Use of High Pressure Processing to Reduce Foodborne Pathogens in Coconut Water

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ABSTRACT

Juices have been implicated in numerous foodborne outbreaks over the last couple of decades. The FDA requires a 5-$\log_{10}$ reduction in juice products, which is most commonly achieved through pasteurization. However, pasteurization deteriorates some sensorial properties and nutritive value. Coconut water (CW; classified as a juice), is rapidly gaining popularity increasing over 300% since 2005. CW has not been implicated in a microbial outbreak, but is thermally processed to achieve the required 5-$\log_{10}$ CFU/ml reduction, which results in negative organoleptic properties. The objectives of this study are to determine whether *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes* can grow in CW, and evaluate the use of High Pressure Processing (HPP) to reduce populations of these bacteria in CW. The three pathogens were inoculated separately into CW and bacterial populations were enumerated over 24 hours. All three bacteria reached at least 8-$\log_{10}$ CFU/ml after 24 hours, which was not significantly different from the control (TSB). CW was then inoculated with each pathogen and processed using HPP (400, 5000, or 600 MPa) for 120 seconds. The D-glucose, D-fructose, sucrose, and phenol oxidase levels in the CW were assessed before and after treatments. Following processing, the pathogens were enumerated from the CW. All three pathogens were reduced by more than 6-$\log_{10}$ CFU/ml following treatments of 500 and 600 MPa, enough to achieve the mandatory 5-log CFu/m reduction. There were no significant changes in the D-glucose, D-fructose, sucrose, and phenol oxidase activity after any of the treatments.

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INTRODUCTION AND JUSTIFICATION

Numerous foodborne outbreaks have been associated with juices over the last couple decades. Between the years 1995 and 2001, there were 15 outbreaks, which led to the FDA forming regulations for juice processing (Vojdani *et al*., 2008). Juice processors now must follow the juice HACCP rule, which mandates that fresh juice must be processed using a method to achieve a 5-$\log_{10}$ CFU/ml reduction in the pertinent pathogen for each specific juice (FDA, 2001). The primary method the juice industry uses to achieve this reduction is through thermal processing, which can alter natural flavors and nutrients in the juice. Researchers are trying to develop alternative methods for processing juice that do not alter its sensory properties.

High pressure processing (HPP) is an alternative to thermal treatment of high water content foods (Jordan *et al*., 2001). Jordan *et al.* suggested that in order to have a realistic chance on the market, the alternative process should last less than 5 minutes to get a high enough level of production for the day. HPP could achieve this goal. A growing number of items on the food market are being treated with HPP. Some of the most common HPP treated foods are guacamole, deli meats, ready-to-eat meals, salsa, and fruit juices (Ramaswamy *et al*., No Year). One of most successful HPP treated food in the USA is guacamole (Patterson *et al*., 2005).

Coconut water (CW) is a fruit juice that is growing in popularity in the US. Companies like Vita Coco®, Zico®, and O.N.E.® have embraced CW as their number one product and sales have increased over 300% to 30 million dollars over recent years (Burkitt, 2009). Forbes estimates that the top selling CW company, Vita Coco®, had over 20 million dollars in CW sales in 2008, and had reported to have increased to almost

$100 million in 2011 (Esterl, 2012). Since CW is classified as a juice, it falls under the Juice HACCP regulation and must receive a process verified to achieve a 5-$\log_{10}$ CFU/ml reduction. Currently, industry achieves this reduction through acidification thermal processing, however, CW is very thermosensitive (Damar *et al.*, 2009) and its properties can be quickly changed by exposure to air. Thermal processing of CW has a fouling effect, similar to milk. This fouling effect produces greasy, white deposits that do not dissolve back into the CW (Narataruksa *et al*., 2010). Thermal processing also destroys many flavors and odors in the juice (Wolbang *et al.*, 2008). In order to get better taste, flavors, and higher nutrient retention, an alternative to thermal processing, such as HPP, could be used.

