.2 ppm O_2) and 1.0% (0.4 ppm O_2) O_2 over 60 days at 30°C. These two models were chosen to represent

potential food products; acidic fruit products, or neutral, non-selective products that can provide essential nutrients for fungal growth. Plates were transferred to anaerobic chambers and flooded repeatedly with nitrogen until target oxygen concentration was achieved. Plates were incubated for 60 days at 30°C. Plates were visually inspected for visible growth. When growth was present, plates were photographed and uploaded to Image J. Surface area covered by growth was measured.

A statistical model predicting when growth may occur could not be formed due to variation between replicates. However, conclusions could still be drawn. *Cladosporium* and *Talaromyces* were unable to grow at either 0.2 ppm or 0.4 ppm O_2 in any 0.85, 0.90 or 0.93 a_w model. *Penicillium* showed an ability to grow at low oxygen concentrations under higher water activity conditions. There was some variation between ability to grow in apple juice concentrate vs. malt extract broth at the same water activity and oxygen concentration. This was likely due to the difference in pH between the two models. More osmophilic fungi showed an ability to grow under all conditions tested. *Byssochlamys* has shown an ability to be essentially facultatively anaerobic. Taniwaki and others (2009) found that *B. fulva* and *B. nivea* were capable of growth in 60% CO_2 and <0.5% O_2 on potato dextrose agar and RCA, an anaerobic bacterial medium. They also found that *P. roqueforti* was capable of growth at 20% CO2 and <0.5% O_2 (Taniwaki et al., 2009).

Low oxygen and low water activity were determined to be ineffective at preventing growth of *Aspergillus* spp. and *Byssochlamys* spp.. These results can provide guidelines for future product development within confectionery products. The results from our study illustrate that headspace oxygen produced by hot filling seems to provide enough oxygen for growth of many fungal species. However, common fungi such as *Cladosporium, Talaromyces,* and

Penicillium can be completely or partially controlled with low oxygen combined with low water activity. Nevertheless, xerophilic species *Aspergillus* and B*yssochlamys* are not easy to control even under very low oxygen and water activity conditions. Growth was not only affected by water activity and oxygen concentration but also pH. Species were more capable of growth in the pH 7.0 malt extract broth than the pH 3.2 apple juice concentrate. Hot fill products may need a steam flush capping system or nitrogen flush to inhibit the growth of spoilage fungi.

The goal of our studies (Chapters 3-5) is to determine the limits of HPP in acidic or neutral, intermediate water activity juices and juice drinks in regard to spoilage organisms. Figure 1.1 describes how the goals were tested. It will follow the risk of spoilage from before HPP processing, immediately after HPP processing, and up to 6 months after. This will provide a reference point for juice processors who may wish to formulate low acid, neutral, or lower water activity beverages with an extended shelf life. pH 4.6 was chosen to represent the limit of *Clostridium botulinum* growth. *C. botulinum* produces a heat labile toxin that affects the nervous system and has very high mortality rates. There are approximately ten outbreaks in the United States per year. When pH is not used to prevent *C. botulinum* growth, multiple hurdles should be implemented. These can include: water activity reduction (<0.93 a_w), addition of preservatives, ensuring aerobic conditions, high salt content, competing organisms, and a 12-log thermal process (FDA - Guidance for Industry: Refrigerated Carrot Juice and Other Refrigerated Low-Acid Juices, Fontana et al., 2008).

Objective 1: *Evaluate the use of high pressure processing to cause a reduction of pressure sensitive spoilage fungi commonly found in juice and determine the effect of water activity and pH*

Objective 2: *Evaluate the use of high pressure processing to cause a reduction of heat and pressure resistant spoilage fungi commonly found in juice and determine the effect of water activity and pH*

Objective 3: *Evaluate the use of high pressure processing to cause a reduction of Alicyclobacillus commonly found in juice and determine the effect of water activity and pH*

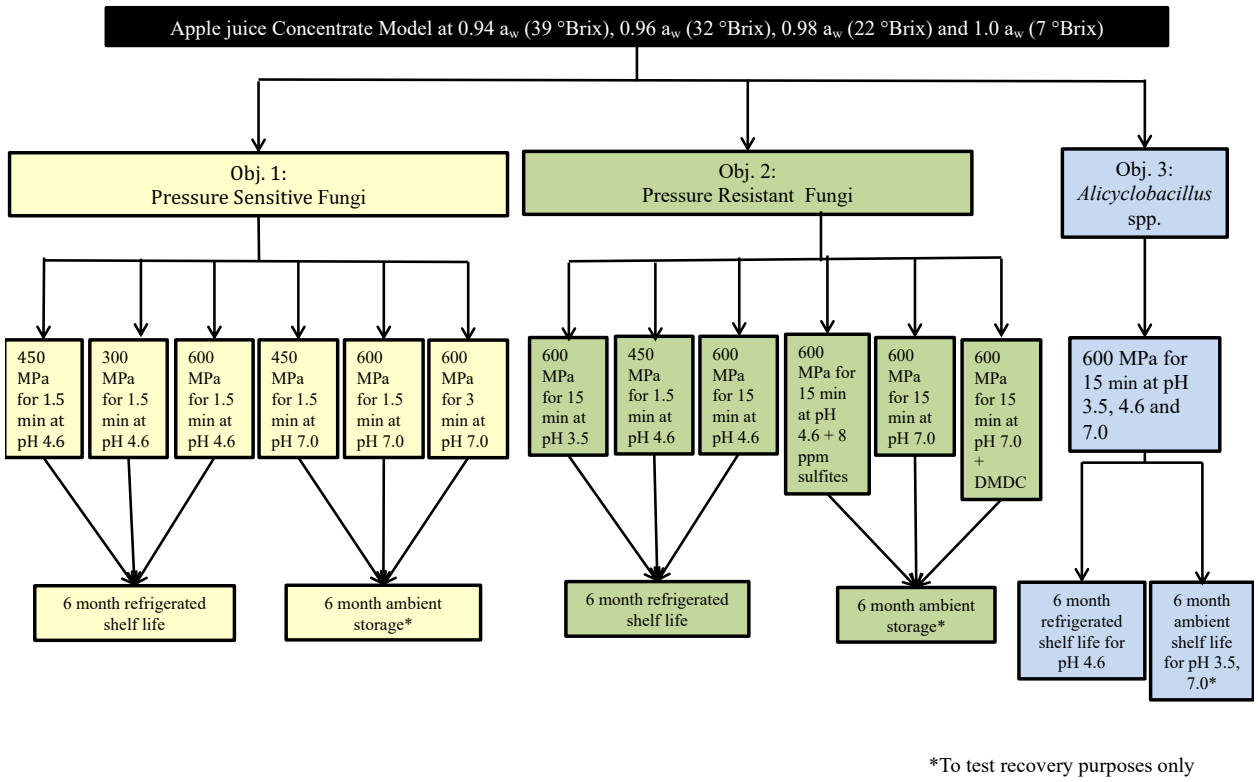

Figure 1.1: Framework of methods used in experiments.

In conclusion, non-thermal methods can provide safe and nutritious food products without resulting in discoloration or nutrient degradation. However, the efficacy of these methods can vary and must be tested with the pertinent organisms in an appropriate product. Oxygen concentration was not an effective method of preventing spoilage, but HPP, UV and PEF can be useful to extend shelf life and reduce pathogens.

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CHAPTER 2

QUALITY CONTROL OF SUPPLIERS AND PREVENTION OF FOOD FRAUD

ABSTRACT

Product quality depends on both ingredients and individuals from farm to fork. Compromise at any point can result in a low quality or unsafe product. When suppliers are economically motivated to adulterate or substitute ingredients, steps must be taken to prevent unsafe choices. Unsafe choices are more likely to occur when there is price pressure or geographical distance. Audits, certifications, and deferred payments can be used to promote ethical production. Additionally, buyers must also be vigilant against quality fades and cutting corners by frequently monitoring products and focusing on the traceability of products to quickly identify where issues originate.

HIGHLIGHTS

- Food fraud is not just an economic issue but can affect safety.
- Effective communication and shared values between suppliers and buyers are crucial for product quality.
- Ensuring that values align, and suppliers are appropriately incentivized and certified should occur before a contract is made.
- Once a partnership is in place, a long term relationship should be emphasized to decrease quality fade.

Quality and safety are a direct result of careful system control from farm to fork. Food, unlike other consumer goods, is extremely sensitive to time, temperature, and environment. Canned foods may have a shelf life of years while fresh produce may have a shelf life as short as days, even under optimal storage. The goal of food companies is to bring the products from farms to the consumers' tables as quickly and efficiently as possible, possibly with processing and packaging in between. Long supply chains, especially with outsourced suppliers, can result in lower quality or unsafe products. These long supply chains can also result in high levels of pesticides in spices, heavy metals in vegetables, adulteration of the product by adding unapproved colorants, or storing products at inappropriate temperatures (Chen et al., 2014). Food fraud, or substituting ingredients or products with cheaper ones, is an economic, quality, and safety issue. In the best-case scenario, substitution is entirely economically based such as replacing extra virgin olive oil with refined olive oil. However, scammers may cause injury or death with dangerous chemical replacements. For example, melamine added to infant formula resulted in six deaths and hundreds of thousands of reported illnesses. Another fraudulent company replaced olive oil with hazelnut oil which can cause allergic reactions and death (Spink, 2013, Arlorio et al., 2009). Today's international food supply chains can increase the economic, nutritional, and safety impact of food fraud. We will discuss several examples of risks that have occurred and discuss solutions to avoid these issues in the future. Noshad and Awasthi (2015) reviewed the quality literature and found the following topics were frequently repeated: supplier quality evaluation, poor quality costs, certification, supplier performance measurement, implementing quality tools, supplier relationships, quality training, committing resources to suppliers and rewarding suppliers (Noshad and Awasthi, 2015). This chapter covers the topics

listed by Noshad and Awasthi with examples of milk adulteration issues and geographical hurdles with suggested solutions of certifications, payments; deferred, bonus or penalty, and training. Some of these methods work synergistically while others are very important no matter what other methods are used. See Figure 2.1 for framework of topics to be subsequently discussed. The most common methods mentioned in management and operations literature in reference to food products are discussed. We also discuss gaps in the literature published by the operations and supply chain journals in the area of food production.

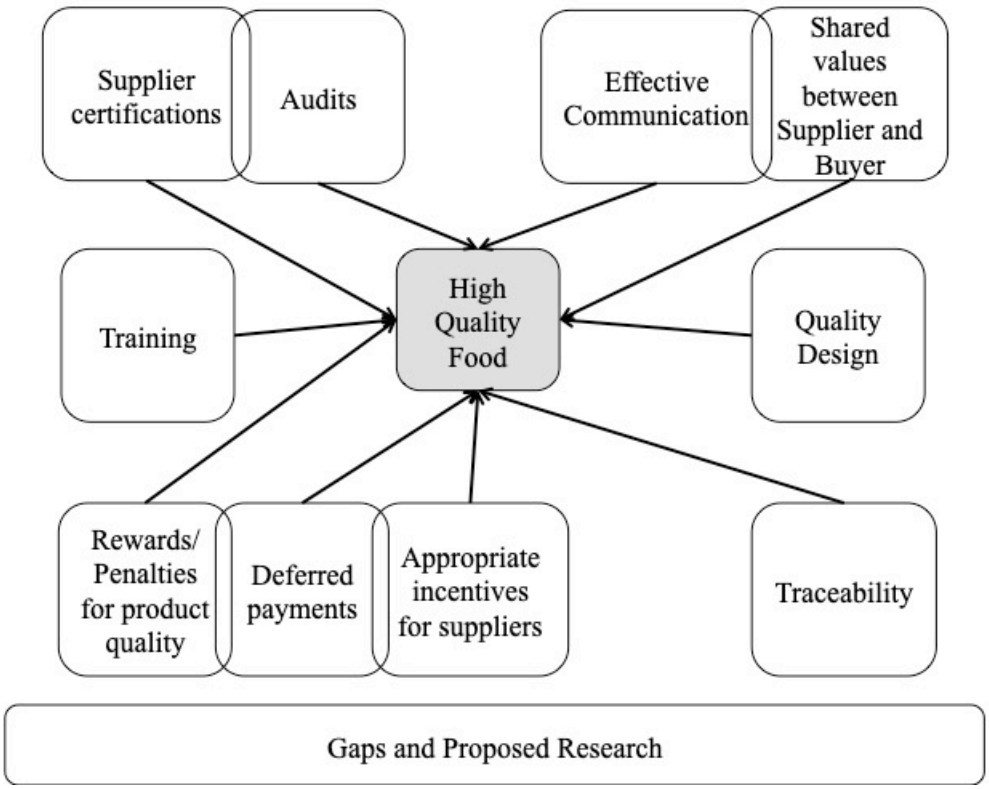

Figure 2.1. Important variables that affect product quality. Overlapping boxes represent variables that are related either by working synergistically or interchangeably.

Milk Adulteration, a localized quality problem. Today's marketplace is very international – shoppers at their local Upstate New York grocery store can purchase strawberries from Mexico in December and strawberries from a local farmer in June with both types of strawberries experiencing different but significant quality issues. Quality issues can happen across huge distances between countries or in the same region. In some developing countries like India, China and Kenya, farmers bring milk from individual farms to collection stations where it is combined and then sold to larger businesses or cooperatives to be pasteurized, bottled, and distributed. Milk from many farms, potentially hundreds, is combined before being sold to consumers. Due to high demand and limited supply, testing of milk, whether individually or combined, is limited. Stations are also likely to accept low quality milk rather than run out of product. Aware of this dilemma, unscrupulous farmers are therefore more likely to deliver milk to the station with the lowest standards. Competing stations are likely to lower standards to better compete for a reliable milk supply. Opportunistic farmers are aware of the stations' constraints and may add adulterants to pass the minimal testing such as adding milk mixed with whey or starch to visibly look like unadulterated milk. Testing combined milk is cheaper for the collection stations but allows free riding – farmers can contribute adulterated milk that gets mixed with pure milk and will not be identified as the free rider. As a result, farmers are highly incentivized to adulterate milk with other ingredients like water, which increases the volume of product they are paid for, but are not necessarily harmful to the public (Mu et al., 2016). The most common form of economically motivated adulteration in these cases is dilution to increase milk quantity (Mu et al., 2018). Farmers are also more likely to deliver milk to stations that accept lower quality deliveries and adulterated milk is not always recognized because testing is limited by expense and time constraints (Mu et al., 2016, Mu et al., 2018). Stations must also

hold the milk at refrigeration temperatures while the test is carried out which may add greater energy and manpower costs as well as a delayed release to consumers. If testing every farmer's shipment, it could be hundreds of tests. Moreover, stations are also not incentivized to test because rejecting milk can be costly and time consuming. Instead, stations are incentivized to attract more farmers. They poach farmers from competing stations by either testing less frequently or lowering their quality standards. However, this paper, by Mu and others (2016) is assuming that farmers are aware of what stations will test for and accept. Such knowledge is not necessarily realistic. Quick quality tests such as only testing fat, visually scanning or smelling samples can easily miss adulterated samples (Mu et al., 2016, Mu et al., 2018).

Mu and others (2016) recommend creating a government requirement of testing mixed milk, but telling farmers that the milk can be individually tested in order to induce fear of receiving low payments in exchange for low quality milk to prevent farmers from adulterating their product. However, milk doesn't need to be tested individually every time. The price paid to each farmer would be based on whether the mixed test met a certain standard (Mu et al., 2016). Yet, this is assuming that the government or dairy buyers are willing to pay more for higher quality milk and that the test used can accurately differentiate between milk qualities. This scenario seems profitable, but how likely are intermediary stations to convince governments to subsidize milk if they aren't already doing so?

Some farmers form cooperatives to cut out the intermediary stations and earn more per unit of milk. However, free riding is still an issue. If the price paid to cooperatives is based on the quality of milk, farmers in said cooperative should be incentivized to provide high quality milk. Another suggestion, could be to pay all farmers a minimum amount but allow them to request individual testing. If the farmer's milk tests higher quality, he could be paid more. This

would require all farmers to pay a small fee to join the co-operative in order to cover testing costs. A penalty could also be charged when low quality milk is identified to discourage farmers from requesting excess testing of low quality product. Alternatively, instead of paying each farmer per shipment of milk, the farmers could be paid periodically based on their combined quality of milk over that period (Mu et al., 2018). A risk with this method is that the farmers may frequently ask to have their milk tested to receive a higher payment.

In general, testing ingredients or final products can be expensive and may not pick up quality or safety issues. For example, melamine adulterated milk and formula in China had been tested for nitrogen content and did not identify an unsafe adulterant (Babich and Tang, 2012). Melamine is used in plastics production but tests similarly in nitrogen levels to milk (Spink and Moyer, 2011). Because melamine allowed diluted milk to pass inspection, the adulteration was actually more widespread than just the brand that needed to be recalled in 2008. Products such as chocolate had to be recalled and other milk brands found proof of melamine adulteration in their own facilities (Tse and Tan, 2011, Chen et al., 2014). The tested product is also typically destroyed in the process, so it is impossible to test every single package for adulteration. This destruction of product due to testing can also result in decreased margins. It is important to have a system in place along the entire supply chain to avoid risks so end product testing is not relied upon (HACCP).

Geographical distance. While the previous issue was very localized, quality and safety issues can cross borders. There are several reasons that issues are not identified before being sold in a foreign market. Traceability is difficult when food ingredients are sourced from all over the world. In fact, 20% of produce is sourced internationally (Roth et al., 2008). While the Food and Drug Administration ("FDA") can audit international factories, it has limited resources. Only 1-

2% of incoming food shipments are checked by the FDA. This is why there was such a delay in identifying the source of adulterated pet foods to suppliers from China. Some buyers use quality programs like Safe Quality Food ("SQF") to ensure uniform safety and quality from their suppliers (Tang and Babich, 2014, Roth et al., 2008).

Just as traceability can be difficult, transparency is limited between buyers and suppliers, usually with asymmetrical information flow. Transparency is necessary for root cause analysis of problems, sharing goals, and understanding the final safety and quality of food products (Roth et al., 2008). Both the buyer and seller need to have the mutual goal of producing a high quality and safe product at a reasonable price (Babich and Tang, 2014, Davis and Hyndman, 2018). This is especially true when suppliers and buyers are in different locations - as quality risks may increase when outsourcing or offshoring. In this case, offshoring is defined as 'staying within the company but in another country'; offshore outsourcing is defined as 'a supplier that is operating as a separate company in another country'; and outsourcing is defined as 'delegating responsibilities to another company that is within the same country as the buyer'. Distance, whether within a company or between different companies, can result in language barriers, cultural barriers, difficulty in auditing suppliers, or observing operations on a regular basis. Distance can have conflicting results as to whether this increases/decreases recalls. For example, "during the 2008 heparin adulteration investigation, FDA officials had to rely on the president of the Chinese company they were investigating to translate documents for them" (Tang and Babich, 2014). Another example: the Mattel Inc. toy recall was due to use of lead paint. Mattel's contractor had subcontracted work out to a company that had not been certified or approved by Mattel. In order to increase profits, the subcontractor used cheaper, lead based paint instead of Mattel approved paint. The Mattel subcontractor had limited risks because Mattel's reputation

was the one in the headlines (Steven et al., 2014). Recalls result in extreme costs to retrieve the product, lost sales, lost future sales, and lowered brand reputation. There is a risk that a company may never recover (Balachandran and Radhakrishnan, 2005, Thirumalai and Sinha, 2011). Mattel had limited visibility and traceability of hundreds of Chinese subcontractors that were not certified by Mattel nor Mattel approved third parties. Mattel's complicated, offshore outsourced supply chain was difficult to control and monitor which resulted in lower quality (Steven et al., 2014).

Similarly, the Chinese melamine in milk and infant formula was due to opportunistic suppliers in a complicated supply chain (Chen et al., 2014). Concentrating suppliers to only those that are geographically nearby can have conflicting results by either decreasing costs, increasing quality and visibility, or "it could increase the firm's dependency allowing the suppliers to engage in opportunistic behaviors" (Steven et al., 2014). Local outsourcing is less risky than offshoring or offshore outsourcing due to the lack of language, cultural, and geographic barriers (Steven et al., 2014). Local regulations can differ as well. If the supplier's government does not consider food fraud a crime, there may be little a buyer can do. Ideally, strong regulations would be in place to deter suppliers from engaging in intentional contamination (Spink, 2013, Tang and Babich, 2014). However, returning all sourcing and production to the US would dramatically increase costs (Tang and Babich, 2014). Concentrating suppliers can also be difficult when ingredients are needed year round. A fruit or vegetable available in the United States during the summer may not be available, at a reasonable cost, during the winter - forcing companies to source internationally (Roth et. al., 2008). Sourcing from multiple farms complicates the supply chain and places buyers at risk. Some locations may lack resources to meet storage and transportation requirements or may treat workers poorly. All of these factors can lead to risk for

the company whose brand name is attached to the product rather than the supplier (Davis and Hyndman, 2018). There are two primary ways suppliers can be unethical: first, adulterating the product with poor ingredients or inappropriate ingredients and processing and second, poor working conditions which are harder to identify but can still affect brand reputation. It is important to certify that suppliers are providing safe environments for workers (Chen et. al., 2016, Trienekens and Zuurbier, 2008). Consumers not only base their purchases on price but also a company's brand reputation, which includes "labor practices, business ethics, responsibility to society at large or environmental impacts" (Roberts, 2003, Lai et al., 2010). In the past, chocolate companies' reputations have suffered based on accusations of child labor in the farming, or processing of cocoa used in their products. It can be difficult for confectionery producers to be able to trace back the source of labor to raw ingredients like cocoa; however, companies who claim to have corporate social responsibility programs should be minimizing ethical risks (Roberts, 2003).