Although there have not been any foodborne outbreaks in the United States linked to CW, the nutritional composition of the juice is one that could theoretically support growth of microorganisms. CW is composed of fats, proteins, carbohydrates, and many minerals. Some of the minerals in CW are iron, magnesium, potassium and sodium (Seow and Gwee, 1997; Prades *et al.*, 2012). CW native pH ranges between 4.2-6.0 and has a water activity of greater than 0.95 (Damar *et al*., 2009; Gabriel *et al*., 2009; Campos *et al*., 1996). The objective of this research is to determine if CW supports the growth of common foodborne pathogens such as *E. coli* O157:H7, *Salmonella spp.*, and *Listeria monocytogenes*, and to evaluate if processing of CW using HPP will achieve a 5-$\log_{10}$ CFU/ml reduction of those pathogens.

LITERATURE REVIEW

JUICE OUTBREAKS

Juices have been implicated in several outbreaks over the past couple of decades. A reported 21 US outbreaks associated with juices occurred between 1995 and 2005 (Vojdani *et al*., 2008). In 1998, legislation was passed requiring that all unpasteurized juices be labeled as raw, in an FDA attempt to lower outbreaks in ciders (FDA, 1998). A continuation of juice-associated outbreaks resulted in legislation requiring all juice processors write and follow a Hazard Analysis Critical Control Point (HACCP) plan, requiring a 5-$\log_{10}$ CFU/ml reduction in the most resistant pathogen (FDA, 2001). For example, orange juice requires a process to reduce *Salmonella enterica*, and apple juice requires a process to reduce *Escherichia coli* O157:H7.

Most juices are acidic (pH<4.6), however low acid juices (pH > 4.6) are considered the most risky, with characteristics to support greater pathogen growth (Pilavtepe-Çelik, 2012). The FDA considers these juices as well as their original fruits to be hazardous because of their association with foodborne outbreaks. One low acid juice that is gaining popularity is coconut water (CW). Even juices with low acid have had aciduric microorganisms such as yeasts, molds, and some bacteria such as *Lactobacillus spp.* growing in them (Ray and Bhunia, 2008). Some strains of acid tolerant *Salmonella spp*. and *E. coli* O157:H7 have been found to be able to survive in low acid juices for over a month (Ray and Bhunia, 2008).

Many of these foodborne outbreaks have identified similar bacteria as the cause. Some of these bacteria include *Salmonella* spp, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. All three of these species are known to cause foodborne infections (Ray

and Bhunia, 2008). A foodborne infection can occur when the pathogen can survive in the food product, is consumed and then thrives in the human gut. When they multiply in the gastrointestinal tract they cause the host to become ill (Ray and Bhunia, 2008). Symptoms of foodborne infections typically happen at least a full day after consumption of the pathogen. Depending on the species of pathogen, infectious doses can range from as little as ten, all the way up to 10^5 cells, or more, to cause an infection.

COMMON FOODBORNE PATHOGENS ASSOCIATED WITH JUICE OUTBREAKS

SALMONELLA ENTERICA

Salmonella enterica is a Gram-negative, rod-shaped bacterium that is non-spore forming (Montville and Matthews, 2008). It cannot ferment lactose and is mesophilic. Most ssp. can grow anywhere from 5-46°C with its optimum temperature being around 37°C (Ray and Bhunia, 2008). *Salmonella* typically does not grow below a pH of 4.5 or A_w of 0.94. The cells are easily recovered after being frozen (Ray and Bhunia, 2008). *Salmonella* can be spread by fecal-oral route, water, and in foods (Doyle and Beuchat, 2007). The organism is typically found in many wild animals, insects, and can even live in humans without incident. The cells are often found in water, soil, and fecal matter (Ray and Bhunia, 2008).

Salmonella belongs to the family of *Enterobacteriaceae.* There are only two species, *S. enterica* and *S. bongori*. The *S. enterica* species is broken up into six subspecies, *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (Doyle and Beuchat, 2007). These subspecies are further classified into serovars. There are over 2,500 serovars of *Salmonella.* Two of these serovars, Typhimurium and Enteritidis are

the most commonly found serovars in Salmonellosis oubreaks (Ray and Bhunia, 2008). *Salmonella spp.* are responsible for causing Salmonellosis (Montville and Matthews, 2008). Salmonellosis is a sickness that causes enteric symptoms, which are local to where the bacteria live in the host (Ray and Bhunia, 2008). It causes diarrhea, abdominal cramping, nausea, chills, and fevers in as little as eight hours after consumption (Montville and Matthews, 2008). At least 10^5 cells must be eaten to cause an infection. The infection is typically isolated to your small and large intestines. It produces an enterotoxin that causes inflammation and tissue damage at the site (Ray and Bhunia, 2008). The infectious does can be much lower and even fatal in the eldery, immunocompromised, and the young (Doyle and Beuchat, 2007).

Salmonella is responsible for approximately 42,000 reported cases and over 1 million suspected cases of foodborne Salmonellosis in the US every year (Scallan *et al*., 2011). Foodborne infections can be lethal, causing an average of 378 deaths a year (Scallan *et al*., 2011). Traditional outbreaks are associated with chicken, pork, turkey, eggs, cantelope, tomatoes, nuts, and seafood (Ray and Bhunia, 2008). Recent outbreaks associated with *S. enterica* serovars have been linked to a variety of products including peanut butter, fresh produce, and juices (CDC, 2013). Many cross contaminations in the kitchen result in cases of Salmonelosis every year. Salmonellosis is recognized as the major cause of all foodborne illnesses by any pathogen in outbreaks and invididual cases since the 1950s in the United States and other developed countries (Ray and Bhunia, 2008).

ESCHERICHIA COLI O157:H7

Escherichia coli is a Gram-negative, rod-shaped bacterium. It is a facultative anaerobe and is motile by means of flagella. *E. coli* is non-spore forming (Doyle and Beuchat, 2007). It can grow well between 11-42°C with its optimum temperature being 30-42°C (Ray and Bhunia, 2008). *E. coli* typically doesn't grow under 10°C and most strains cannot grow below a pH of 4.5 (Ray and Bhunia, 2008). The bacterial cells can easily survive after being frozen. *E. coli* is typically found as a harmless member of the intestinal tract in humans and many other animals. It is typically used as a test bacterium to see whether or not a food or water supply is contaminated. Typically, it is spread through the fecal-oral route (Ray and Bhunia, 2008).

E. coli is a member of the *Enterobacteriaceae* family. It is broken up into six groups based on their adherence to cells and the type of toxins that they produce: Enterotoxigenic, Enteropathogenic, Enteroinvasive, Enterohemorrhagic, Enteroaggregative, and Diffuse-adhering (Ray and Bhunia, 2008). This study will be using a serogroup strain from the group Enterohemorrhagic *E. coli* (EHEC) called *Escherichia coli* O157:H7. *Escherichia coli* O157:H7 is a member of a group of *E. coli* species called Shiga toxin producing *Escherichia coli* (STEC). Shiga toxin, the toxin produced by *E. coli* O157:H7, adds to the virulence of the pathogen (Montville and Matthews, 2008). *E. coli* serogroups are broken up according to three of their major surface antigens present. These three are the O antigen (somatic), the H antigen (flagella), and the K antigen (capsule). There are over 100 O and K antigens and over 50 H antigens, which make an enormous number of possible combinations (Doyle and Beuchat, 2007).

E. coli O157:H7 is the most commonly found shiga toxin-producing bacteria in North America (Montville and Matthews, 2008). It takes as little as 100 cells to cause symptoms of the infection (Ray and Bhunia, 2008). The bacteria can cause severe diarrhea and cramping. The cells start their growth in the intestine and start to spread their toxin into the bloodstream and all around the body. This can cause damage all over the body including other organs and the brain. The most severe cases can lead to hemolytic uremic syndrome (HUS) and thrombocytopenic purpura (TTP) (Montville and Matthews, 2008; Ray and Bhunia, 2008). HUS is an extremely dangerous progression of the sickness, which produces bloody diarrhea and can lead to kidney damage or failure, and TTP can cause blood clotting in the brain (Ray and Bhunia, 2008). Symptoms typically occur 3-9 days after being consumed. All of these symptoms can be fatal, especially in the elderly, the young, and the immunocompromised (Doyle and Beuchat, 2007).