Certifications and Quality Management Programs. Consumers may also base their purchases on certifications advertised on packing. Furthermore, certification is important for many aspects, not just marketing. Quality and safety systems (GAPs, HACCP, ISO, SQF, Codex, Six Sigma, Root Cause Analysis) exist to help suppliers and manufacturers produce acceptable products, avoid problems and failures, and be mindful of consumer complaints. These systems can also be used to help buyers differentiate potential suppliers, rather than choosing the cheapest supplier (Chen and Lee, 2016, Babich and Tang, 2012).

Some of these certification systems include:

- Codex Alimentarius - established as an international set of safety standards by the Food and Agricultural Organization and the World Health Organizations. It includes standards

from the field such as appropriate limits for pesticide residues to scientific methods for testing, to appropriate package labeling (Roth et al., 2008, Trienekens and Zuurbier, 2008).

- HACCP – Hazard Analysis and Critical Control Points focuses on preventing or reducing physical, biological, chemical, or radiological food safety risks throughout production. It is endorsed by Codex Alimentarius. There are seven steps to following this program: 1. conduct a hazard analysis, 2. determine the critical control points, 3. establish critical limits, 4. critical control point monitoring, 5. corrective actions, 6. verification procedures and 7. record keeping. HACCP is mandatory for the juice, meat and seafood categories in the United States (Trienekens and Zuurbier, 2008).
- GAPs - Good Agricultural Practices set guidelines for growing and storing food including pest management, worker training, and water quality (Trienekens and Zuurbier, 2008).
- ISO - International Organization for Standardization focuses on customer satisfaction by publishing international standards to ensure products are safe and of good quality. It requires companies to be able to trace the life of the product (ISO, 2018, Trienekens and Zuurbier, 2008).
- SQF - Safe Quality Food is partially based on HACCP. All SQF certified companies must first have an appropriate HACCP plan in place. SQF focuses on continuous improvement in food safety and quality and requires explicit support of management in meeting these goals (SQF Code, Trienekens and Zuurbier, 2008).
- Six Sigma – uses statistics to reduce variation in manufacturing and focus on continuous improvement. Uses phases – “Definition”, “measurement”, “Analysis”, “Improvement” and “Control” to reduce failure rates (Scott et al., 2009).

- Root Cause Analysis programs – these programs work backwards to identify the cause or causes of a failure in order to prevent the same failure from occurring again. This investigation process may detect issues that are not immediately identified during the initial correction phase (Strong, 2015).
- Lean Initiatives – Increase efficiency by decreasing waste, excess spending and increasing processing speeds (Higgins, 2014).
- 5S – "Sort, Set in order, Shine, Standardize and Sustain" is a method of keeping tools in the manufacturing plant organized which can save time in the workflow (Higgins, 2014).

There are many others that may be used on packaging such as "rainforest alliance" or "fair trade" certified. However, some of these may be difficult to use as differentiation between possible suppliers if they are not robust on more than one category. Without certification or audit, opportunistic suppliers are hard to identify compared to ethical ones (Chen et. al., 2016). However, certifications cannot protect against unethical choices to adulterate foods nor prevent certified contractors from contracting out work (Babich and Tang, 2014). In some cases, the cost of certification may be higher than frequent inspections of products. While this seems counterintuitive, there may be other benefits. Reducing inspection may increase production efficiency by reducing delays, increasing output and decreasing inventory holds. With high inspection rates, the supplier is able to identify high quality products, but may run out which would result in a penalty just as sending defective products would. The size of penalties can result in differing outcomes. When defective products result in small penalties, the supplier is not incentivized to increase inspection. When certification effectively validates high quality suppliers and costs less than inspection, it is preferred (Hwang et al., 2006).

Payments. Certifying appropriate suppliers does not solve all issues. Suppliers occasionally cut corners after a buyer/supplier relationship has been built (Chen and Lee, 2016). There may be price pressure to offer the lowest price to entice buyers, followed by pressure to increase profits in the short term by cutting corners, substituting ingredients, or hiring subcontractors without notice to the buyer (Tang and Babich, 2014). As a result, many companies have zero tolerance policies when suppliers are caught adulterating product. Audits can also be used to screen potential suppliers, identify unethical behavior on site, or used to test the end product (Chen and Lee, 2016, Babich and Tang, 2012).

Several payment systems can be put into place to limit low quality product. A bonus or deferred payment may be paid upon successful inspection of product or a penalty if a violation is found. In the case of adulterated milk, Mu and others (2016) recommended giving bonuses to high quality milk stations so they are incentivized to only accept high quality milk from farmers, or base payment on quality of milk supplied. The bonus based policy rewards farmers to deliver the highest quality milk to earn the bonus (Mu et. al., 2016). This can be applied to other food products that have extreme demand and limited supply. Recognition, and increased business with the supplier can also be used to reward high quality production (Chen and Lee, 2016). However, the increased monitoring and testing will result in increased costs for the buyer. Depending on the product, it may be easy or difficult to audit. For example, auditing many geographically dispersed farms could be more costly for the auditing company than a few local factories.

Rewards, certification, and auditing are all complementary, but potentially costly for the buyer (Chen and Lee, 2016, Babich and Tang, 2012). Delayed payments are less costly for the buyer but put the onus on the supplier. With deferred payments, the buyer pays a fee upfront, before production and pays a second payment if no violations have been found within a specified

period. However, the supplier is required to have enough funds to produce the product and the original payment may not be enough to cover all costs. If the supplier is incentivized to cheat by adulterating or cutting corners, the buyer should use deferred payment and have a larger contingent payment (decreasing buyer's liability) to retain more control. Suppliers will be induced to produce quality foods for fear of not receiving the contingent payment (Babich and Tang, 2012, Tang and Babich, 2014).

Long term relationships between supplier and buyer are more likely to result in higher quality products by the supplier because the supplier wants to continue working with the buyer and will exert more effort. When there is a threat that the buyer will end a relationship, the supplier is induced to produce higher quality product, which also results in better profits for both (Davis and Hyndman, 2018). It is important for companies to form these relationships with suppliers who have similar values in regard to quality production. While certifications and audits can help identify these value systems, it is also important to reward cooperation, transparency and communication (Tang and Babich, 2014).

Between 2006 and 2007, Mattel had to recall many toys due to either lead paint or small, detachable magnets, which posed a choking hazard. The toys included: American girl jewelry, Fisher Price toys, sarge, Geo Trax, Barbie, Polly Pockets, Batman, One Piece, Tanner, and Doggie Day Care which were produced in China. The lead paint issue was caused by a third party subcontractor (another contractor hired by Mattel approved contractor) who used cheap paint not approved by Mattel in an attempt to increase profits. There was no relationship between Mattel and the subcontractor. In fact, Mattel was unaware that an unapproved supplier had been hired by the supplier. The loose magnets were a result of poor product design. Mattel performed monthly audits and reviewed records but failed to identify the lead. Mattel's solutions to the

problems were to recall products, re-design appropriately, increase monitoring of paint, apologize to parents, test more toys coming from Asia, and create a corporate responsibility program (Gilbert and Wisner, 2010). It was important to find the root cause of the design issue as blaming the supplier would have ended a relationship, when the fault was from Mattel.

Similarly, complicated supply chains are common in the food industry as well. "Originating company is widely thought to have the final legal and ethical responsibility" - however, the supplier may be at fault (Gilbert and Wisner, 2010). The Mattel example is very similar to unsafe substitutions that have occurred in the food supply sector and similar lessons can be applied. Mattel's poor toy design resulting in loose magnets should have been caught during quality assurance during stage gate or pre-production. In Mattel's two recalls there were two root causes. Regarding lead paint - the subcontractor was to blame, but regarding the magnets - Mattel's design team was to blame. The root cause is important to determine where and how the problem was caused. Cost sharing contracts between the supplier and manufacturer can be used to discourage suppliers from making unethical decisions. The manufacturer must typically pay a higher price to the supplier to agree. However, when costs are shared, the supplier is more likely to exert effort to produce a higher quality product. The contract can have terms that place a higher cost on the one who is blamed for the source of a recall in root cause analysis (Chao et al., 2009). These penalties can be incurred after testing before product release or based on consumer complaints (external failures) (Balachandran and Radhakrishnan, 2005).

Social Justice. The British Pesticides Safety Directorate has an unusual way of preventing companies from using too many pesticides. They publicly share the names of companies that test too high (Trienekens and Zuurbier, 2008). Websites, apps and social networks use similar methods to allow consumers to publicly share quality and safety concerns.

These can spread very quickly. In 2013, Chinese consumers learned that KFC's suppliers had used unreasonable amounts of drugs and growth hormones. Consumers used micro blog Sina Weibo to spread the word and resulted in a 25% drop in earnings at KFC (Tang and Babich, 2014).

Training. While rewards, payments or penalties work on a large scale, it is important to ensure that all individuals are properly trained. Safety and quality issues can stem from poor decision-making. Poor raw ingredients can result in a low quality final product, but people can cause poor quality as well (Luning and Marcelis, 2006, Luning and Marcelis, 2007). It is important to ensure that managers and suppliers are trained to the same standards, especially when food is sourced internationally. Some cultures have differences in hygiene standards and may be resistant to changing cleanliness standards even with training. This difference can result in workers taking safety risks (Roth et. al., 2008). In some countries, government regulation may be less strict resulting in limited enforcement of food standards (Tang and Babich, 2014). While the intent may not be to produce low quality products, there are many variables that depend on human behavior including choosing proper critical control points and meeting them, appropriate corrective actions, consistent and complete record keeping etc. Training can help predict how people make decisions, although there will always be variability (Luning and Marcelis, 2006). It is important that suppliers, contractors, or subcontractors have all of the relevant information and are motivated to make the right decision. Workers in factories can have high turnover, which makes training difficult as well (Roth et al., 2008). Management must also be supportive if product needs to be put on hold or destroyed. Management should not push to release product or to use inappropriate resources to improve quality (Luning and Marcelis 2006, Luning and Marcelis, 2007). The proper technologies to produce food and store properly should also be in

place (Luning and Marcelis, 2007). Food fraud is an economic, quality, and safe issue. Moore, Spink and Lipp created a database of economically motivated adulteration of products from 1980 to 2010. They found that 95% of the recalls were substitutions (Moore et al., 2012). See Table 2.1 for examples. Even when these substitutions do not pose immediate health threats these could result in long term deficiencies when the contaminated foods do not contain beneficial ingredients assumed to be present. Certified organic, natural, local or fair trade is also easy to replicate and hard for buyers to identify when counterfeit products are circulating the market. Fraudulent suppliers may also provide the same food, but not sourced as claimed. To deter unethical behavior, buyers may track via paperwork, barcodes or radio frequency labeling to easily identify legitimate products and for traceability (Spink, 2013).

Table 2.1. Recent Food Recalls

Safety Recall	**Citation**	**Quality Recall**	**Citation**
Dioxin in Irish pork	Tse and Tan, 2011	Replacing tuna with other fish	Moore et al., 2012
Melamine in chocolate	Tse and Tan, 2011	Using water and citric acid to replace juice	Moore et al., 2012
Heparin - Blood thinner medication substitute	Babich and Tang, 2014	Replacing sheep or goat milk with cow milk	Moore et al., 2012
Melamine in milk or wheat gluten	Babich and Tang, 2014, Moore et al., 2012, Tse and Tan, 2011	Substituting Italian olive oil with olive oil from another location	Moore et al., 2012
Replacing turmeric or paprika with lead based chemicals	Moore et al., 2012	Replacing natural vanillin with synthetic vanillin	Moore et al., 2012
Substituting Chinese star anise with Japanese star anise (which can cause seizures)	Moore et al., 2012	Purposefully mislabeling fish	Moore et al., 2012
Adding diethylene glycol to Austrian wine	Moore et al., 2012	Adding sugar to low quality juice	Moore et al., 2012
Using Sudan Red dyes to brighten color of chilies	Moore et al., 2012		
Using diethylene glycol instead of glycerin in cough syrup and toothpaste	Moore et al., 2012, Spink, 2013		
Salmonella in pepper	Chen et al., 2012		

Gaps and Proposed Research. There are still several gaps between food science needs and supply chain or operations studies. First, there are limited data used to develop or test the

theories posited by operations publications. This limit results in very theoretical recommendations that may not translate well to real world applications. Other publications simply refer to "products" but do not specify shelf life or handling of the product. Foods can be extremely perishable: fresh fruits and vegetables are difficult to distribute and sell due to their short shelf lives that are affected by temperature, time, relative humidity, and ethylene (Broekmeulen, 1998). A complicated distribution system may be required to transport, store and sell fruits at their peak ripeness because consumers have come to expect ideal pieces of fruit and vegetables at the grocery store, year round. It is estimated that 30-50% of all food produced ends up as waste (Parfitt et al., 2010, Chapman, 2010). This can happen anywhere along the supply chain from harvest to the consumer's home refrigerator. Product loss can occur during inappropriate storage, processing, or age (Parfitt et al., 2010). Temperature especially can pose hurdles: narrow temperature requirements for storage, having to keep loading/unloading docks a consistent temperature, refrigerated trucks, training of employees to understand temperature requirements, temperature monitoring, tracking of ingredients (Smith and Sparks, 2004). Finding a balance between cost and shelf life extension is crucial to reducing food waste.

In order to maintain financially and environmentally sustainable, modeling is considered to optimize distribution. These consider many variables:

- Shelf life – whether fixed (products discarded after a predetermined amount of time) or random (unknown but discarded when considered spoiled)
- Initial Quality level – fresh fruits and vegetables that are subpar will have a shorter shelf life
- Inventory and consumer demand
- Season – is the product available locally or will it be transported internationally

- Transportation – can account for 66% of logistics cost
- Warehouse locations
- Retail locations
- Storage
- Cooling costs (Smith and Sparks, 2004, Rong et al., 2011, Akkerman et al., 2010).

Many of the operations papers list assumptions or variables but generally do not include all of the above. Food science publications may include many of the above variables but do not build appropriately refined models; instead they overly simplify to case studies.

These models, both operations and food science, do not always take into account unexpected human behavior. For example, Luning and Marcelis (2007) considered a case study of cocoa that is sterilized but has frequent post process contamination. The manager set strict limits and increased the number of air filters to reduce contamination to no avail. There were a number of contributing problems: low air pressure to pull air in the direction of post process product towards raw ingredients, poor cleaning procedures, and poor air filter replacement training leading to infrequent replacement (Luning and Marcelis, 2007). This emphasizes the need for training, educated decision-making and quality culture in a company. If employees are not properly trained and committed to creating safe, quality foods, they cannot properly carry out their responsibilities (Kafetzopoulos and Gotzamani, 2014). Quality also needs to be part of the organization's culture. It needs to be part of the workplace norms, employee training, and objectives (Hurley et. al., 2013). Employees must also see managers making quality centric decisions. When all employees and managers show a quality-centered strategy, the culture is stronger than using monetary incentives. Maintaining quality products can also avoid recalls. Recalls have direct costs: communicating the recall to distribution centers, customers, and

consumers; disposition of product; product loss; transportation costs, and indirect costs: loss of reputation and brand trust which can lead to sales loss. This is especially a risk in today's online social media as a single quality issue can be shared thousands of times (Srinivasan and Bryan, 2014). There are limited publications with supporting data outlining how to control a culture of quality and responsible decision-making. It is also important that workers understand that their decisions to take short cuts affect the company overall and consumers who have the potential to become ill: "Worker line of sight regarding how their actions affect outcomes enhances performance" (Boudreau et al., 2003).

Another difference between fields is the use of terms such as "quality". The definition of quality varies between supply chain/ operations and food science. While Mu and others (2016), defined quality contamination as "nonpoisonous adulteration", Tang and Babich (2014) define low quality to include the potential to cause harm (Mu et al., 2016, Tang and Babich, 2014). It's important to use uniform language when describing situations and issues in operations and food production. In general, food science journals refer to quality as an attribute that cannot cause harm, and safety as an attribute that may cause illness, physical harm or death whether acutely or from chronic exposure. If operations journals use the same definitions as food science journals, it will be easier to share information across categories.

Some operations publications propose an optimized method to incentivize suppliers and then back up their suggestions with a case study. Food science journals also tend to use case studies, such as describing Tesco's solutions to delivering groceries at different temperatures (Smith and Sparks, 2004). A best-case scenario would also include testing these methods and collecting current data to see if they are accurate. If multiple manufacturers update their methods, they could compare cause and effect data. It would be important to compare different categories

of foods to see if there are differences between perishable fresh produce or say, canned goods. Currently, the literature available is either very narrow i.e. milk cooperatives in developing countries or extremely vague i.e. “products” or “food products” without definition. Instead categorized papers separated by distinguishable qualities such as shelf life or storage temperature would be very useful for the food manufacturing industry. The FDA already has a legal framework of such categories such as “fruits, vegetables, deli meats, cooked ready-to-eat crustaceans – breaking down the products by safety and quality” (Appendix 5, 2018). Each of these categories will have individual quality issues. For example, fresh vegetables which may not receive a heating step, require controlled cold chain transportation and may need surface treatment to reduce initial contamination or modified atmospheric packaging to control the growth of pathogenic and spoilage organisms during shelf life. On the other hand, ready-to-eat crustaceans will have a cooking step to reduce pathogens, but will be at risk for post process contamination. Deli meats, another category that has issues with post process contamination, will often be processed twice – once during production and once after packaging (Hiperbaric).

Statistical process control programs can be used to track deviations but are the “least used QC technique” according to Lim and others (2015) when compared to other quality tracking methods such as sampling (Lim et al., 2015). This may have to do with the level of competency and knowledge in the workforce.

Much of the data shown in operations papers involves complicated calculus that may be difficult for food scientists and producers to understand. Summary literature explaining the results in more simple terms may make the results easier to share with the people who can benefit. There is a need to build a communication toolbox that succinctly shares ideas between both areas without sacrificing information.

Suggestions by operations management authors to change government regulations or subsidies are not always feasible. Easier to reach, short term recommendations would be very useful for food producers. For example, Mu and others (2016) suggested subsidizing milk based on quality, which is a practice in some countries. While this is a good long term solution, others may be implemented more quickly. Perhaps there needs to be a milk quality test that is cheaper or quicker to implement (Mu et al., 2016).

The Food Safety Modernization Act (FSMA) was signed in 2011 and has a rolling implementation date based on the size of companies. FSMA now requires companies to identify hazards and preventative controls for foods, much like a HACCP program. Many of the papers written before 2014 do not consider the new food safety requirements and some of their suggestions may no longer be relevant now that food safety is required to be built into the production system (FDA Food Safety Modernization Act).

Decision making on an individual scale should also be studied. If a supplier as a whole is not choosing to cut corners and release low quality products, why are individuals choosing to? Behavioral studies could be implemented to better understand decision making and when quality fade occurs.

There is potential for the use of real time data tracking systems in the form of blockchain to increase traceability and validation through food systems. Blockchain works by assigning an individual code to each activity. For example, a code will be formed when a farmer records that he has harvested 1,000 bushels of apples. When these bushels arrive at the juicing facility, a new code will be formed that will be compared against the information from the farmer. See Figure 2.2. A continuous record in real time can validate where each bushel of apples is going and where they came from, with input from each individual or tracking device involved at that

particular step. Validated thermometers with uploading capabilities can record the thermal processing steps. Non-smart technologies can be recorded and uploaded by operators. Not only does this allow for manufacturers to identify that each control point met the target specifications, it will also allow for quick identification of contamination in foodborne outbreaks. The data uploaded to the system cannot be erased and thus, faults cannot be covered up (communication with Bruno Xavier, The potential of Blockchain technology application in the food system, IBM Food Trust Solution Brief 2.0). Certificates and contracts can also be part of the blockchain system to help track whether the ingredients came from an approved farm or if that farm was organic. While blockchain is still in the early days of implementation in the food system, it is constantly being updated by IBM and large food manufacturers (IBM Food Trust Solution Brief 2.0). Because this is still in the early stages, there is limited data available for smaller producers not involved in the IBM Food Trust.

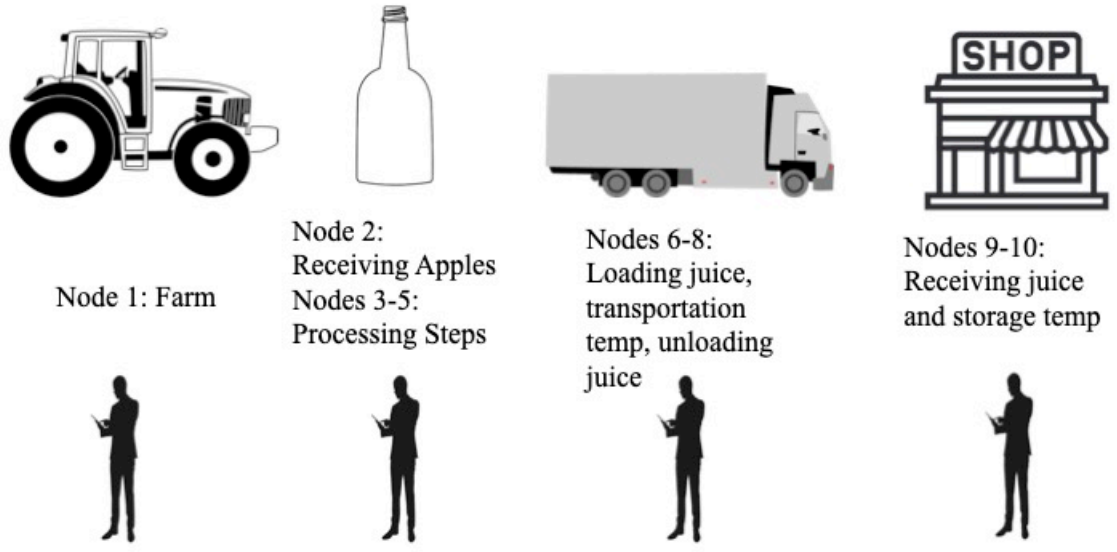

Figure 2.2. A simplified explanation of how Blockchain can be applied to a food system.

Conclusions. Brands utilize audits, certifications, payments or penalties to deter suppliers from engaging in duplicitous behavior. However, there are many examples of recalls where this behavior was not recognized until product had been sold to consumers. It is important for manufacturers to update methods to appropriately entice suppliers to make the right decisions. Integrated studies between the supply chain and food science areas are needed to identify these methods.

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CHAPTER 3

HIGH PRESSURE PROCESSING OF SPOILAGE FUNGI AS AFFECTED BY PH AND WATER ACTIVITY IN A DILUTED APPLE JUICE CONCENTRATE

Key words: spoilage fungi, High Pressure Processing, storage

ABSTRACT

High Pressure Processing (HPP) is a method used to extend shelf life of foods by subjecting products in their final packaging to extreme pressure. This leads to protein degradation in microbial cells, membrane degradation, and eventual cell death. Survival of spoilage organisms, especially spore-formers, has not been closely considered in regard to the effect of water activity (a_w). The purpose of this study was to determine the effect of pH and a_w on HPP apple juice to prevent fungal spoilage during shelf life.

Apple juice concentrate was adjusted to a_w 0.94 (39.3°Brix), 0.96 (32.2°Brix), 0.98 (22.0°Brix), or 1.00 (7.1°Brix) and pH 4.6 or pH 7.0. Polyethylene terephthalate bottles were filled with concentrate and inoculated with *Penicillium* spp*., Aspergillus niger, Byssochlamys spectabilis, Rhodotorula mucilaginosa*, *Candida parapsilosis*, *Torulaspora delbrueckii,* or *Brettanomyces bruxellensis.* Samples were HPP treated at 450 MPa for 1.5 min and for pressure-resistant species, at 600 MPa for 1.5 min or 3 min to resemble industrial processing conditions. Fungi were more resistant to HPP at lower a_w and higher pH. *C. parapsilosis* was more resistant to HPP than the other organisms tested. Fungi, with the exception of *C. parapsilosis*, experienced a 4.26- or greater log reduction at a_w of 0.98 and above at either pH. Their reductions ranged from 0.5 to 5.3 log reduction at 0.94 a_w and 0.96 a_w with pH 4.6. The reductions were 0.1 to 4.5-log at 0.94

and 0.96 a_w at pH 7.0. In general, shelf life was a matter of weeks at ambient temperatures and 7.0 pH, even when processing pressures were increased to 600 MPa. To reduce risk of fungal spoilage, HPP products should be at or above 0.98 a_w and below 4.6 pH and stored at refrigerated conditions.

HIGHLIGHTS

- Fungi were able to recover from HPP treatment and spoil diluted apple juice concentrate.
- High a_w and pH 4.6 caused higher levels of inactivation than lower a_w and 7.0 pH.
- *Candida* exhibited much higher pressure resistance than other tested fungi.

High Pressure Processing, a nonthermal technology to extend shelf life and enhance food safety, can meet consumer demand for clean label ingredients and provide safe, convenient foods. Commonly referred to as "cold pasteurization," it subjects products in their final packaging to extreme pressure (450 – 600 MPa), typically at refrigeration (5°C) temperatures (Lado et al., 2002, Black et al., 2007). This leads to protein degradation in vegetative microbial cells, cell membrane degradation, and eventual cell death (Lado et al., 2002). However, spores are more resistant and therefore not affected, and products must be refrigerated to prevent or slow growth of survivors (Rendueles et al., 2011). The composition of the food has a direct relationship to the efficacy of the preservation method (Georget et al., 2015). The composition and physicochemical characteristics of foods, especially water activity and pH, have an effect on the survival of spoilage organisms. Higher survival rates of fungi have been observed in juices with higher sugar concentrations (Black et al., 2007).

Until recently, most HPP challenge studies have focused on the safety of products to meet FSMA regulations (US Drug and Food Administration, 2004). However, there has been limited work focusing on the reduction of spoilage microorganisms in HPP products. Spoilage fungi can cause unwanted clouding, swelling, and growth in juices and juice drinks so it is important to determine the limits of HPP processing in a juice model. Generally, lower water activity can be protective to fungal cells, while lower pH can be more detrimental to fungal survival. This effect has been exhibited in an experiment by Sokolowska and others (2013). *Saccharomyces cerevisiae* was subjected to HPP in both buffer and beet root juice. A 5-log reduction was achieved in buffer but only a 3.5-log reduction was achieved in juice (Sokolowska et al., 2013). Basak and others (2002) studied the effect of HPP on the survival of yeast *Saccharomyces cerevisiae* in orange juice and concentrate. Twenty min of 250 MPa in single

strength orange juice caused a 3-log reduction of *S. cerevisiae* while 60 min at 400 MPa was necessary to achieve approximately the same result in orange juice concentrate (Basak et al., 2002). Hocking and others (2006) found that *Saccharomyces* and *Pichia,* both yeasts, experienced a 3- or 4- log reduction in a 20°Brix, 4.2 pH sucrose solution at 400 MPa for 2 minutes. *Penicillium* and *Fusarium,* both heat sensitive filamentous fungi, experienced inactivation after processing at 400 MPa for 2 min in the same sucrose solution (Hocking et al., 2006). The composition of the food product can also affect surviving organisms' ability to recover and grow after processing (Sokolowska et al., 2013). It is clear that the protective effect from low a_w or high sugar content varies between species.

Of the few publications available on the survival of spoilage fungi after HPP, several have focused on the effect of HPP on pressure sensitive fungi. Yen and Lin (1996) studied the shelf life of high pressure processed and thermally pasteurized guava puree. The purees which were processed at 400 - 600 MPa for 15 min at 25°C or 88-90°C for 24 s, along with an unprocessed control, were stored for 2 months at 4°C. The 600 MPa variable initially reduced naturally occurring yeast and mold populations to non-detectable levels. The thermal pasteurization and 400 MPa variables both reduced populations by only 1-log. During the 4°C shelf life, the samples processed at 600 MPa did not show recovery of cells, but the control, 400 MPa, and thermally pasteurized samples demonstrated growth after the first 10 days (Yen et al., 1996).

The goal of this study was to evaluate the use of high pressure processing to cause a reduction of spoilage fungi commonly found in juice and determine the effect of water activity and pH. Spoilage organisms were chosen based on common occurrence in spoiled foods. *Paecilomyces variotti*, the asexual, and less heat resistant form of *Byssochlamys spectabilis,* is

able to grow at low water activities and low oxygen concentrations (Houbraken et al., 2008). *Penicillium* spp. and *Aspergillus* spp. are the two most common spoilage genera of food and thus both of these are represented in the study. *Brettanomyces bruxellensis* has been known to cause off-odors and flavors in beverages including ciders. *Torulaspora delbrueckii* is capable of growth at extremely low water activities and is somewhat resistant to preservatives. *Rhodotorula mucilaginosa* has been isolated from fruit products including applesauce and pies. *Candida parapsilosis* has been found in foods from meats, seafood, and dairy, to fruits and vegetables (Pitt et al., 2009). Initial pressurization levels of 450 MPa were selected based on previous studies: Varela-Santos and others (2012) in addition to Hsu and others (2008) found significant reductions of yeasts and molds in juices with pressures above 350 MPa and 400 MPa respectively, using benchtop models. Samples held at refrigeration temperatures in both studies were expected to have longer shelf lives than those tested (35 and 28 days respectively) so we chose to target 26 weeks or 6 months (Varela-Santos et al., 2012, Hsu et al., 2008). Ambient storage was also tested in some cases to see if recovery was possible.

MATERIALS AND METHODS

Inoculum. The apple juice solutions were prepared by raising the water activity of apple juice concentrate (Williamson, NY) to reach the targeted 0.94 – 1.00 a_w after inoculation. The solutions were pH 4.6 or 7.0 adjusted by adding NaOH (Chem-Impex, Wood Dale, IL). A pH of 4.6 was initially chosen because it is the critical limit to prevent growth of *Clostridium botulinum,* a pathogenic organism that can produce toxin and withstand HPP. pH 7.0 was selected to determine the effect of neutral pH on HPP inactivation of spoilage fungi. If results proved HPP was successful at pH 7.0, more neutral products may be possible if hurdles were in place to prevent *C. botulinum* growth. Diluted apple juice concentrate was heated to 95°C and

held for 30 min to inactivate organisms present in the concentrate. Initial physicochemical values of apple juice concentrate were: 0.72 a_w, 3.3 pH, and 70.4°Brix. Water activity measurements were made by an Aqua Lab Dew Point Water Activity Meter 4TE (Decagon, Pullman, WA). pH was determined with an Oakton pH 5 Acorn Series pH meter (Oakton Instruments, Vernon Hills, IL). Total soluble solids were determined with an Abbe refractometer (Leica Inc., Buffalo, NY). All analyses were conducted in triplicate.

Spoilage Organism Preparation. *Aspergillus niger* "S11-0041"*, Paecilomyces variotii* "26"*, Penicillium* spp. "GM"*, Brettanomyces bruxellensis* "CE262"*, Torulaspora delbrueckii* "G9"*, Rhodotorula mucilaginosa* "F9"*,* and *Candida parapsilosis* "I7"*,* were obtained from the collection at Cornell AgriTech (Dr. R. Worobo's laboratory). All organisms were isolated from spoiled fruit juices, beverages, or foods. A plug of each organism was plated onto malt extract agar (BD, Franklin Lakes, NJ) and grown for 30 days at 30±2°C. Organisms were harvested by flooding plates with Tween 80 (Baker Analyzed, Philipsburg, NJ) and gently scraping with sterile spreader (Butz et al., 1996). Filamentous fungi; *A. niger, P. variotii,* and *Penicillium* spp.*,* were frozen at 0°C until use. Thawing occurred at ambient temperature. Survival at 0°C was confirmed in preliminary studies. Yeasts *B. bruxellensis, T. delbrueckii, R. mucilaginosa,* and *C. parapsilosis* were used immediately.

Inoculation. Sixty grams of apple juice concentrate model was added to flexible 2 oz Polyethylene terephthalate (PET) bottles (Captiva Containers, FL). One mL of target organism was added to each bottle to achieve a 4-5 log CFU/mL population. In several cases, there was a population of 3-log CFU/mL. Samples were refrigerated until processing and used in less than 24 h. Immediately before processing, initial fungal samples were enumerated on DRBC Agar (BD, NJ) in duplicate and incubated for 7 days at 30°C.

HPP. The flexible PET bottles filled with model and inoculum were placed into PET bags. Bags were vacuum sealed for 1.8 s, double bagged and re-sealed, all to prevent the risk of container leakage inside the HPP unit. All samples were initially processed at 450 MPa for 1.5 min at 5°C in a 55 L HPP unit (Hiperbaric, Miami, FL) to determine average log reduction. Samples that showed resistance were repeated at higher times and pressures (up to 3 min and 600 MPa) and samples that achieved at least a 3-log reduction at 450 MPa were repeated at 300 MPa for 1.5 min. Processing samples in pH 7.0 apple juice concentrate at 300 MPa was deemed inefficient based on initial results processed at 450 MPa in pH 7.0. Due to relatively low reductions in pH 7.0 samples processed at 450 MPa, pressures at 600 MPa were tested. *C. parapsilosis* showed pressure resistance at both pH 4.6 and pH 7.0 so pressures and processing times were increased in comparison to the other yeasts tested. Lower pressures can cause less wear and tear on the HPP unit and therefore may be appealing to processors. All samples were processed in triplicate, using separate runs. In the case of the filamentous fungi, samples with a pH of 4.6 were repeated again in triplicate in order to obtain shelf life results at ambient temperature. This allowed for a better comparison between treatments. Samples were plated on DRBC (BD, NJ) immediately after processing.

Shelf Life and storage. Figure 1 represents the framework used for this study. pH 4.6 samples were stored at 5°C for 6 months (26 weeks) to determine refrigerated shelf life and plated periodically (4 – 6 weeks). When 4.6 pH samples tested <1.0 CFU/mL for two months in a row, sample testing was discontinued. However, samples were kept for a visual inspection. The second set of filamentous fungi at 4.6 pH were stored for 6 months at ambient temperature in order to compare results with samples at pH 7.0. Inoculated samples with 7.0 pH were stored at ambient temperatures (20-23°C) and plated periodically for 6 months to represent a worst-case

scenario. Unprocessed controls at 0.94 or 1.0 a_w were inoculated at a 2-log level and stored at 5°C temperatures to determine ability to grow at refrigeration. Samples were observed for visible mycelia or gas production.

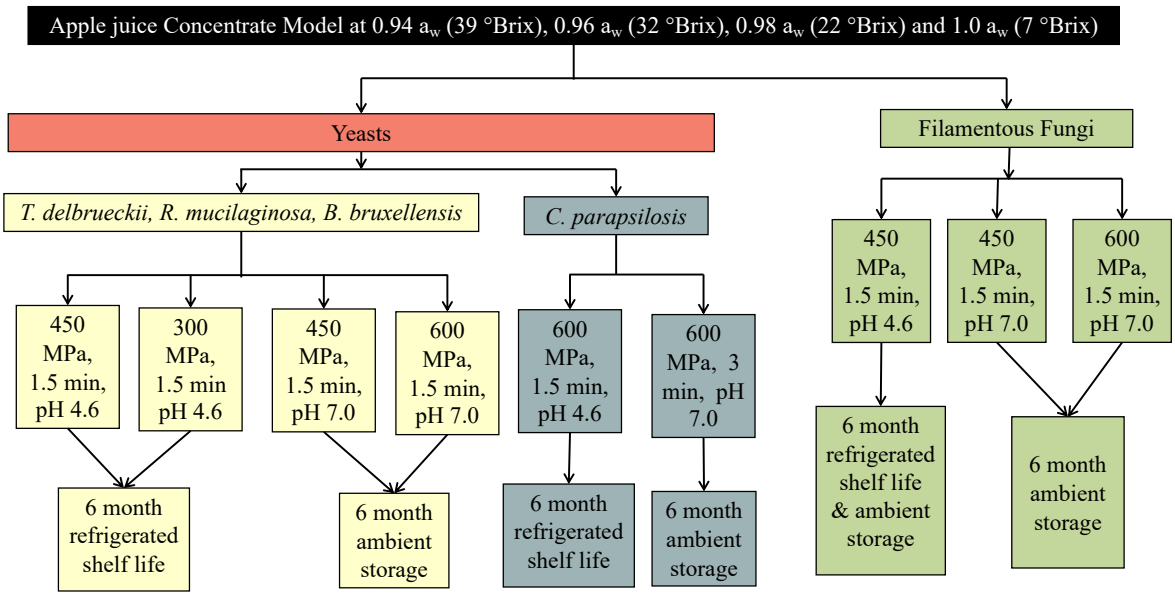

Figure 3.1. Framework of study with processing and storage conditions.

Statistical Analyses. Each experiment was performed in triplicate. Log reductions were calculated by taking the log of initial and subsequent populations. Growth over shelf life was determined by comparing after HPP levels to periodic plating counts, visible growth or gas production. A model was created to compare estimated marginal means using R version 3.2.2. A Type III sums of square test was used to determine a three way interaction (R Core Team, Vienna, Austria). Differences were considered significant at a P value of 0.05. Values <1 CFU/mL (undetectable when 1 mL was plated) were recorded as "1.0" for conservative statistical purposes. Actual log reductions may be greater than those reported in these cases.

RESULTS AND DISCUSSION

Physical and chemical analyses. Physicochemical measurements of diluted apple juice concentrate model at 20°C were as follows: targeted pH 4.6 was measured as 4.59±0.01 and targeted at 7.0 was measured as 7.01±0.03. The 0.94 a_w model was measured as 0.940±0.001 a_w and 39.3±1.9 °Brix. The 0.96 a_w model was measured as 0.961±0.003 a_w and 32.1±0.7 °Brix. The 0.98 a_w model was measured as 0.979±0.002 a_w and 22.0±0.5 °Brix. The 1.0 a_w model was measured as 0.997±0.003 a_w and 7.1±0.9 °Brix.

Statistics. Statistical comparisons could only be made between samples processed at 450 MPa since subsequent time and pressure combinations varied between genera. There was a significant interaction between a_w and pH ($P < 0.01$) as well as between a_w: pH: fungal genera ($P < 0.01$). Overall, log reductions were greater at higher water activities and lower pH. Results are shown in Figures 2, 3, and 4 as well as Tables 1, 2, and 3. In comparisons made within all tested species, but between pH values, higher log reductions were achieved with 0.94 or 0.96 a_w and pH 4.6 rather than pH 7.0. At or above 0.98 a_w, there was little distinction in reductions between pH 4.6 and pH 7.0 for *A. niger, P. variotii, Penicillium* spp*., B. bruxellensis, and T. delbrueckii* due to high levels of inactivation that were achieved. In pairwise tests, pH was not significantly different for *A. niger* at 0.98 – 1.0 a_w*, P. variotii* at 0.98 – 1.0 a_w, *Penicillium* spp. at 1.0 a_w, *B. bruxellensis* at 0.96 – 1.0 a_w, and *T. delbrueckii* at 0.94 – 1.0 a_w. *C. parapsilosis* and *R. mucilaginosa* behaved differently than the other species tested. They had the opposite outcomes with no pairwise statistical differences in results at either pH 4.6 or 7.0 at 0.94 a_w, but significant differences at higher a_w. Due to these differences, the results and discussion will be divided by species.

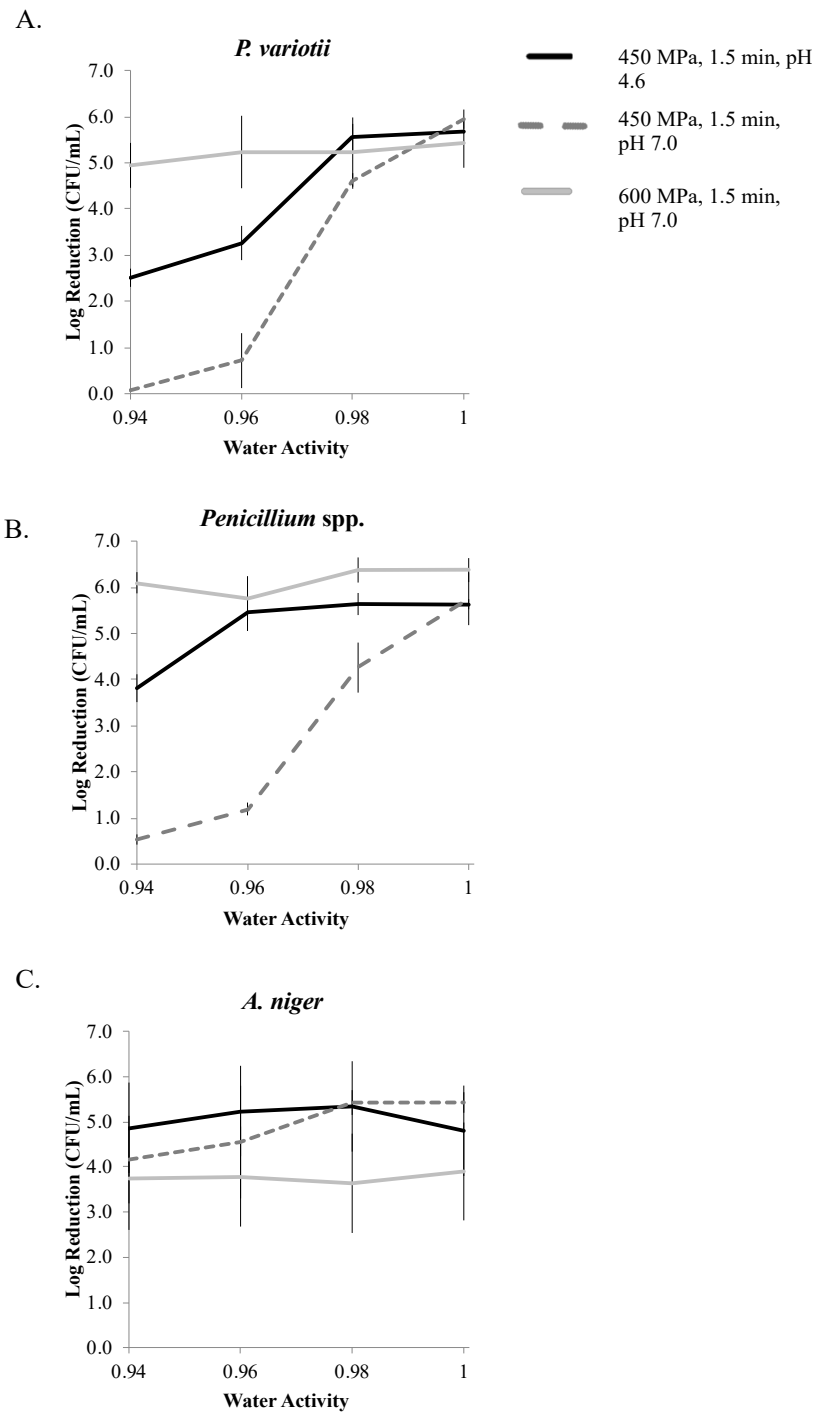

Figure 3.2. Log reduction of filamentous fungi after HPP at 450 and 600 MPa for 1.5, min in pH 4.6 and 7.0 apple juice concentrate diluted to 0.94-1.0 a_w.[a] (A.) *P. variotii,* (B.) *Penicillium* spp. and (C.) *A. niger.*

[a] Error bars show standard deviation

Table 3.1. Survival of filamentous fungi during storage.