E. coli O157:H7 causes an estimated 63,000 foodborne infections and 20 deaths a year in the US (Scallan *et al*., 2011). Recent studies have also found there to be an increase in antibiotic resistant strains of EHEC (Doyle and Beuchat, 2007). Outbreaks associated with the bacterium have been linked to contamination of meat products, juices, ciders, water supplies, and even cheeses (Montville and Matthews, 2008). The largest outbreaks have occurred in spinach and in ground beef (Ray and Bhunia, 2008). Since the bacterium has been identified in the 1980's, the number of outbreaks of *E. coli* O157:H7 has drastically gone up in the United States (Doyle and Beuchat, 2007).

LISTERIA MONOCYTOGENES

Listeria monocytogenes is a Gram-positive, rod-shaped bacteria that is facultatively anaerobic (Montville and Matthews, 2008). It is non-spore forming, and is motile by means of flagella in a tumbling motion (Ray and Bhunia, 2008). The bacterium is psychrotrophic and has adapted to growing and thriving in colder, refridgerated temperatures that food is often stored in (Ray and Bhunia, 2008). Its growth range is 1-44°C with its optimum temperature at around 37°C. Due to its psychrotrophic nature, *L. monocytogenes* grows extremely well compared to other bacteria from 7-10°C (Ray and Bhunia, 2008). It is a glucose fermenter and is hemolytic. *L. monocytogenes* is resistant to high salt contents, low pH levels, and is acid tolerant, which helps survival in the gut (Ray and Bhunia, 2008). It can be found in animals, sewage, water, plants, and is found in the soil. Humans are often carriers without side effects (Doyle and Beuchat, 2007).

There are six species of *Listeria*: *monocytogenes, ivanovii, seeligeri, innocua, welshimeri, and grayi* (Doyle and Beuchat, 2007). *L. monocytogenes* is the only one that is pathogenic to humans and some animals. *L. ivanovii* is pathogenic to animals, especially cattle and sheep (Ray and Bhunia, 2008). The other species are nonpathogenic. *L. monocytogenes* is divided into 13 serogroups, all of which have been isolated in different regions around the world.

L. monocytogenes causes Listeriosis. This bacterial infection caused by *L. monocytogenes* was not recognized until the 1980s (Ray and Bhunia, 2008). Listeriosis is a sickness that causes non-enteric symptoms and spreads rapidly. It is typically isolated to the small intestine during initial growth, and then can spread to other organs via the bloodstream (Ray and Bhunia, 2008). Listeriosis causes severe fever, cramps, and

muscle aching (Montville and Matthews, 2008). The bacterium can cause septicemia in the non-pregnant and abort fetuses in pregnant women (Montville and Matthews, 2008). The infectious dose of *L. monocytogenes* is unknown, but is estimated to be fairly low (100 cells) (Ray and Bhunia, 2008).

L. monocytogenes causes almost 1,600 foodborne infections and is responsible for an estimated 255 deaths a year in the US (Scallan *et al*., 2011). The most at-risk groups are those who are immunocompromised, the elderly, pregnant women, and fetuses (Montville and Matthews, 2008; Doyle and Beuchat, 2007). Outbreaks associated with the bacterium have been linked to contamination of meats, ready-to-eat foods, cheeses, seafood, vegetables, juices, and fruit in recent years (Doyle and Beuchat, 2007; Montville and Matthews, 2008). Many of the outbreaks can be isolated to improperly sanitized storage areas and food processing equipment (Doyle and Beuchat, 2007). *L. monocytogenes* has a fatality rate of over 25%. Because of the high fatality rate, there is a zero tolerance policy for foods in the United States (Ray and Bhunia, 2008).