Storage Conditions	***P. variotii***	***Penicillium* spp.**	***A. niger***
450 MPa, pH 4.6, Refrigerated	26 weeks without growth	26 weeks without growth	26 weeks without growth
450 MPa, pH 4.6, Ambient	Growth at 4 weeks	Growth at 4 weeks	Growth at 8 weeks
450 MPa, pH 7.0, Ambient	Growth at 4 weeks	Growth at 4 weeks	Growth at 4 weeks
600 MPa, pH 7.0, Ambient	Varied from 4 – 26 weeks	Varied from 12 – 26 weeks	Varied from 8 – 26 weeks

***P. variotii* results.** *P. variotii* was relatively sensitive to HPP at the tested conditions. Initially, complete inactivation appeared to occur when *P. variotii* was processed at 600 MPa for 1.5 min in pH 7.0 apple juice concentrate at all water activities, this effect was also seen after 450 MPa for 1.5 min at 0.98 and 1.0 a_w, pH 4.6 apple juice concentrate and 1.0 a_w pH 7.0 apple juice concentrate. However, samples inoculated with *P. variotii* held at ambient temperature showed recovery, proving that injured cells remained present after processing. *P. variotii* held at refrigeration did not show an ability to grow. There was a greater survival of *P. variotii* in 0.94 and 0.96 a_w at either pH, processed at 450 MPa for 1.5 min.

HPP results for the reduction of *Paecilomyces* in juices are limited in the literature. However, the effect of HPP on yeasts and molds as a whole has been studied. Aaby and others (2018) subjected strawberry puree (pH 3.3, 8.3 °Brix) to HPP. They also found low levels of

yeasts and molds remained in refrigerated strawberry puree samples over the 49 days of incubation. Puree samples processed at 400 MPa for 3 min showed increases in fungal populations even when held at 6°C (Aaby et al., 2018). Our results at a slightly higher pressure and higher pH did not show increases, but populations did not decrease over time either.

Penicillium spp. **results.** *Penicillium* spp. were also more sensitive to HPP at higher water activities. *Penicillium* spp. had very similar results to *P. variotii* but were more sensitive to 450 MPa for 1.5 min in 0.96 a_w, pH 4.6 apple juice concentrate. Again, *Penicillium* spp. showed recovery and growth at ambient temperatures, but not at refrigeration conditions. One control inoculated with *Penicillium* spp. at 1.0 a_w and pH 4.6 was capable of growth at refrigeration. It appears that the high pressure was able to reduce *Penicillium* levels enough to prevent this after processing. Arroyo and others (1999) found that *Penicillium* spp. processed at 400 MPa, for 30 min at 5°C was reduced to non-detectable levels in tryptic soy broth (Arroyo et al., 1999).

Goh and others (2007) subjected spoilage yeast and filamentous fungi to HPP in sucrose syrups. At lower total soluble solids, no significant protection was observed. As the soluble sugar content increased from 50 to 60°Brix, the survival rates of tested microorganisms also increased (Goh et al., 2007). Our results were not tested above 39 °Brix, but a slight protective effect was observed in *Penicillium* spp. in apple juice concentrate at the lower tested water activities and processed at 450 MPa for 1.5 min. A greater baroprotective effect was seen with *P. variotii* as well as *R. mucilaginosa.*

A. niger **results.** Some initial populations of *A. niger* that were processed in pH 7.0 at 600 MPa were only 3-log CFU/mL, while all of the *A. niger* samples processed in pH 7.0 at 450 MPa were 5-log CFU/mL. However, many of these samples were reduced to non-detectable levels after process, regardless of initial inoculation level. Additional work should be conducted

to better compare reductions rates. Nevertheless, conclusions can still be drawn from these experiments. *A. niger* was sensitive to HPP at all tested conditions with trends almost completely horizontal at all tested water activities. Samples of *A. niger* held at refrigeration temperature showed no ability to recover and grow over shelf life. Conversely, *A. niger* samples held at ambient temperature were able to grow. Even *A. niger* samples in pH 4.6 apple juice concentrate were capable of growth when held at ambient temperatures. Other studies have not investigated the effect of HPP on *A. niger* in juices. Arroyo and others (1999) found that *A. niger* was reduced to non-detectable levels in broth after 400 MPa for 30 min (Arroyo et al., 1999).

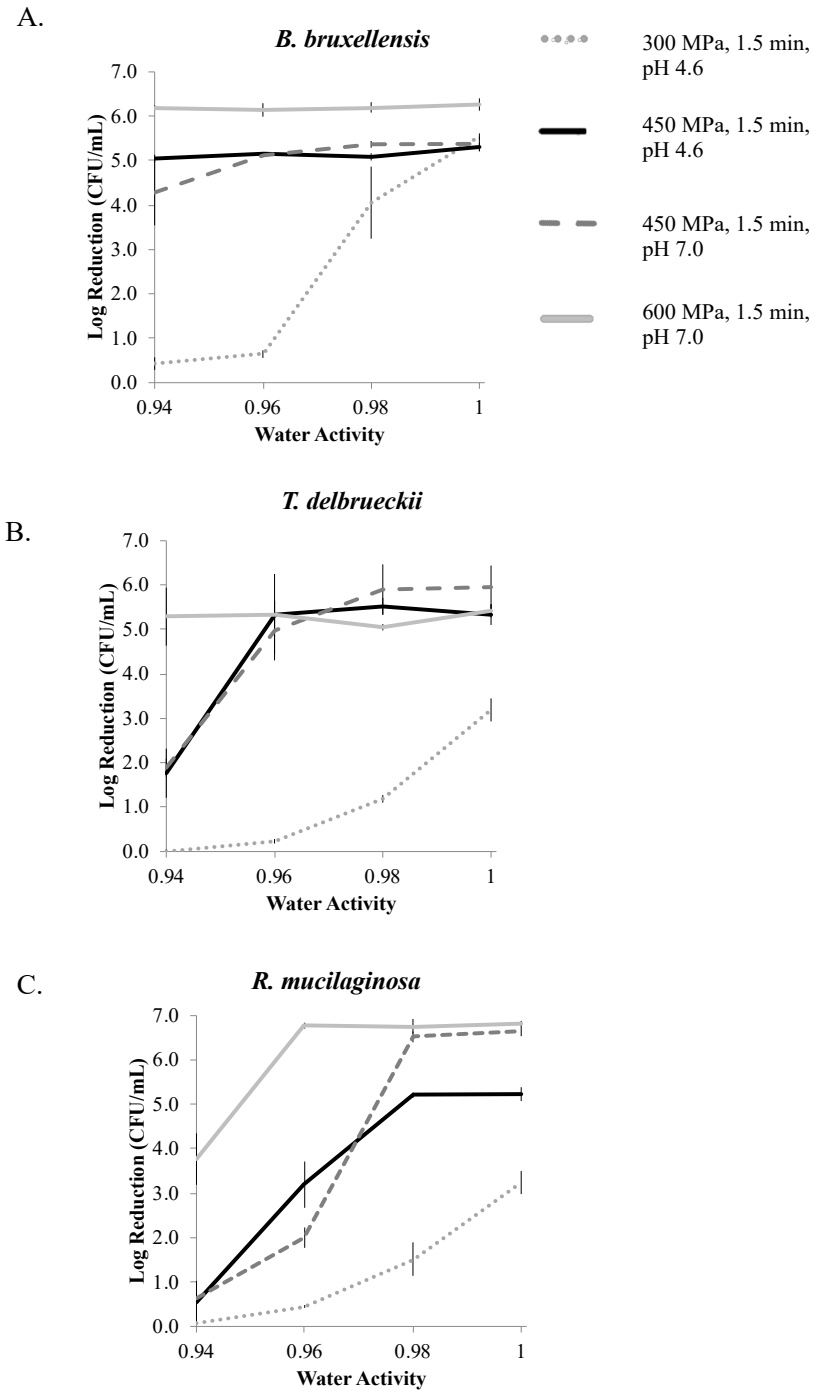

Figure 3.3. Log reduction of yeasts after HPP conditions of 350, 450, and 600 MPa for 1.5 min in pH 4.6 and 7.0 apple juice concentrate diluted to 0.94-1.0 a_w. [a] (A.) *B. bruxellensis,* (B.) *T. delbrueckii,* and (C.) *R. mucilaginosa.*

[a] Error bars show standard deviation

Table 3.2. Survival of yeasts during storage.

Storage Conditions	***B. bruxellensis***	***T. delbrueckii***	***R. mucilaginosa***
450 MPa, pH 4.6, Refrigerated	26 weeks without growth	20 - 26 weeks	26 weeks without growth
300 MPa, pH 4.6, Refrigerated	26 weeks without growth	Growth at 11 weeks	26 weeks without growth
450 MPa, pH 7.0, Ambient	Growth at 4 weeks	Growth at 4 weeks	4 weeks without growth
600 MPa, pH 7.0, Ambient	Growth at 4 weeks	Varied from 4– 26 weeks	Varied from 4 – 26 weeks

***B. bruxellensis* results.** Processing *B. bruxellensis* was very efficient at 450 MPa in 0.94-1.0 a_w, pH 4.6 diluted apple juice concentrate. Raising the pH to 7.0 and the pressure to 600 MPa achieved similar results across the water activities tested. The difference in survival of *B. bruxellensis* across water activities processed at 450 MPa in pH 7.0 apple juice concentrate was limited; a lower reduction occurred at 0.94 a_w than at 0.96-1.0 a_w. Comparing reductions between pH 4.6 and 7.0 only showed a significant difference at 0.94 a_w ($P = 0.017$). Processing *B. bruxellensis* at 300 MPa in pH 4.6 apple juice concentrate showed the greatest difference between water activities. Less than 1-log reduction occurred at 0.94 and 0.96 a_w, but a 4-log reduction was observed at 0.98 a_w and 5-log at 1.0 a_w.

Wine can be adversely affected by many processing methods typically used for juices, but HPP is less harsh. *Brettanomyces* in particular has been of interest to the wine community. Relatively low levels of pressure have been effective at reducing these yeasts. A 5-log reduction

of *Brettanomyces* in Chardonnay wine was achieved by just 15 s at 200 MPa but only a 1-log reduction was achieved in a Dolcetto Syrah variety. The sulfur dioxide concentration as well as alcohol concentration worked synergistically with HPP to achieve larger reductions: with white wines typically having higher concentrations of sulfite than reds (Van Wyk et al., 2017). Another study used synthetic wine (pH 3 and 4, ethanol 10, 12, and 14%) to process *B. bruxellensis* at 100 – 300 MPa for 1 to 7 min at ambient temperature. Processing at 300 MPa was more effective than lower pressures and total inactivation was achieved in all samples after only 1 min. No recovery after 1 week was observed for samples at 300 MPa. However, samples at lower pressures that had initial surviving populations increased over the one week of storage (González-Arenzana et al., 2016). We observed similar results with survivors capable of growth during ambient storage.

***T. delbrueckii* results.** Processing *T. delbrueckii* in pH 4.6 apple juice concentrate at 450 MPa for 1.5 min resulted in a 5-log reduction at 0.96, 0.98, and 1.0 a_w. Processing under the same conditions at 0.94 a_w, was less successful: with a 2-log CFU/mL reduction being achieved. Refrigerated storage prevented *T. delbrueckii* from growing, even in the 0.94 a_w samples. Processing *T. delbrueckii* in pH 4.6 apple juice concentrate at 300 MPa showed a gradient of inactivation across increasing water activity, but all 300 MPa samples exhibited growth over storage. Processing *T. delbrueckii* in pH 7.0 required increasing processing parameters. 450 MPa for 1.5 min was not sufficient to limit growth during storage.

Increasing the pressure to 600 MPa proved successful at decreasing spoilage for most samples of *T. delbrueckii* at pH 7.0 and 0.98-1.0 a_w, with a single instance of recovery and gas production. Similar to our results at 0.98 a_w and 22°Brix at pH 4.6, Kaushik and others (2014) achieved inactivation of yeasts and molds, initially at a 4.2-log population, when processing

mango pulp (pH 4.0 and 17.4°Brix) at 600 MPa for 5 min at ambient temperature, but only a 2.9-log reduction at 500 MPa for 5 min (Kaushik et al., 2014).

HPP dressing products are currently popular: there are several commercially available dressings that have been high pressure processed. However, *T. delbrueckii* can also cause spoilage in ranch dressing. Waite and others (2009) challenged a model ranch dressing with a pH of 4.4 and water activity of 0.975 by inoculating it with *T. delbrueckii.* Samples were processed at 200, 400 or 600 MPa for 3 min and stored at 26°C for 3 weeks. Processing of the dressings at 200 MPa did not cause any significant decreases in *T. delbrueckii* populations. Both 400 MPa and 600 MPa caused reductions greater than 4-log. *T. delbrueckii* did not recover and grow during storage. These results are similar to our study: pH 4.4 and 0.975 a_w dressing vs. pH 4.6 and 7.0, 0.98 – 1.0 a_w apple juice samples reached a 6-month shelf life, with one exception that exhibited growth (Waite et al., 2009).

***R. mucilaginosa* results.** At 0.98 and 1.0 a_w, a greater log reduction of *R. mucilaginosa* appeared to have occurred in the pH 7.0 apple juice concentrate. However, the initial concentration of *R. mucilaginosa* in pH 7.0, processed at 450 MPa was at 6-log CFU/mL and the initial concentration of *R. mucilaginosa* in pH 4.6, processed at 450 MPa was at a high 5-log CFU/mL which may explain the difference in reductions. For both conditions, populations were reduced to non-detectable levels after processing. *R. mucilaginosa* populations remained constant in samples at 0.94-0.96 a_w during shelf life even though a significant reduction was not observed with *R. mucilaginosa* and the population levels remained consistently at log 5-6 CFU/mL. Increasing the pressure to 600 MPa proved successful at extending the shelf life for most samples of *R. mucilaginosa* at 0.98-1.0 a_w. While most high water activity samples processed at 600 MPa were maintained at non-detectable levels for 26 weeks at ambient temperatures, *R.*

mucilaginosa had a single instance of recovery, growth, and gas production, indicating that juices should be stored at refrigerated temperatures; all controls showed an inability to grow at refrigeration temperatures after a low level inoculation even in pH 7.0 model with one exception. *Penicillium* spp. was capable of growth at month 4 at refrigeration in the 1.0 a_w, pH 4.6 sample.

One of the first HPP studies comparing water activities and yeast reduction was done by Oxen and Knorr in 1993. They inoculated sucrose, fructose, glucose, or NaCl solutions with *Rhodotorula rubra* and processed at 5-45°C with pressures ranging from 0.1 to 400 MPa. Results at 5 and 25°C were very similar, but increasing the processing temperature caused greater inactivation. The results of *R. rubra* processed in 30 and 40% sucrose, fructose, or glucose at 5°C and 400 MPa can be compared to our results of *R. mucilaginosa* at 0.94 and 0.96 a_w diluted apple juice at pH 7.0 and 450 MPa. With a 30% sugar solution, there was total inactivation of *R. rubra* at 400 MPa at 5°C, regardless of solute. At 40% sugar there was total inactivation in sucrose, but only a 3.5-log reduction in glucose or fructose. The present study also shows a greater reduction at higher water activity. The results may diverge slightly due to medium composition and species differences (Oxen et al., 1993).

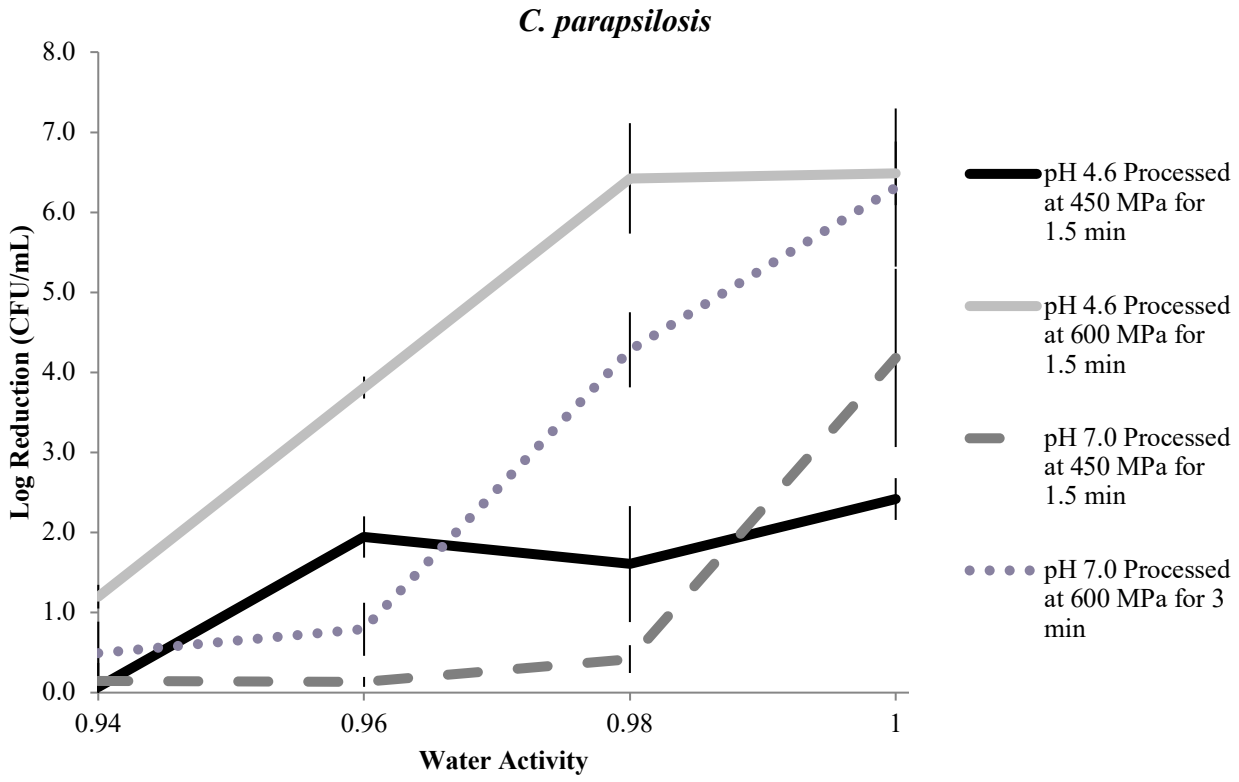

Figure 3.4. Log reduction of *C. parapsilosis* after HPP at 450 and 600 MPa for 1.5 or 3 min in pH 4.6 and 7.0 apple juice concentrate diluted to 0.94-1.0 a_w. [a]

[a] Error bars show standard deviation

Table 3.3. Survival of *C. parapsilosis* during storage.

Storage Conditions	***C. parapsilosis***
450 MPa, pH 4.6, Refrigerated	26 weeks without growth
600 MPa, pH 4.6, Refrigerated	26 weeks without growth
450 MPa, pH 7.0, Ambient	Growth at 4 weeks
600 MPa, pH 7.0, Ambient	Growth at 4 weeks

***C. parapsilosis* results**. At 0.96 and 0.98 a_w, 450 MPa for 1.5 min in pH 4.6 apple juice caused at least a 1-log reduction of *C. parapsilosis*. However, at 1.0 a_w, 450 MPa HPP was more effective at reducing *C. parapsilosis* in pH 7.0 apple juice concentrate. This may be due to ideal conditions for these organisms. Yeasts generally prefer acidic conditions and *B. bruxellensis,* for example, is capable of growth as low as pH 1.8 (Pitt et al., 2009). At more neutral conditions, further from their optimum conditions, yeasts may be more susceptible to pressure. *C. parapsilosis* has been isolated in more neutral foods such as milks and thus may prefer the pH 7.0 condition (Pitt et al., 2009). *C. parapsilosis* also exhibited much higher levels of resistance, as it was able to recover during ambient storage even after 3 min at 600 MPa. This higher resistance led to our decision to use different processing parameters for *C. parapsilosis.* Due to its resistance at 450 MPa, there seemed to reason to test at 300 MPa. Initial tests in pH 7.0 apple juice concentrate showed that reductions were limited below 3 min. This increased resistance compared to other yeasts may be due to Candida's dimorphic ability to switch between a single celled yeast form and a pseudohyphae form. It has been isolated in a wide range of environments from soil to human skin and has been identified as an opportunistic pathogen in immunocompromised patients. *C. parapsilosis* has also shown resistance to antimicrobials used to treat these infections (Trofa et al., 2008).

There is limited information showing the ability of *Candida* to survive HPP. Daryaei and others (2010) subjected *C. zeylanoides* and *C. lipolytica* to HPP at 600 MPa for 5 min at 20°C in fermented milk at pH 4.30, 5.20 and 6.50. Populations were reduced to non-detectable levels and stayed below detection levels during shelf life at 4°C. *C. lipolytica* was able to grow at 4°C after processing at 300 MPa. However, these *Candida* species are common to cheeses, and were only incubated for 48 hours before processing (*9*). The age of spores can have an effect on the

resistance to HPP which is why we chose to only use 30-day old spores (Black et al., 2007). Thirty day incubations are common because resistance increases with age. A study by Beuchat (1988) found that the heat resistance of *Talaromyces flavus* increased as the age of ascospores increased, up to 30 days, at which point, it plateaued (Beuchat, 1988). Our results showing higher pressure resistance may be due to a more ideal environment and older spores.

General Trends. The protective effects of low a_w observed in our experiments were similar to those observed by others. Several hypotheses have been proposed to explain why increasing sugar or decreasing water activity may allow increased resistance. At lower water activities, cells may become dehydrated, shrinking the cell, reducing pore size and thickening the cell membrane. Alternatively, sucrose may replace the water in the lipid head groups of the membrane and prevent phase transition, thus preserving proteins and protecting the membrane during pressure (Goh et al., 2007). Our results have shown this water activity effect across both filamentous fungi and yeasts, with the exception of *A. niger* which was similarly susceptible to pressure at 0.94 and 1.0 a_w ($P = 0.66$ at pH 4.6). Due to the ability of spoilage organisms to recover at warmer temperatures, processors should use HPP only to process products at 0.98 and 1.0 a_w.

Overall, processing juices at pH 4.6 caused greater reductions than at pH 7.0. However, there were differences in survival between genera tested, with the lowest reductions achieved by *C. parapsilosis*. Processing at 450 MPa, 1.5 min, 5°C in pH 4.6 and 0.98 or 1.0 a_w caused a 4.0-log or greater reduction of *Penicillium* spp., *P. variotii, A. niger, T. delbrueckii, R. mucilaginosa,* and *B. bruxellensis.* Even when total inactivation appeared to occur, in some instances these samples were able to recover and grow at ambient temperatures.

Processing samples at 300 MPa proved unsuccessful; while some log reductions were observed, the majority of the fungi achieved recovery and subsequent growth when held at 5°C for only a few weeks. Samples processed at pH 4.6 and 450 MPa and held at 5°C showed no growth over the 6-month shelf life. However, the filamentous fungi processed under the same conditions were able to recover at room temperature, regardless of water activity. Controls that were not processed show that these organisms are unable to grow in diluted apple juice concentrate under refrigeration conditions when inoculated at a 2-log CFU/mL level. It is recommended to store HPP juices at refrigeration temperatures to prevent fungal growth during storage.

Almost all samples processed at pH 7.0 and 450 MPa showed recovery and growth when held at room temperature. Our shelf life is significantly longer than those testing microbial stability in other HPP studies. Cao et al. (2012) studied HPP strawberry juice stored for six months at 4 and 25°C, but only followed physicochemical characteristics. They found that storage at 4°C was considerably better in preventing color, ascorbic acid and total phenols deterioration than at 25°C (Cao et al., 2012). Similarly, Bull and others (2004) found that HPP for 1 min at 600 MPa was effective at reducing initial yeast and mold levels in orange juice from 4.8-log CFU/mL to non-detectable levels. However, populations increased at the 8-week mark when stored at 10°C (Bull et al., 2004).

HPP is known to extend the shelf life of products and our refrigerated shelf life results validated this concept. Both yeasts and filamentous fungi proved relatively sensitive to pressures at or above 450 MPa for 1.5 min or longer. This extended shelf life may enable producers to use later sell by dates; potentially leading to lower economic losses and food waste.

Future Work. Our results are in line with others' showing that lower water activity provides a protective effect. Processing juices at higher water activities and lower pH could result in a 6-month or greater refrigerated shelf life. Future work should be conducted to directly compare all conditions tested to both refrigerated and ambient storage. The greater reductions observed at pH 4.6 may allow for a long shelf life at ambient temperatures. Lower pH may also be evaluated to see if there is a greater synergistic effect in high acid juices.

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CHAPTER 4

HIGH PRESSURE PROCESSING OF HEAT AND PRESSURE RESISTANT FUNGI AS AFFECTED BY PH, WATER ACTIVITY, SULFITES AND DIMETHYL DICARBONATE IN A DILUTED APPLE JUICE CONCENTRATE

Key words: spoilage fungi, High Pressure Processing, storage

ABSTRACT

High pressure processing (HPP) is a popular method of processing juices to retain "fresh" sensorial properties. However, heat and pressure resistant fungi, *Byssochlamys* and *Aspergillus*, can survive processing and cause visible spoilage in juices. The goal of this study was to evaluate the pressure and time necessary to cause a reduction of heat and pressure resistant spoilage fungi commonly found in juice and determine the effect of water activity (0.94 – 1.0 a_w) and pH (3.5 – 7.0). The effect of sulfites on the reduction of fungi at pH 4.6, and dimethyl decarbonate (DMDC) on the reduction of *A. fischeri* at pH 7.0 was also determined as a proof of concept to see if either aided in fungal inactivation. Storage up to 26 weeks at refrigeration temperatures was also evaluated after HPP. Processing *A. pseudoglaucus* in pH 3.5 diluted apple juice concentrate at 600 MPa for 15 min at 5°C was more effective than in pH 4.6, 7.0, or 4.6 with sulfites. Processing *A. fischeri* at pH 3.5 or 7.0 for 15 min at 600 MPa, 5°C or for 4.6 for 1.5 min at 450 MPa resulted in a 1-log reduction. The addition of DMDC to pH 7.0 apple juice inoculated with *A. fischeri* and processed at 600 MPa for 15 min resulted in a 1-log activation rather than a reduction. Processing at 600 MPa for 15 min at 5°C caused activation of *P. niveus* (*B. nivea* Westling) at pH 3.5, 4.6 and 7.0 diluted apple juice concentrate at 0.94-1.0 a_w. In

general, a greater reduction of all fungi was observed at 1.0 a_w than at 0.94 a_w. A 17 to 26 week storage period could be achieved, but only at refrigeration temperatures.

HIGHLIGHTS

- HPP for 15 min at 600 MPa was necessary for an extended refrigerated shelf life.
- *P. niveus* was the most resistant to HPP processing.
- *A. pseudoglaucus* was relatively sensitive to HPP but capable of growth at 5°C.
- *A. fischeri* could be reduced by 1-log with HPP at 450 or 600 MPa.

High pressure processing (HPP) is a popular method of processing juices. HPP is appealing to consumers looking for minimally processed products; consumers have been shown to prefer HPP treated juices and products over thermally treated similar products. It uses extreme pressures (400-600 MPa) to cause cell membrane and protein damage to microorganisms and thus inactivation (Mujica – Paz et al., 2011). Products are generally in their final packaging during processing, reducing the risk of post process contamination. HPP treated products retain better sensorial and nutritive properties compared to thermal processed products. Color loss is generally minimal but may increase during shelf life. Vitamin retention is usually improved as compared to thermal processing (Castro et al., 2014).

Heat resistant molds can be hard to inactivate and difficult to control during shelf life. Some fungi have been capable of growth at low temperatures (10°C), low oxygen concentrations and low pH (Butz et al., 1996). Heat resistant fungi have been known to withstand typical HPP parameters (Chapman et al., 2007). High pressure can trigger germination of dormant cells that subsequently grow during shelf life and cause spoilage as well as economic losses. The effect of HPP is dependent on many variables: the composition of the media can provide protective effects to the spores or synergistic effects by providing hurdles along with the processing. Low pH has been proven in some cases to increase reductions while higher pH and media rich in nutrients can improve survival. Low water activity (a_w) products offer baroprotection as well, with differing results based on the solute. In general, vegetative cells are sensitive to HPP while ascospores are resistant due to thick cell walls and a dehydrated spore core (Black et al., 2007). As a result, HPP products are typically kept refrigerated to avoid outgrowth of spoilage organisms (Mujica – Paz et al., 2011).

Some strains of heat resistant filamentous fungi, *Byssochlamys* and *Aspergillus*, can survive at a wide range of temperatures, oxygen concentrations and pH and have been known to produce mycotoxins (Evelyn et al., 2016). For example, *N. fischeri (A. fischeri)* has been demonstrated to survive in temperatures above 100°C and both *N. fischeri* and *B. nivea* have survived canning processes (Pitt et al. 2009, Evelyn et al., 2016; Evelyn et al., 2015). *Byssochlamys* can cause large economic losses from apple harvest to juice. It produces enzymes that can cause unwanted softening of the whole fruit as well as patulin, which is a toxin that must be controlled in juice production (Panagou et al., 2010). *B. nivea* can also produce CO_2; causing swelling in packaging (da Rocha Ferreira et al., 2009). Evelyn and others (2015, 2016) studied the effect of HPP at temperatures above 38°C on *N. fischeri* survival in apple juice and *B. nivea* in strawberry puree (Evelyn et al., 2016). They found that the samples required long hold times at 600 MPa for 10 min or greater at 75°C to cause reductions (Evelyn et al., 2016). Raising the processing temperature can cause shortened processing times. However, using HPP at high temperatures can cause degradation of nutrients and color, especially carotenoids and anthocyanins (Dijksterhuis et al., 2006, Castro et al., 2014). Thermal processing alone would require heating times of 20 minutes at 90°C or 60 min at 85°C for a 3-log reduction of *N. fischeri* in apple juice (Evelyn et al., 2016, Evelyn et al., 2015). In contrast, Voldrich and others tested log of *Talaromyces avellaneus,* another heat resistant, ascospore forming fungi, in apple juice processed at 600 MPa at varying temperatures. They found that processing at 17°C caused a 2-log reduction of *T. avellaneus* and increasing to 25°C did not cause a significant difference (Voldrich and others, 2004).

The effect of pH may also enhance survival, even though fungi are known to be acid tolerant. Reyns and others (2003) subjected *Talaromyces macrosporus* to HPP in buffer ranging

from 3.0 to 6.0 pH. However, processing at 400 MPa for 20 min did not exhibit a significant inactivation differences at the range of pH values tested. Increasing the pressure to 600 MPa resulted in higher inactivation of spores at pH 3.0 than compared to pH 6.0 (Reyns, 2003). Due to the differences observed, it is important to study the effect of pH in other HPP treated foods.

Additional research is needed to identify strategies to control heat and pressure resistant fungal spoilage in intermediate water activity beverages, including the exploration of processing aids to increase inactivation. Dimethyl dicarbonate (DMDC) and sulfur dioxide have been used as preservatives in beverages, most commonly wine (Chavan and Tupe, 2014). DMDC hydrolyzes to methanol and carbon dioxide in the presence of water. Presence of DMDC cannot be detected within hours of treatment, resulting in a "clean label" preservative. The use of DMDC on the survival of *Byssochlamys fulva* in apple juice and strawberry nectar has been explored. 75 ppm of DMDC caused inactivation of vegetative cells of *B. fulva* at 30°C within 10 min and at 10°C within 60 min in apple juice. Ascospores of *B. fulva* were resistant to DMDC at concentrations of 500 – 1000 ppm but vegetative cells were not (van der Riet et al., 1989). The synergistic effect of DMDC combined with HPP has not yet been explored. There is potential that DMDC could increase inactivation rates or extend the shelf life of HPP products.

Sulfites have also been used to control spoilage. They are most active as an antimicrobial at lower pH values (Chavan and Tupe, 2014). Sulfites react with proteins, aliphatic aldehydes, coenzymes, vitamins, hormones and can cause damage to DNA and unsaturated lipids resulting in inhibition to fungal growth and reproduction (Maier et al., 1986). Puig and others (2008) studied the effect of high pressure homogenization on grape must (one red without SO_2, one white with SO_2 added) that was fermented into wine. Their goal was to reduce the natural microbiota present on grapes that may cause off flavors in the subsequent wine. High pressure

caused a 3-log reduction with yeast and molds reduced to non-detectable levels. Due to the differences in initial populations due to SO_2 additions, and the reduction of yeasts and molds to non-detectable counts, it is hard to determine the synergistic effect of SO_2 combined with high pressure. However, it is clear that that the 200 MPa treatment was effective at reducing unwanted yeasts and molds (Puig et al., 2008). More work should be done evaluating the effect of sulfites combined with high pressure processing.

The goal of this study was to evaluate the pressure and time necessary to cause a reduction of heat and pressure resistant spoilage fungi commonly found in juice and determine the effect of water activity and pH. The effect of sulfites and DMDC was also determined to see if either aided in reductions. Shelf life and storage potential up to 26 weeks was also evaluated after HPP.

MATERIALS AND METHODS

Inoculum. Apple juice concentrate (Williamson, NY) was diluted with water to 0.94 – 1.00 a_w. Initial physicochemical values of apple juice concentrate, measured at 20°C, were: 0.72 a_w, 3.3 pH, and 70.4°Brix. Apple juice concentrate pH was raised to 3.5, 4.6 and 7.0 with NaOH (Chem-Impex, Wood Dale, IL). Models were hot filled at 95°C and held for 30 min to inactivate vegetative cells present in the concentrate.

Water activity measurements were made by an Aqua Lab Dew Point Water Activity Meter 4TE (Decagon, Pullman, WA). pH was determined with an Oakton pH 5 Acorn Series pH meter (Oakton Instruments, Vernon Hills, IL). Total soluble solids were determined with an Abbe refractometer (Leica Inc., Buffalo, NY). All analyses were conducted in triplicate.

Spoilage Organism Preparation. *Aspergillus pseudoglaucus,* and *Aspergillus fischeri* and were obtained from Cornell AgriTech (Dr. R. Worobo's laboratory). *Paecilomyces niveus* (*Byssochlamys nivea* Westling) was obtained from the Cornell University Plant Pathology Department (isolated and identified by Dr. M. Biango-Daniels). This particular strain was isolated from an apple orchard, found to be extremely heat resistant and capable of patulin production in apple juice as well as *Paecilomyces* rot in apples (Biango-Daniels et al., 2019). Plugs of filamentous fungi were plated on malt extract agar (BD, Franklin Lakes, NJ) and grown for 30 days at 30°C. Ascospore age can have an effect on the survival of pressure resistant fungi, with resistance increasing with age so 30-day incubations are common (Chapman et al., 2007, Butz et al., 1996).

Spores were harvested by flooding plates with Tween 80 and gently scraping with a sterile spreader (Butz et al., 1996). Mycelium mixture was filtered with 3 layers of sterilized cheesecloth (Evelyn et al., 2015). Filtered spores were frozen at 0°C until use. Survival of spores under these conditions was confirmed in preliminary studies. Asci and ascospores were confirmed with microscopy.

Inoculation. Inoculation conditions and subsequent processing and storage conditions are summarized in Figure 4.1. Sixty grams of apple juice concentrate model was added to flexible 2 oz Polyethylene terephthalate (PET) bottles (Captiva Containers, FL). One mL of target organism was added to each bottle, samples were refrigerated until processing and used in less than 24 h. Immediately before processing, initial fungal samples were enumerated on DRBC Agar (BD, NJ) and incubated for 7 days at 30°C. Unprocessed controls at 0.94 and 1.0 a_w were also inoculated at a 2-log CFU/mL concentration and stored at 5°C during storage for comparison. Controls were checked periodically for visual mycelia growth.

As a proof of concept to attempt to control fungal spoilage at neutral pH, 7.0 pH, 0.94 and 1.0 a_w apple juice samples inoculated with *A. fischeri* also had 15 uL DMDC (Velcorin, Pittsburgh, PA) added to them to achieve a 250 ppm concentration, the FDA allowed maximum (21CFR172.133, 2018). DMDC was added and samples were shaken vigorously for 1 min immediately before Time 0 plating (van der Riet et al., 1989). As a proof of concept to attempt to control fungal spoilage in acidic juices, 10 uL potassium metabisulfite (Alfa Aesar, Ward Hill, MA) stock solution (842 ug/ 10 mL water) was also added to 0.94 and 1.0 a_w, pH 4.6 samples of all tested species to achieve an 8 ppm concentration with a margin of error to not exceed the FDA maximum of 10 ppm to avoid mandatory label declaration (21CFR130.9, 2018). After initial results proved to be somewhat effective for *A. pseudoglaucus*, 0.96 and 0.98 a_w samples for that species were also processed.

HPP. PET bottles with juice and inoculum were placed into PET bags and vacuum sealed for 1.8 s, double bagged and re-sealed, all to prevent the risk of leakage inside the HPP unit. All 4.6 pH apple juice samples without processing aids or preservatives were initially processed at 450 MPa for 1.5 min at 5°C in a 55 L, 200 mm diameter HPP unit with a cooling component to maintain target temperature (Hiperbaric, Miami, FL) to determine average log reduction. Samples were plated on DRBC immediately after processing. Due to pressure resistance, all subsequent processing times were increased to 600 MPa for 15 min. Longer processing times were not considered to be financially feasible for commercial production (Mujica – Paz et al., 2011).

pH 3.5, 4.6 and 7.0 samples without added microbial agents were stored at 5°C for 26 weeks (6 months) to determine refrigerated shelf-life. Samples at 3.5, and 7.0 pH were also stored at ambient temperatures (19-23°C) for up to 26 weeks to determine survival of fungi.

Samples with added microbial agents were only stored at ambient temperatures to assess any synergistic effects with HPP.

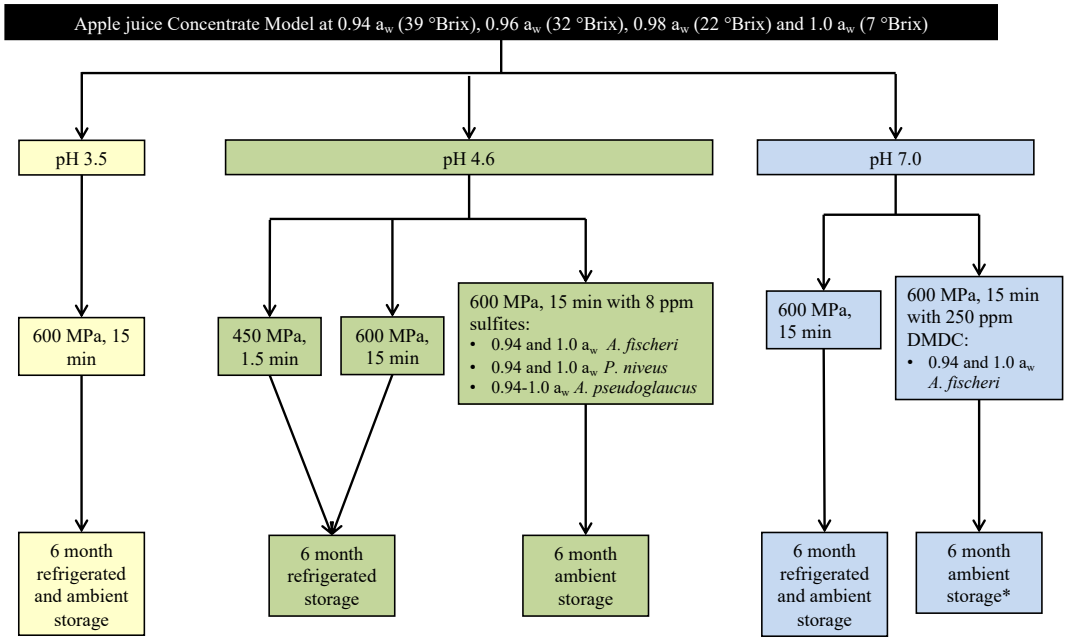

Figure 4.1. Framework for experimental conditions: HPP processing conducted at 5°C.

*pH 7.0 ambient storage to evaluate fungi survival.

Statistical Analyses. Each experiment was performed in triplicate, except for proofs of concept with sulfites and DMDC – which were performed in duplicate. Log reductions were calculated by taking the log of initial and subsequent populations. Growth during storage was determined by comparing after HPP levels to periodic plating counts. A model was created to compare estimated marginal means using R version 3.2.2. A Type III sums of square test was used to determine a three way interaction (R Core Team, Vienna, Austria). Values <1 CFU/mL (undetectable when 1 mL was plated) were recorded as "1.0" for conservative statistical purposes.