COCONUT WATER

FUNDAMENTAL COCONUT

The coconut tree is a perennial plant that flowers year round. Typically it flowers every month. The tree can survive over 70 years and produces coconuts up to 13 times a year (Jackson *et al*., 2004). A coconut tree (*Cocos nucifera*) produces fruit, which are hard, cantaloupe-sized drupes. Inside each coconut's outer shells is a white meaty layer, and inside that is the central cavity Gabriel, 2009). This central cavity is coated with a jelly-like substance. This jelly is translucent and very soft when the coconut is young

(Jackson *et al*., 2004). The cavity is filled up with a liquid endosperm, which is cytoplasmic in origin (Chowdhury *et al.*, 2005; Yong *et al*., 2009). This cavity can be filled with up to 600ml of liquid (Chowdhury *et al.*, 2005). Yong *et al.* stated that the liquid part of the coconut's endosperm is the coconut water (CW), and the coconut milk is the product gathered by squeezing or cutting up the pulp and adding water. Coconut milk is typically used as an ingredient in cooking or baking and not consumed as a drink, whereas coconut water is served as a refreshing beverage. Coconut milk is used for the production of coconut oil and cooking products because of its high levels of lipids and protein. CW is mainly composed of water (Yong *et al*., 2009).

COCONUT COMPOSITION

CW is considered a low acid (>4.6) and high water activity (~0.995) beverage (Walter *et al.*, 2009). CW contains fats, proteins, carbohydrates, and minerals (Narataruksa *et al*., 2010; Prades *et al.*, 2012). Some of these minerals include calcium, iron, magnesium, phosphorus, potassium, and sodium (Prades *et al.*, 2012). Other types of nutrients found in CW include zinc, copper, manganese, selenium, chlorine, sulphur, aluminum, and boron. Vitamin content includes C, B_1, B_2, B_3, B_5, B_6, B_7, and B_9 (Yong *et al*., 2009). The mineral and vitamin amounts of the CW can vary due to variety and geography (Seow and Gwee, 1997). The main carbohydrates found in CW are glucose, fructose, and sucrose (Jackson *et al*., 2004).

Many studies have been done to understand the nutritional content changes of the coconuts during their stages of maturity. Fat and protein content were found to increase month to month until about nine months, which is believed to be due to the development of the endosperm (Jackson *et al*., 2004). This is also when the soft jelly coating

disappears and begins to harden to become more of the white, meaty part of the coconut. As an immature green coconut, the sugar levels are low (1.5%) and increase as it matures (5%). Also, at immature stages, the CW is almost entirely reducing sugars such as glucose and fructose. When the nut matures, non-reducing sugars such as sucrose appeared and content increased until maturity (Jackson *et al*., 2004; Yong *et al*., 2009). The CW pH levels also seemed to become more basic as the coconut matured until about 10 months. The pH went from ~4.5 up to almost 6 (Jackson *et al*., 2004; Murasaki-Aliberti *et al.*, 2009; Damar *et al*., 2009; Gabriel *et al*., 2009; Campos *et al*., 1996).). The soluble solids and total solids increased as the nut matured as well. The volume of CW in the central cavity increases until about the ninth month, and then it starts to decrease (Jackson *et al*., 2004). Coconut water also contains enzymes that cause browning in both the water and the coconut meat, one of which is phenol oxidase (PPO) (Matsui *et al.*, 2008). Because of the changes over time and the presence of enzymes, the CW producer must select the perfect month to harvest the coconuts and generally allow for no longer than one week on the shelf at refrigeration temperatures if the CW has not been processed or treated at all (Jackson *et al*., 2004; Walter *et al.*, 2009).

COMMERCIAL COCONUT WATER

CW is mostly consumed and commercially produced in Indonesia, Philippines, Thailand, and Brazil (Damar *et al.*, 2009). Production of CW for human consumption typically includes acidification, sweetening, and processed thermally with Ultra High Temperature (UHT) (Damar *et al.*, 2009; Gabriel *et al., 2009*). The CW must be acidified down to a pH of at least 4.6 to prevent the possible growth of *Clostridium botulinum*, and then thermally processed to avoid other pathogenic growth (Damar *et al., 2009)*. The risk

of bacterial contamination is increased due to the coconuts typically being placed on the ground during harvest, as well as its high pH, and high water activity (Walter *et al.*, 2009). The typical coconut is harvested at about nine months of maturity (Jackson *et al*., 2004).