RESULTS AND DISCUSSION

Physical and chemical analyses. Physicochemical measurements of diluted apple juice concentrate model at 20°C were as follows: targeted pH 3.5 was measured as 3.5±0.08. pH 4.6 was measured as 4.59±0.01 and targeted at 7.0 was measured as 7.01±0.03. pH 4.6 was chosen as a midpoint because it inhibits the growth of *Clostridium botulinum,* a spore forming, toxin producing pathogen capable of surviving high pressure processing. *C. botulinum* produces a toxin that can cause death at low concentrations (Black et al., 2007). The lower limit of pH 3.5 was chosen to represent apple juice, which has a wide pH range from 3.35 – 4.0 (Bridges and Mattice, 1939). Apple juice concentrate at pH 7.0 was selected to see HPP results at a neutral pH. The 0.94 a_w model was measured as 0.940±0.001 a_w and 39.3±1.9 °Brix. The 0.96 a_w model was measured as 0.961±0.003 a_w and 32.1±0.7 °Brix. The 0.98 a_w model was measured as 0.979±0.002 a_w and 22.0±0.5 °Brix. The 1.0 a_w model was measured as 0.997±0.003 a_w and 7.1±0.9 °Brix.

HPP effect. Overall, the genera of filamentous fungi tested proved to be very pressure resistant. Statistical comparisons showed a significant interaction between species, water activity and pH. *A. pseudoglaucus* was the most pressure sensitive of the three species tested while *P. niveus* proved to be the most resistant. Log reductions are shown in Figures 4.2, 4.3 and 4.4. Storage was best for all three species at refrigeration temperatures with results shown in Tables 4.1, 4.2 and 4.3. Plating alone did not always indicate a failed shelf life – visible mycelia was also considered a failure, even if population counts did not increase. Due to differences in outcomes, the results and discussion will be presented by fungal genera tested in this study.

FIGURE 2

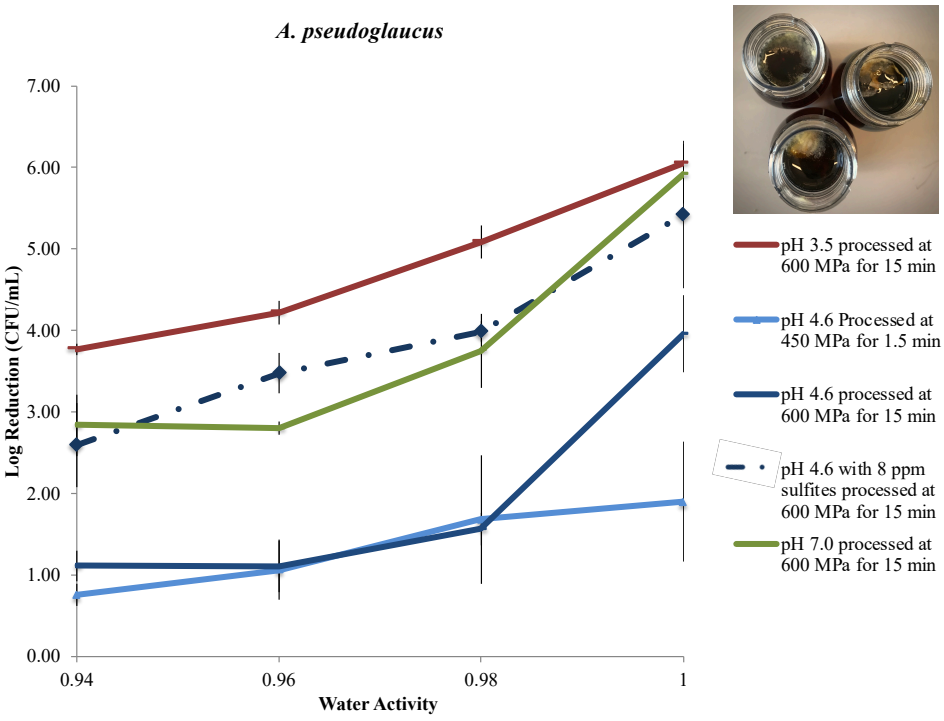

Figure 4.2. *A. pseudoglaucus* processed at 450 and 600 MPa for 1.5 - 15 min in 3.5, 4.6, and 7.0 pH apple juice concentrate at varying water activities. Inset: Refrigerated *A. pseudoglaucus* samples with visible mycelia at week 17. [a]

[a] Error bars show standard deviation

Table 4.1. Visible growth of *A. pseudoglaucus* during storage at 0.94-1.0 a_w.

Processing Conditions	**Ambient storage**	**Refrigerated storage at 5°C**
450 MPa, 1.5 min, pH 4.6	Not tested	Visible mycelia at 6 weeks at 0.94 – 1.0 a_w
600 MPa, 15 min, pH 4.6	Not tested	Visible mycelia at 20 weeks at 0.94 – 0.98 a_w, no growth observed for 1.0 a_w for 26 weeks
600 MPa, 15 min, pH 7.0	Visible mycelia at 4 weeks at 0.94 – 1.0 a_w	No growth observed for 26 weeks
600 MPa, 15 min, pH 3.5	Visible mycelia at 2 weeks 0.94 – 1.0 a_w	Visible mycelia at 17 weeks at 0.94 – 0.96 a_w, no growth observed for 0.98 – 1.0 a_w for 26 weeks
600 MPa + sulfite, 15 min, pH 4.6	Visible mycelia at 4 weeks at 0.94 – 1.0 a_w	Not tested
pH 4.6 control	Visible mycelia at 1 week at 0.94 – 1.0 a_w	Visible mycelia at 16 weeks
pH 7.0 control	Visible mycelia at 1 week at 0.94 – 1.0 a_w	No growth observed for 26 weeks
pH 3.5 control	Visible mycelia at 1 week at 0.94 – 1.0 a_w	No growth observed for 26 weeks

***A. pseudoglaucus* results.** The highest reduction of *A. pseudoglaucus* was achieved in pH 3.5 apple juice concentrate processed at 600 MPa, 5°C for 15 min and the lowest was in pH 4.6, with no sulfites added, processed at 450 MPa for 1.5 min (Figure 4.1). There was a significant statistical interaction between the log reductions achieved at 3.5, 4.6 and 7.0 pH (P=0.007). Even when complete inactivation (>5-log) of *A. pseudoglaucus* appeared to occur at pH 3.5 and 7.0, at 1.0 a_w recovery was observed at ambient temperature and visible growth occurred within a few weeks. *A. pseudoglaucus* in refrigerated pH 3.5 apple juice samples showed recovery and visible growth at 0.94 and 0.96 a_w at week 17 (Table 4.1). This was most likely due to the higher survival rates of *A. pseudoglaucus* at lower water activity.

The baroprotective effect seen with higher survival rates of *A. pseudoglaucus* at 0.94 and 0.96 a_w is similar to the effect seen by Goh and others (2007). Fungi: *Saccharomyces cerevisiae, Pichia anomala, Hanseniaspora uvarum, Penicillium expansum, Rhizopus stolonifera,* and *Fusarium oxysporum* were inoculated into sucrose syrups and NaCl solutions at 4.2 pH and at 0.932, 0.903 and 0.866 a_w. Samples were processed at 600 MPa, 20°C for up to 3 min. Higher survival rates were observed at lower water activities. For example, *P. expansum* was reduced by more than 4-log in the 0.932 a_w sucrose syrup, but only by 2-log in the 0.866 a_w syrup. At low water activities, and in a dehydrated or semi-dehydrated condition, the spore proteins may be more stabilized than in a fully hydrated state. Alternatively, or potentially synergistically with the previous theory, the low water availability may cause pore size and cell content shrinkage leading to stabilization. This would allow for less damage during HPP treatment (Goh and others, 2007, Georget 2015).

Eicher and others (2002) studied the effect of HPP on the survival of *E. repens* (*A. pseudoglaucus*) in isotonic NaCl, both with a heat shock and without a heat shock before

processing. Heat shocking at 60°C for 15 min caused greater inactivation rates of *E. repens* processed at 500 MPa, 25°C for 30 min. Under the same processing conditions, but without the heat shock, *E. repens* was reduced by approximately 0.5-log CFU/mL. These limited inactivation results are very similar to our results observed at 0.94 a_w (Eicher, 2002).

Visible *A. pseudoglaucus* growth in diluted apple juice concentrate occurred at week 6 in pH 4.6 apple juice concentrate processed at 450 MPa for 1.5 min, 0.94 – 1.0 a_w, and stored at refrigeration while visible growth was delayed until week 20 in samples processed at 600 MPa for 15 min, 0.94-0.98 a_w and stored at refrigeration. *A. pseudoglaucus* samples at 1.0 aw, pH 4.6, and processed at 600 MPa for 15 min did not grow for 26 weeks at refrigeration. In comparison, the 0.94 a_w, pH 4.6 unprocessed control stored at refrigeration took 16 weeks to show visible mycelium. It appears that the 450 MPa processing step extended the shelf life by an additional 10 weeks, even if the reduction caused by high pressure was less than 1-log. Adding sulfites to apple juice concentrate and processing at 600 MPa for 15 min was significantly better at reducing *A. pseudoglaucus* populations in 4.6 pH apple juice concentration when compared to the 4.6 pH condition without sulfite. However, even *A. pseudoglaucus* samples with sulfite exhibited visible mycelia within a few weeks at ambient storage. Processing *A. pseudoglaucus* in pH 3.5 at 0.94 – 1.0 a_w and in pH 7.0 at 0.94 and 1.0 a_w apple juice concentrate was better at reducing populations than adding 8 ppm sulfite to pH 4.6 apple juice concentrate. Pairwise tests at 0.94 a_w show significant differences ($P < 0.05$) in reductions of *A. pseudoglaucus* at all conditions tested except between 0.94 a_w with sulfites at pH 4.6, processed at 600 MPa for 15 min and 0.94 a_w at pH 7.0, processed at 600 MPa for 15 min ($P = 0.82$).

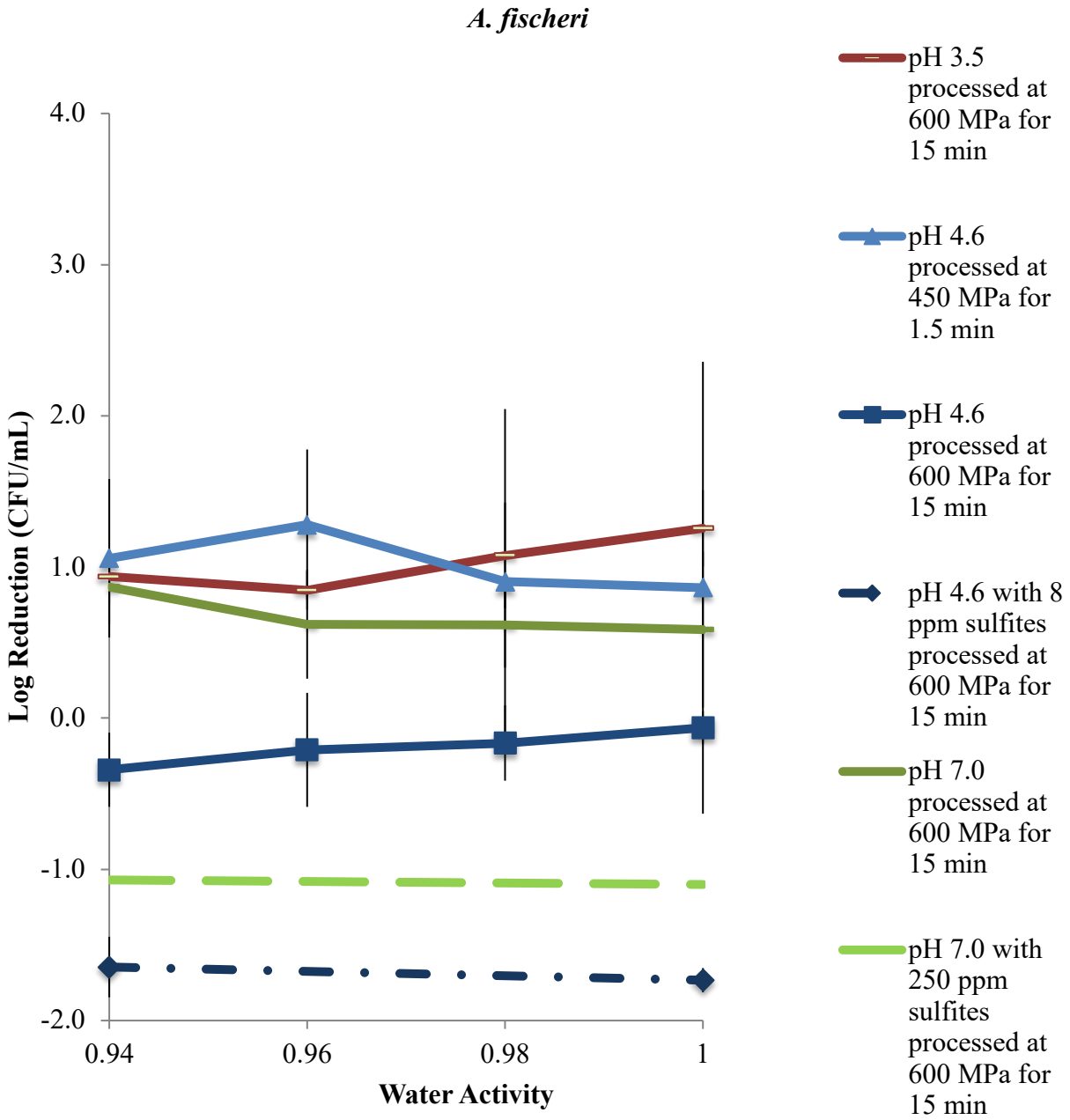

Figure 4.3. *A. fischeri*: Processed at 450 and 600 MPa for 1.5 - 15 min in 3.5, 4.6, and 7.0 pH apple juice concentrate at varying water activities.[a]

[a] Error bars show standard deviation

Table 4.2. Visible growth of *A. fischeri* during storage.

Processing Conditions	**Ambient storage**	**Refrigerated storage at 5°C**
450 MPa, pH 4.6	Not tested	No growth observed for 26 weeks
600 MPa, pH 4.6	Not tested	No growth observed for 26 weeks
600 MPa, pH 7.0	Visible mycelia at 4 weeks at 0.94 – 1.0 a_w	No growth observed for 26 weeks
600 MPa, pH 3.5	Visible mycelia at 2 weeks at 0.94 – 1.0 a_w	No growth observed for 26 weeks
600 MPa + sulfite, pH 4.6	Visible mycelia at 4 weeks at 0.94 – 1.0 a_w	Not tested
pH 4.6 control, at 0.94 and 1.0 a_w	Visible mycelia at 1 week at 0.94 and 1.0 a_w	No growth observed for 26 weeks
pH 7.0 control, at 0.94 and 1.0 a_w	Visible mycelia at 1 week at 0.94 and 1.0 a_w	No growth observed for 26 weeks
pH 3.5 control, at 0.94 and 1.0 a_w	Visible mycelia at 1 week at 0.94 and 1.0 a_w	No growth observed for 26 weeks

***A. fischeri* results.** pH differences were less distinct with *A. fischeri.* At 0.94 and 1.0 a_w, there were statistical differences between pH 3.5 and 4.6 ($P<0.01$) as well as 4.6 and 7.0

($P<0.01$), but not between 3.5 and 7.0. At 0.96 and 0.98 a_w, only pH 3.5 and 4.6 were statistically different. Hocking and others (2006) also high pressure processed *A. fischeri*. First, heat resistant *Byssochlamys fulva* and *Neosartorya fischeri* (*A. fischeri*) were either heat shocked, or not heat shocked and then all samples were processed at 600 MPa for up to 2 min at ambient temperatures in a sucrose solution adjusted to pH 4.2. *N. fischeri* spores were more pressure resistant than *B. fulva.* A 2-log reduction was achieved for non-heat shocked *B. fulva,* and a 3-log reduction was achieved for heat shocked *B. fulva* after 2 min. Approximately a 1-log reduction was achieved for non-heat shocked *N. fischeri* with a slight increase in reductions caused by processing heat shocked *N. fischeri* under the same conditions. While Hocking and others processed similar species to the present study, the sucrose model does not represent a real food. In comparison, our study used a diluted apple juice concentrate with more variables, older spores and a commercial HPP unit. The ascospores were only 2 weeks old, and older ascospores are typically more resilient (Hocking et al. 2006). Other HPP experiments have shown activation of dormant spores after processing (Dijksterhuis et al., 2006). Differences in results could be due to age of spores, composition of medium, and spore incubation and inoculation methods. Typically, high sugar content, older spores and higher pH contribute to better survival (Dijksterhuis et al., 2006, Evelyn et al., 2016). Despite these differences, both Hocking and others and our results show approximately a 1-log reduction of *A. fischeri* achieved at 600 MPa for 15 min. However, as seen in Figure 4.4, we observed *P. niveus* was more pressure resistant than *A. fischeri* (Hocking and Pitt, 2006).

Statistical comparisons were not made with sulfites or DMDC since these microbial control agents were only added at 0.94 and 1.0 a_w samples and were performed in duplicate. However, a clear trend can still be observed. Instead of improving the reductions, both the

addition of 8 ppm sulfite and 250 ppm DMDC caused a greater activation of spores at the conditions tested. All ambient storage conditions tested showed growth within a few weeks, but all refrigeration shelf life conditions maintained a constant level of *A. fischeri,* up to 26 weeks without visible mycelia. Whitney and others (2008) have evaluated the effect of adding 62.5 or 125 ppm DMDC to pathogens *E. coli* O157:H7 in apple juice and *Salmonella* Agona in orange juice and processing at 400 or 550 MPa for 2 min at 6°C. The addition of DMDC to *E. coli* inoculated apple juice caused a synergistic effect with 550 MPa HPP. HPP alone caused a 1.92-log reduction of *E. coli,* while HPP combined with 125 ppm DMDC caused a 4.97-log reduction. HPP (400 MPa for 2 min) alone caused a 1.97-log reduction of *Salmonella,* while HPP combined with 62.5 ppm DMDC caused a 5.96-log reduction immediately after processing in orange juice. Testing populations of *Salmonella* 24 hours after processing showed some recovery: there was a population reduction of 2.86-log with 400 MPa for 2 min, and a reduction of 5.77-log with 62.5 ppm DMDC combined with HPP (Whitney, 2008). This is in direct contrast to our results. However, bacterial vegetative cells are considerably more sensitive to pressure than fungal ascospores. Spores are protected by thick cell walls and concentrated cell contents which allow them to better survive HPP (Georget, 2015). More work should be conducted to determine the differences between DMDC aided HPP of microorganisms and potential recovery during shelf life.

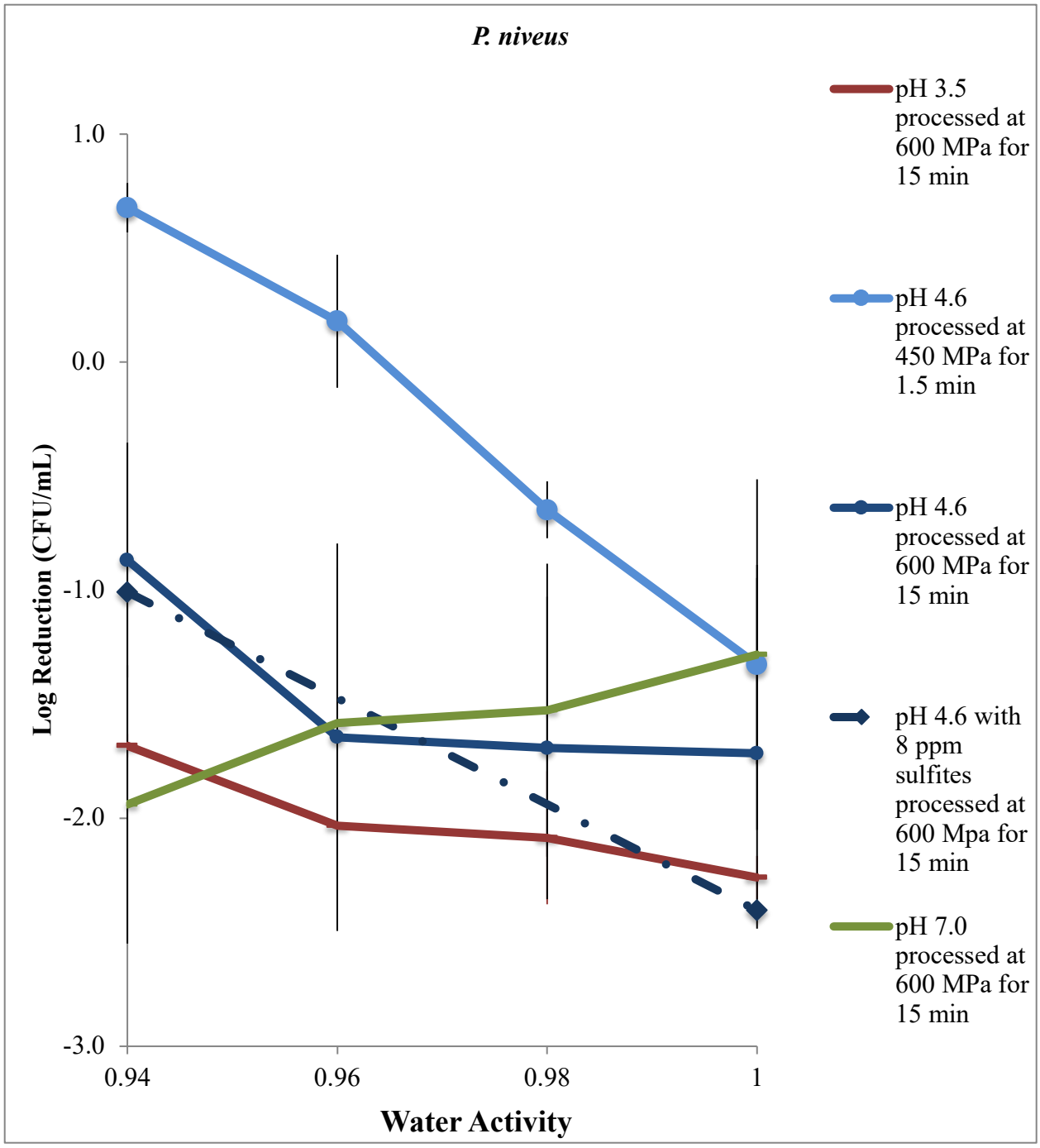

Figure 4.4. *P. niveus* processed at 450 and 600 MPa for 1.5 - 15 min in 3.5, 4.6, and 7.0 pH apple juice concentrate at varying water activities. [a]

[a] Error bars show standard deviation

Table 4.3. Visible growth of *P. niveus* during storage.

Processing Conditions	**Ambient storage**	**Refrigerated storage at 5°C**
450 MPa, pH 4.6	Not tested	No growth observed for 26 weeks
600 MPa, pH 4.6	Not tested	No growth observed for 26 weeks
600 MPa, pH 7.0	Visible mycelia at 1 week at 0.94 – 1.0 a_w	No growth observed for 26 weeks
600 MPa, pH 3.5	Visible mycelia at 2 weeks at 0.94 – 1.0 a_w	No growth observed for 26 weeks
600 MPa + sulfite, pH 4.6	4 weeks for 1.0 a_w, no visible growth for 0.94 a_w	Not tested
pH 4.6 control, 0.94 and 1.0 a_w	Visible mycelia at 1 week at 0.94 and 1.0 a_w	No growth observed for 26 weeks
pH 7.0 control, 0.94 and 1.0 a_w	Visible mycelia at 1 week at 0.94 and 1.0 a_w	No growth observed for 26 weeks
pH 3.5 control, 0.94 and 1.0 a_w	Visible mycelia at 1 week at 0.94 and 1.0 a_w	No growth observed for 26 weeks

***P. niveus* results.** *P. niveus* was the most pressure resistant species tested, with only a few pairwise significant differences in results between pH values occurring only at 0.94 and 1.0 a_w. Almost all conditions caused spore germination, with the only reduction of *P. niveus*

occurring at 0.94 a_w, 4.6 pH, 450 MPa for 1.5 min. Higher activations at higher pressures may have been due to the spore activation mechanism caused by HPP. It is believed that higher pressures increase the spore wall permeability, leading to the hydration of the spore core. This can trigger the germination of the spore (Reyns et al., 2003). The survival of *P. niveus* after HPP was also reported by Chapman and others (2007). They grew pressure resistant *B. fulva, B. nivea,* and *N. fischeri* ascospores for 3–15 weeks. Ascospores were inoculated into pH 4 or 6 citrate phosphate buffer or pH 5.0 mango puree. Samples were processed at 600 MPa for 10 min at ambient temperature. Older ascospores of all species had greater survival rates. Eleven-week-old (or older) *B. nivea* ascospores exhibited germination in both buffers. *B. fulva* ascospores that were 11 weeks or greater were unaffected by HPP. *N. fischeri* ascospores that were 9 weeks or older were only reduced by 1-log CFU/mL. This slight reduction of *N. fischeri* and activation of *B. nivea* were very similar to our results using 30-day old ascospores. This increased survival at 30 days versus 9-11 weeks may be due to better protection in apple juice concentrate than buffer. The ascospores inoculated into the mango puree and processed at 600 MPa had greater survival than in buffer. *B. nivea* ascospores that were 5-weeks old better survived processing than 3-week old ascospores in mango puree (Chapman et al., 2007).

P. niveus was capable of growth during ambient storage, but not during refrigerated storage. The addition of sulfites to the 0.94 a_w samples of *P. niveus* may have extended the ambient storage by several weeks. However, the addition of sulfites to the 1.0 a_w samples did not aid in storage extension. *P. niveus* was not capable of growth under refrigeration conditions, even when activation of ascospores was triggered by high pressure.

Future work. Overall, the species tested were highly resistant to HPP. However, the two most resistant species, *P. niveus* and *A. fischeri,* were incapable of growth during shelf life at

refrigeration. *A. pseudoglaucus* was capable of growth during refrigeration, but was more susceptible to high pressure. Previous results have shown that increasing the processing temperature or cycling pressure treatments repeatedly may increase reductions of spoilage fungi. However, more work should concentrate on the effect of pH, processing time and targeted shelf life. A smaller initial population reduction caused by cycling treatments may still result in enough injury to spores to extend refrigerated shelf life without needing to increase the treatment temperature. Alternatively, additional studies on processing aids that may be strain dependent could be conducted. This approach may be more successful at reducing the incidence of *B. nivea* spoilage since Butz and others (1996) found *B. nivea* is also resistant to pressure cycling (Butz et al., 1996). In contrast, da Rocha Ferreira and others (2009) subjected *B. nivea* to pressure cycling in juice. Processing below 80°C at 600 MPa for 15 min did not cause significant reductions of *B. nivea* in juice, while cycling caused a greater reduction than a single 600 MPa for 15 min treatment. Clearly, more research focused on the reduction of these pressure resistant organisms is needed.

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CHAPTER 5

EFFICACY OF HIGH PRESSURE PROCESSING, WITH VARYING PH AND WATER ACTIVITY, ON SURVIVAL OF *ALICYCLOBACILLUS* SPP. IN APPLE JUICE CONCENTRATE MODEL

Key words: *Alicyclobacillus*, High Pressure Processing, water activity, pH

ABSTRACT

Alicyclobacillus spp. is a common spoilage organism found in fruit juices, especially apple. The spores of *Alicyclobacillus* spp. have extreme heat resistance and has been known to survive typical juice pasteurization steps, causing later off flavors and odors in juices with long shelf lives (Steyn et al., 2011). High pressure processing (HPP), a non-thermal treatment, using pressures from 400-600 MP, typically from 1.5-3 min and has been shown to be promising in reducing levels of *Alicyclobacillus* spp. in juice. However, previous studies have focused on combining HPP with thermal treatments to achieve higher log reductions. They have also mostly used bench top units which may have different results than using those that are commercially available. The goal of this study was to determine the effect of water activity and pH on the efficacy of HPP and subsequent survival of *A. acidoterrestris* using a commercially available unit. In general, industrially significant log reductions of three strains of *A. acidoterrestris* were not seen after processing at 5°C, 600 MPa for 15 min at 3.5, 4.6 or 7.0 pH and 0.94, 0.96, 0.98 and 1.0 a_w. However, populations remained relatively stable for the first 12 - 19 weeks of ambient shelf and during a 26-week refrigerated shelf life after processing, even in high water activity samples. This shows potential for using HPP to process both juices and concentrates.

HIGHLIGHTS

- Using HPP at 5°C, 600 MPa for 15 min did not cause significant reductions of *A. acidoterrestris.*
- After HPP, *A. acidoterrestris* did not grow in diluted 0.94, 0.96, 0.98 and 1.0 a_w apple juice concentrate over at 5°C for 6 months.
- In most cases, *A. acidoterrestris* did not grow during ambient storage, after HPP at 600 MPa for 15 min until the 19 week mark.

Alicyclobacillus spp. is a spore forming, thermophilic and acidophilic spoilage bacterium commonly found apple and orange juice, most likely coming from soil. This genus was identified in 1982 when it was found to be causing spoilage in a German apple juice. Since then, 20 species have been recognized with *A. acidiphilis, A. acidoterrestris, A. fastidiosus,* and *A. pomorum* found specifically in juices (Steyn et al., 2011). Several studies have found between 6 and 100% of fruit juice samples tested were positive for *Alicyclobacillus* spp. (Chang, et al. 2004, Tianli et al. 2014, Sokolowska et al. 2013, Silva et al. 2012). The high occurrence of *Alicyclobacillus* spp. may be due to its occurrence in the soils of orchards and fruit groves, which is why drop fruits should not be used. However, *Alicyclobacillus* spp. has also been isolated from the surface of apples and citrus fruits and as a result, fruit processing facilities and wash water (Steyn et al., 2011).

Alicyclobacillus spp. produces guaiacol over the shelf life of juice which causes medicinal flavors and odors, as well as cloudiness (Alpas et al. 2003, Lee et al. 2006). Guaiacol causes taint in a wide range of food products; wine, dairy and confectionery products although it may also be advantageous in roasted or smoky flavor products. When *Alicyclobacillus* spp. concentrations reach 10^4 or 10^5 CFU/mL, guaiacol odor and flavor can be detected in juices (Chang et al. 2004, Tianli et al. 2014). The off flavor of guaiacol in juice can be recognized at lower concentrations than odor (Huang et al., 2015). Electronic noses can identify the presence of *A. acidoterrestris* with up to 100% accuracy (Hartyáni et. al. 2013). Juice with *Alicyclobacillus acidoterrestris* has been tested on guinea pigs without ill effects and is therefore not considered pathogenic to humans (Chang et al. 2004).

Current juice pasteurization methods (86-96°C, 2 min) do not inactivate *Alicyclobacillus* spp. spores (Steyn et al., 2011). Germination and growth of spores surviving pasteurization can

occur within days (Silva et al., 2012). Growth can occur at a wide range of temperatures and pH values: 20-70°C and 2.5-6.0 pH. Its thermal resistance may be due to the presence of densely packed, hydrophobic ω-cycloheptane fatty acids in the membrane which moderate membrane permeability (Uchida and Silva, 2017, Chang et al. 2004, Steyn et al., 2011). Use of high pressure processing (HPP), a non-thermal process, may be promising to overcome the hurdles that *Alicyclobacillus* spp. poses. HPP uses pressures between 400 MPa and 600 MPa to inactivate vegetative cells but does not cause sterilization. It works by damaging cell membrane and proteins but does not adversely affect nutrient content and color as much as thermal processing (Rendueles et al., 2011). HPP has a wide variety of applications from seafood de-shelling, to gelatinization and to meat tenderization (Uchida and Silva, 2017). Since heat is not used, sensorial properties are less likely to deteriorate during processing unlike thermal pasteurization (Silva et al., 2012, Uchida and Silva, 2017). For example, Hartyáni and others (2013) found that the color of HPP apple juice stored for 4 weeks remained very stable (Hartyáni et al., 2013). Pressure can be applied to foods after packaging, reducing the risk of post process contamination.

Bacterial spores, such as those formed by *Alicyclobacillus* spp. can be extremely pressure resistant – with some types able to withstand pressures greater than 1200 MPa, which is higher than the commercial processing pressure limit (Zhang and Mittal, 2008, Vercammen et al., 2012). Under harsh conditions, these bacterial cells form endospores which are dormant and protective until conditions are improved. Dormant spores can survive extreme pH, chemical, temperature and dry conditions, potentially for years (Casas et al., 2012). Generally, presence of nutrients can induce spore germination. When nutrients are present, the spore's core pH rises, hydrates, and swells, leading to enzymatic activity. Finally, this leads to spore outgrowth and the

loss of the spore coat. High pressure can also induce spore germination, potentially by nutrients produced by nearby lysed cells (Setlow, 2003). The medium composition can have a clear effect on the germination of spores. Low water activity may impede the hydrolysis step during spore germination leading to a retained pressure or heat resistance (Black et al., 2007).

Combining HPP with higher temperatures has been promising for reducing *Alicyclobacillus* spp. populations (Sokolowska et al. 2013). Combined treatment is believed to be synergistic by causing the spores to be activated out of dormancy (the most resistant phase) in the first step and then killed in the second after becoming more sensitive (Rendueles et al., 2011). Silva and others found 1-log reductions of *Alicyclobacillus acidoterrestris* occurred within 13 min at 45°C and 600 MPa in orange juice. However, Silva and others did not test continued survival of *A. acidoterrestris* during shelf life after processing (Silva et al., 2012). In general, bacterial spores are very resistant to HPP, however there are limited studies determining whether *Alicyclobacillus spp.* is capable of growth during shelf life after processing. Many of the HPP studies focusing on bacterial spores have also been limited to *Clostridium* spp. and *Bacillus* spp. (Zhang and Mittal, 2008). These studies have found that incubation temperature and medium composition can affect spore pressure resistance. Germination of *B. subtilis* can be induced by moderate (<150 MPa) to high pressures (>400 MPa) at low to moderate temperatures (<50C) (Reineke et al., 2013).

Sokolowska et al. (2013) and Lee et al. (2006) determined the effect of total soluble solids on *Alicyclobacillus* spp. survival in apple juice concentrate. Lee inoculated *A. acidoterrestris* into apple juice concentrate at 0.776 a_w (pH 3.90, 70°Brix), 0.946 a_w (pH 3.91, 35°Brix), 0.975 a_w (pH 3.91, 17.5°Brix) and apple juice 0.982 a_w (pH 3.54, 13.0°Brix) and processed samples at 207 - 621 MPa at 22 - 90°C (Lee et al., 2006). Sokolowska and others

(2013) inoculated *A. acidoterrestris* into apple juice ranging from 11.2 °Brix to 77.1 °Brix and processed at 200 MPa at 50°C for up to 45 min (Sokolowska et al., 2013). Both Lee and Sokolowska observed that *A. acidoterrestris* survival increased with increasing soluble solids (Sokolowska et al. 2013, Lee et al. 2006). Sokolowska and others also performed their experiments at a relatively low pressure (200 MPa) using a bench top unit so results may not translate clearly to commercial processing (Sokolowska et. al., 2013, Rendueles et al., 2011). pH can also play a large role in spore survival. Depending on the strain, spores have shown greater thermal resistance in higher pH mediums (Chang et al. 2004). This may show potential for HPP in high acid foods when targeting *Alicyclobacillus* spp. spoilage. The goal of this study was to determine the effect of water activity and pH on the efficacy of high pressure processing and subsequent survival of *A. acidoterrestris* using a commercially available unit.

MATERIALS AND METHODS

Media. Inoculum media was prepared by raising the water activity and pH of apple juice concentrate (Williamson, NY) to 0.94, 0.96, 0.98 or 1.00 a_w and pH 3.5, 4.6 or 7.0 by adding water and NaOH (Chem-Impex, Dale Wood, IL). 4.6 pH was chosen as a midpoint since it limits the growth of *Clostridium botulinum. C. botulinum* can survive HPP and produce a lethal toxin. pH 3.5 was chosen since *Alicyclobacillus* spp. can grow at very low pH (Black et al., 2007). We also chose 7.0 pH to see if there was an effect at neutral pH, since it is outside of *Alicyclobacillus's* optimum pH range of 3.5-5.5 (Vercammen et al., 2012). Models were heated to 95°C and held for 30 min to inactivate any vegetative organisms present in concentrate. Media was kept refrigerated until use. Initial physicochemical values of apple juice concentrate were: 0.72 a_w, pH 3.3 and 70.4 °Brix. Water activity measurements were made by an Aqua Lab Dew

Point Water Activity Meter 4TE (Decagon, Pullman, WA). pH was determined with an Oakton pH 5 Acorn Series pH meter (Oakton Instruments, Vernon Hills, IL). Total soluble solids were determined with an Abbe refractometer (Leica Inc., Buffalo, NY). All analyses were measured three separate times.

***Alicyclobacillus* spp. preparation.** Three *A. acidoterrestris* strains (35, 39, and VF) were obtained from Cornell AgriTech (Dr. R. Worobo's laboratory). All organisms were isolated from spoiled fruit juice concentrates with "35" and "VF" from apple juice concentrate and "39" from orange juice concentrate. "VF" is the same as the "VF" strain tested in Splitstoesser et al.'s *Growth Characteristics of Aciduric Sporeforming Bacilli Isolated from Fruit Juices* (1994) and identified as *A. acidoterrestris* (Silva and Gibbs, 2001). Single colonies were plated onto potato dextrose agar (BD, Sparks, MD) acidified with 10% tartaric acid to pH 3.5 and incubated at 45± 5°C for 30 days. Spores were harvested by flooding plates with 0.1% Tween 80 (Baker Analyzed, Philipsburg, NJ) and gently scraping with sterile spreader and used immediately.

Inoculation. Sixty grams of apple juice concentrate model were added to flexible 2 oz Polyethylene terephthalate (PET) bottles (Captiva Containers, FL). One mL of target organism was added to each bottle and samples were refrigerated until processing and used in less than 24 h. Immediately before processing, each sample was plated on acidified potato dextrose agar (pH 3.5) for initial enumeration and incubated for 7 days at 45± 5°C. Bottles were placed into PET bags and vacuum sealed, which were then double bagged and re-sealed; all to prevent the risk of leak inside the HPP unit. Untreated controls were also inoculated into 0.94 and 1.0 a_w apple juice concentrate at levels between 10^2 and 10^3 CFU/mL.

HPP. Based on results from previous HPP experiments, *Alicyclobacillus* spp. was expected to survive short processes. Samples were processed at 600 MPa for 15 min at 5°C

(Hiperbaric, 55 L, Miami, FL) in an attempt to see log reductions. The pressure limit achieved by the commercial Hiperbaric unit at the Cornell HPP Validation Center (Geneva, NY) is 600 MPa. Times longer than 15 minutes did not seem to contribute valuable information for commercial processors. Samples were plated on acidified potato dextrose agar immediately after processing.

Shelf life testing. pH 4.6 samples were stored at 5°C for 6 months and 3.5 and 7.0 pH samples were stored at ambient temperature (22± 2°C) for 6 months to determine storage limits. Ambient temperatures were chosen to represent a worst-case scenario since growth of *Alicyclobacillus* spp. occurs above 20°C (Huang et al., 2015). Ambient temperatures for 7.0 pH samples were to determine *A. acidoterrestris* survival only. All samples used for storage testing were the same as those used for log reduction calculations. All tests were performed in triplicate. Controls were stored under the same conditions as the treatment groups.

Statistical Analyses. Log reductions were calculated by taking the log of initial and subsequent populations. Growth over shelf life was determined by comparing after HPP levels to periodic plating counts. A model was created to compare estimated marginal means using R version 3.2.2. A Type III sums of square test was used to determine a three way interaction (R Core Team, Vienna, Austria). Values <1 CFU/mL (undetectable when 1 mL was plated) were recorded as "1.0" for conservative statistical purposes. Statistical significance was considered achieved when $p<0.5$.

RESULTS AND DISCUSSION

TABLE 5.1. Measured a_w, total soluble solids, and pH values of diluted apple juice concentrate model used to determine the survival of spoilage fungi[a]

Nominal a_w	**a_w**	**Total soluble solids content (°Brix)**	**Average 3.5 pH**	**Average 4.6 pH**	**Average 7.0 pH**
0.94	0.940±0.001	39.3±1.9	3.52±0.08	4.59±0.01	7.01±0.03
0.96	0.961±0.003	32.2±0.7			
0.98	0.979±0.002	22.0±0.5			
1.0	0.997±0.003	7.1±0.9			

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

Overall, HPP caused a limited effect in all three *A. acidoterrestris* strains tested (see Figures 5.1-5.3). Negative log reductions meant that spores were activated by the pressure and caused a higher count after processing than initial enumeration. There was a significant interaction between water activity, pH and strain ($P<0.01$). However, there weren't clear trends between tested strains. This may be due to the source of strains. Both *A. acidoterrestris* 35 and *A. acidoterrestris* VF, originally isolated from apple juice concentrate, achieved the least amount of spore germination at pH 4.6. *A. acidoterrestris* 39, originally isolated from orange juice concentrate, was activated the most at pH 4.6. The limited reduction by HPP was similar to the results observed by Lee et al. (2006) with no significant reduction of *A. acidoterrestris* occurring at 22°C in un-diluted apple juice concentrate, diluted apple juice concentrate, and apple juice at 207 – 621 MPa for up to 10 min. However, up to 2.5-log reductions were achieved in 0.975 and 0.982 a_w diluted apple juice concentrate or apple juice when the same test was performed at 45°C. Lee and others did not see reductions in 0.946 a_w apple juice concentrate without raising

the processing temperature to 71°C (Lee et al., 2006). However, Lee did not test the subsequent shelf life of processed *A. acidoterrestris* spores. There have been concerns over the survival of spores in apple juice concentrates that will later be diluted into juices and the ability of these dormant spores to germinate at higher water activities (Huang et al., 2015). Our results show that in most cases tested, the spores did not germinate and grow until week 19 at the variables tested which shows there is still potential for using HPP to reduce spoilage by *A. acidoterrestris*. There was an exception of growth of *A. acidoterrestris* 39 at pH 7.0, 1.0 a_w apple juice concentrate held at ambient temperatures for 12 weeks. The 1.0 a_w *A. acidoterrestris* controls of all three strains showed exponential growth when held at ambient temperatures within 2 weeks. No 0.94 a_w controls or controls held at refrigeration showed growth; highlighting the effect of HPP on the treatment groups. The following results and discussion are separated by tested strains.

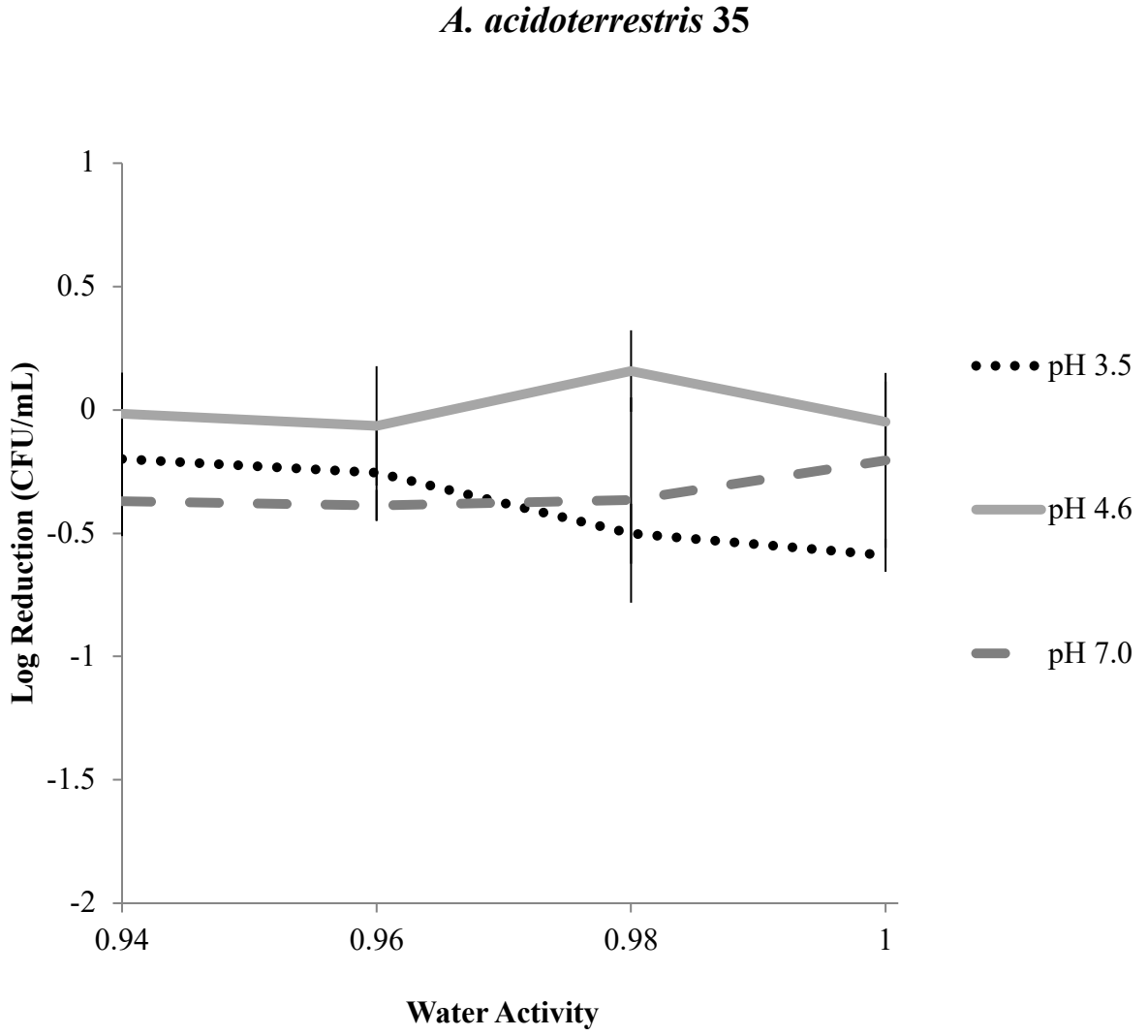

Figure 5.1. *A. acidoterrestris* 35 from apple juice concentrate: Processed at 600 MPa for 15 min in apple juice concentrate at 0.94, 0.96, 0.98 and 1.0 a_w and 3.5, 4.6 and 7.0 pH. [a]

[a] Error bars show standard deviation

***A. acidoterrestris* 35.** In general, processing *A. acidoterrestris* 35 in diluted juice concentrate at pH 4.6 was statistically better than processing at 3.5 or 7.0 pH juice concentrate, with the greatest difference observed at 0.98 a_w ($P<0.01$). Statistical differences between pH

values were in contrast to Vercammen and others' results (2012). Vercammen inoculated tomato sauce (pH 4.2 or 5.0), citric acid buffer (pH 4.0 or 5.0), and potassium phosphate buffer (pH 7.0) with *A. acidoterrestris* and *Bacillus coagulans.* Samples were processed at pressures ranging from 0.1 to 800 MPa for 10 min at temperatures ranging from 25°C to 60°C with a benchtop HPP unit. *B. coagulans* was reduced by 1-log after 10 min of 800 MPa at pH 4.0. Vercammen and others did not find clear pH trends in *A. acidoterrestris* survival in buffer during HPP treatments at 40°C. At these tested parameters, no significant *A. acidoterrestris* inactivation occurred but the greatest germination rates occurred at lower pressures. They also determined that pH 5.0 in buffer produced higher *A. acidoterrestris* germination rates than 4.0 buffer however, results were reversed with the tomato sauce conditions. pH 4.2 tomato sauce produced higher germination rates than pH 5.0 sauce. Finally, they found that *A. acidoterrestris* spore inactivation was limited at 600 MPa unless temperatures were at 60°C. *B. coagulans* was more pH sensitive, with declines in population in pH 4.0 buffer processed at 800 MPa. *B. coagulans* was activated in pH 7.0 buffer by pressures 100 – 800 MPa at 40°C but required high pressures for germination in lower pH buffers and sauce. Our results were the opposite to those observed in the tomato sauce conditions, with less of a germination effect in the pH 4.6 range (Vercammen et al., 2012).

During storage, populations of *A. acidoterrestris* 35 remained constant in pH 4.6 diluted apple juice concentrate at 5°C for 26 weeks and pH 7.0 apple juice concentrate at ambient temperatures for 19 weeks. At the 19 week point for *A. acidoterrestris* 35 in pH 7.0 apple juice concentrate, one sample at 0.94 a_w and two samples at 1.0 a_w, grew as much as 1-log. For 19 weeks at pH 3.5, populations of *A. acidoterrestris* 35 mostly declined except for a few anomalies with growth at higher water activities and appeared to be mold contamination. At the 19 week

mark in pH 7.0 apple juice concentrate, *A. acidoterrestris* 35 began to grow (increase of up to 1-log CFU/mL) across all four tested water activities. It appears that while HPP did not cause significant reductions, this strain required up to 19 weeks to recover and grow in the model. This was a relatively long recovery period since *A. acidoterrestris* has been shown to grow in media within a week and growth of 1.0 a_w controls held at ambient temperatures was observed within 2 weeks (Silva et al., 2012). There was not a continuing increase of HPP treated *A. acidoterrestris* after 19 weeks and at no point did the population level exceed the 4-log CFU/mL range. Sinigaglia and others (2003) tested the water activity, temperature and pH limits to *A. acidoterrestris* spore germination. Malt extract agar prepared with water activities 0.960 – 0.992, and with pH 3.5 – 5.5 were inoculated and incubated at 35 - 55°C. Plate growth was measured regularly. Results were validated by inoculating juices (orange, pineapple, pear, apple and apricot) as well as tomato sauce and incubating at the same temperatures. The juice conditions showed that *A. acidoterrestris* growth was optimal at 45°C. The most optimal water activity for growth was 0.992 a_w, but 0.960 a_w caused spore viability loss at 5.5 pH and 55°C. (Sinigaglia et al., 2003). In contrast, our results show growth at both 0.94 and 1.0 a_w. The long recovery period does show promising results for using HPP to control *A. acidoterrestris* 35 in apple juice and apple juice concentrate.

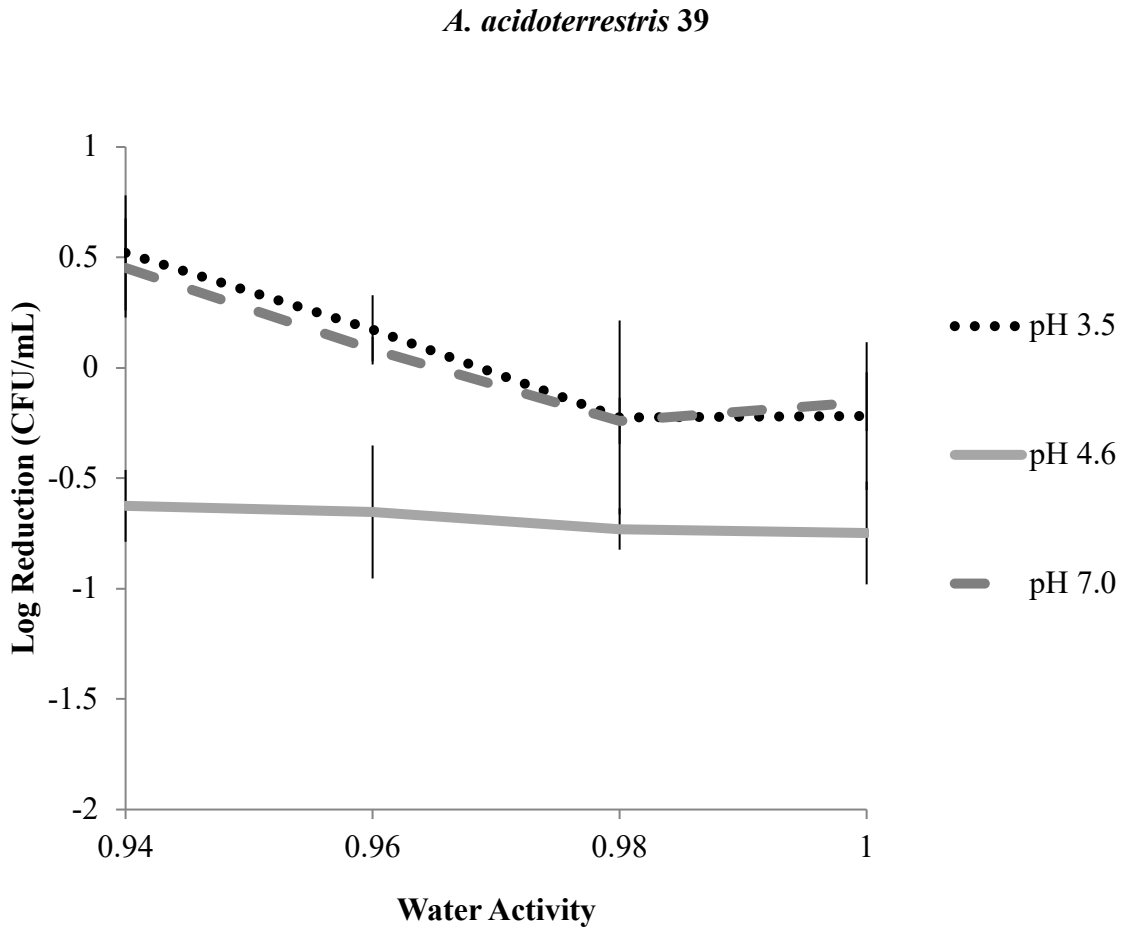

Figure 5.2. *A. acidoterrestris* 39 from orange juice concentrate: Processed at 600 MPa for 15 min in apple juice concentrate at 0.94, 0.96, 0.98 and 1.0 a_w and 3.5, 4.6 and 7.0 pH. [a]

[a] Error bars show standard deviation

***A. acidoterrestris* 39.** Results from processing *A. acidoterrestris* 39 at pH 3.5 and 7.0 were similar and significantly better than processing at pH 4.6, which caused the most activation of *A. acidoterrestris* 39 ($P<0.01$ when compared to pH 4.6 at all water activities tested). Processing *A. acidoterrestris* 39 at 0.94 a_w was the most effective, albeit with less than a 1-log reduction. Uchida and Silva (2017), Lee (2006), and Sokolowska (2013) saw the opposite effect with higher soluble solids creating a protective effect at lower water activities (Uchida and Silva

2017, Lee et al. 2006, Sokolowska 2013). We also observed differences with both strains "35" and "VF". Our differences in results between strains may be due to trouble enumerating *A. acidoterrestris* 39 at pH 3.5 and 7.0. Time 0 counts were regularly enumerated at <100 CFU/mL even after repeating the experiments up to 6 times (strains "35" and "49" had initial counts in the 3- or 4-log CFU/mL range). Since the spore suspension was opaque, the spores were most likely dormant and not growing on the media. It is highly unlikely that the spores were dying out after suspension in the juice models since *Alicyclobacillus* spp. can grow at a wide pH range. We chose not to heat shock the spores before HPP because spores are shown to be more susceptible after a previous processing step and did not want to change experimental methods after completing the pH 4.6 condition (Silva et al., 2006). However, failing to heat-shock may lead to an underestimation of viable spores (Steyn et al., 2011).

Almost all storage conditions tested showed consistent or decreased populations of *A. acidoterrestris* over 19 weeks. Samples processed at pH 4.6 did not show recovery during refrigerated shelf life. Samples processed in pH 3.5 apple juice concentrate showed a slight decline to non-detectable levels. However, *A. acidoterrestris* 39 grew in pH 7.0 at 1.0 a_w at week 12 or later at ambient temperatures. These results also confirmed that samples had been inoculated with viable spores, regardless of initial low plating results. Due to this growth, it is recommended to keep samples refrigerated if shelf life is expected to be greater than 3 months.

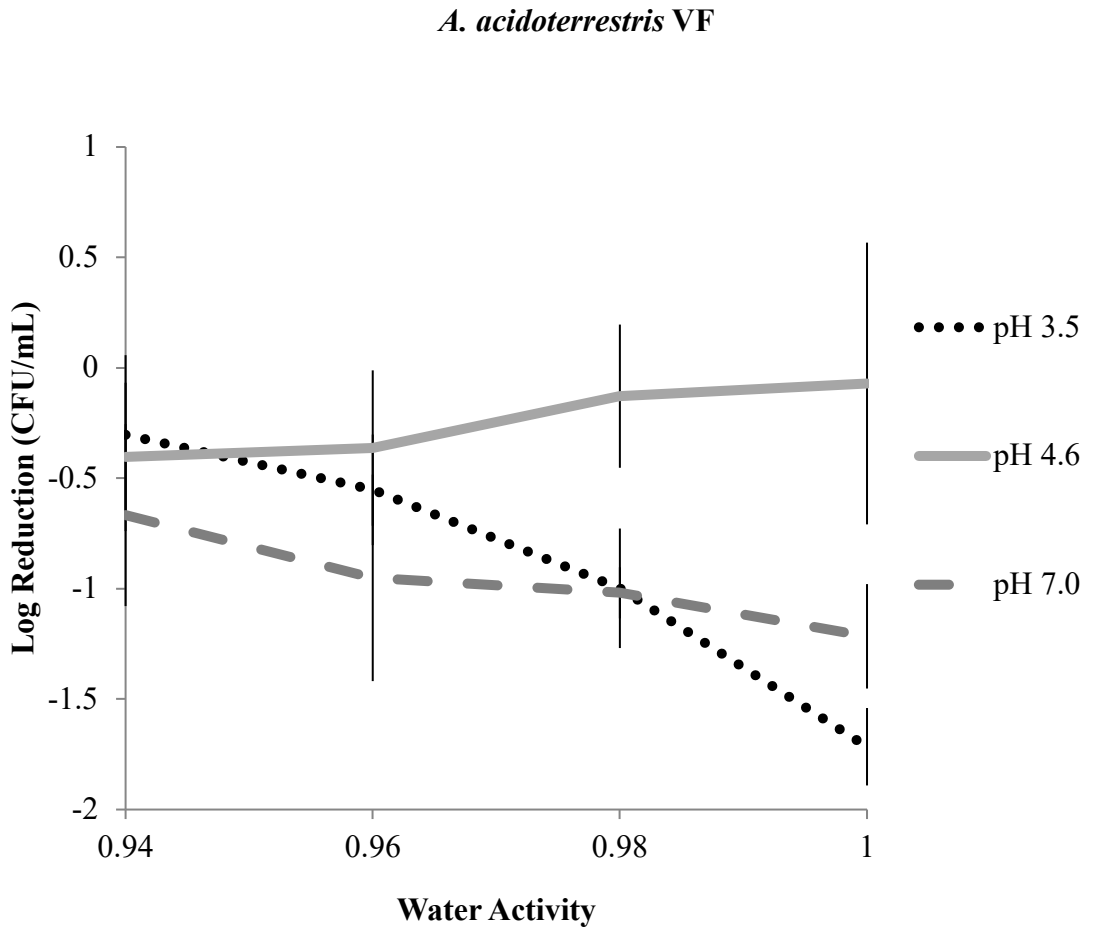

Figure 5.3. *A. acidoterrestris* VF from apple juice concentrate: Processed at 600 MPa for 15 min in apple juice concentrate at 0.94, 0.96, 0.98 and 1.0 a_w and 3.5, 4.6 and 7.0 pH. [a]

[a] Error bars show standard deviation

***A. acidoterrestris* VF.** No reductions caused by HPP were observed for *A. acidoterrestris* VF in any of the tested conditions. At 0.94 and 0.96 a_w, the least amount of activation occurred in samples at 3.5 or 4.6 pH rather than 7.0 pH. At 0.98 and 1.0 a_w, it is best to process *A. acidoterrestris* VF at 4.6 pH than 3.5 pH apple juice concentrate. Splittstoesser and others (1994) found that this strain was better isolated at 3.5 and 4.0 pH potato dextrose agar than neutral or low pH (2.5) which may explain the greater resistance at pH 3.5. It was also able to grow over 1-

log in apple juice at 11.4°Brix, 3.5 pH within 2 days at 43°C (Splittstoesser et al., 1994). *A. acidoterrestris* VF was incapable of growth during ambient shelf life in pH 7.0 juice model or 5°C shelf life at pH 4.6. *A. acidoterrestris* VF initially showed recovery after HPP in pH 3.5 but declined slightly over ambient storage. Hartyáni and others (2013) also processed *Alicyclobacillus* spp. in apple juice and orange juice at pressures up to 600 MPa for 10 min, 20°C. Their results showed decreasing populations of both *Alicyclobacillus* spp. vegetative cells and spores over a period of 4 weeks (Hartyáni et al., 2013).

Alpas, Alma and Bozoglu (2003) found that *A. acidoterrestris* vegetative cells in BAM broth could be reduced by approximately 2 logs when HPP at 450 MPa, at 35°C for 20 min. Moreover, they found that after processing for 20 min at 350 MPa, at 50°C, the greatest reduction was seen in apple juice (pH 3.01) when compared to orange juice (pH 2.44) and tomato juice (pH 4.16). We also observed that *A. acidoterrestris* VF had greater reductions in 4.6 pH apple juice concentrate when compared to higher and lower variables. Alpas and others also observed that *A. acidoterrestris* did not significantly increase in the HPP treated fruit juices over the shelf life held at 30°C for three weeks. While their treatment parameters were unusually long, this holds potential since *Alicyclobacillus* spp. has the ability to reach 10^5 CFU/mL in juices within several days at higher temperatures (Alpas et al. 2003, Chang et al. 2004).

Future Work. Previous studies have shown that raising the temperature to 60 or 71°C can increase the efficacy of HPP (Lee et al., 2006, Vercammen et al., 2012). Repeatedly subjecting juices to multiple rounds of HPP may also be effective. The first round of HPP would cause germination of the spores and the subsequent round or rounds would theoretically inactivate the vegetative cell (Zhang and Mittal, 2008). Additional work should also focus on

shelf life after processing. Injury to spores may be able to extend shelf life, even when reductions are minimal.

In conclusion, HPP at 600 MPa, 5°C for 15 min does not cause significant decreases in the population of *A. acidoterrestris* inoculated in diluted apple juice concentrate. However, depending on the strain, *A. acidoterrestris* may be incapable of growing in apple juice concentrate at varying water activities after HPP for 12 to 19 weeks. Only *A. acidoterrestris* 39 was capable of growth at ambient temperatures in pH 7.0, 1.0 a_w for 12 weeks. All other conditions tested showed stable or declining populations of *A. acidoterrestris* for 19 weeks after HPP. The lack of inactivation is in line with other authors' results, however, a long shelf life after processing has not been studied before. Other ways of reducing *Alicyclobacillus* populations should be implemented before juice processing occurs. Drop fruits should not be used, fruits should be properly washed before processing and good manufacturing practices should be used (Steyn et al., 2011).

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