Traditionally, CW has been used for other sources than just a refreshing beverage. In remote locations where there are few hospitals, CW be used as a replacement for intravenous fluid bags. The saline content and pH allow the CW to safely replenish ions and liquid in the body (Campbell-Falck *et al.*, 2000). CW is currently used in laboratory settings to help encourage plant tissue culturing. CW makes a good growth-promoting component (Yong *et al*., 2009). CW is also being tested out as an antimicrobial ingredient. Wang and Ng (2005) have found an antifungal peptide within the coconut fibers. There have also been three antimicrobial peptides found in CW that appear to be inhibitory to Gram-positive and negative human pathogens (Mandal *et al.*, 2009).

CURRENT JUICE PROCESSING

REASONS FOR THERMAL PROCESSING

Most juice on the market is processed thermally. This process is thorough in destroying pathogens, spoilage microbes, and is every effective in inactivating enzymes like pectin methylesterase and phenol oxidase (Rawson *et al.*, 2011). These enzymes cause browning or reduce cloud formation in the drink, which is problematic for consumers (Farnworth *et al*., 2001). Processes like pasteurization, blanching, and retort sterilization are very common because they are recognized by the FDA as approved methods to treat the product, and prevent spoilage. Because thermal processing prevents

spoilage microorganisms from surviving, the juices treated thermally often have extended shelf lives, even at room temperature (Ferrari *et al.*, 2011). Blanching is often considered the most effective method to control enzymatic browning (Weemaes *et al*., 1998).

NEGATIVE CONSEQUENCES

Thermal treatments can negatively affect the sensorial and nutritional qualities of the juices (Wolbang *et al.*, 2008). Juices that are often harmed the most by the thermal treatment are low acid and thermosensitive juices. Some of these noted changes in specific drinks include a loss of lycopene in watermelon juice, odor and ascorbic acid loss in cantaloupe juice, loss of beta-carotene, color, carotenoids, ascorbic acid and free radical scavenging activity in carrot juice, and a rapid decrease in ascorbic acid content in tomato juice following thermal treatment (Ma *et al*., 2010; Dede *et al*., 2007; Patterson *et al.*, 2012).

Many of the bioactive compounds found in the juice are affected by thermal treatments (Ferrari *et al.*, 2011). In fruit beverages, one of the biggest losses comes from ascorbic acid, which is very thermosensitive (Keenan *et al*., 2012). This ascorbic acid is used as an antioxidant to prevent the enzymatic browning in fruits. If the ascorbic acid is gone, and not all of the enzymes have been inactivated, browning can occur. Keenan *et al*. also found that the color in thermally treated fruit smoothies appeared significantly darker than the color of fresh fruit smoothies. Temperature increases were also found to change the color of guacamole when given a treatment (Palou *et al.*, 2000). Many producers consider thermal processing to be unacceptable in anthocyanin containing juices. The high temperatures that are so effective in inactivating the PPO enzymes also cause anthocyanin degredation (Weemaes *et al*., 1998). Many customers and sensory

panels have indicated a clear preference for fresh beverage compared to thermally treated beverages (Walter *et al*., 2009).

THERMAL PROCESSING OF COCONUT WATER

Thermal processing of CW is shown to cause a fouling effect. This fouling is shown by a formation of white, greasy deposits (Narataruksa *et al*., 2010). The deposits are composed of proteins (albumins and globulins), fats, and minerals. These deposits can begin to form at 50°C in coconut milk and as the treatment temperature increases, so does the mass of the deposit formation (Narataruksa *et al*., 2010). Narataruksa *et al*. found that many of the deposits were responsible for the loss in many of the minerals in the remaining coconut milk. These detrimental sensory effects are why CW is often sold without being thermally treated in regions where coconuts are grown, such as Indonesia and the Philippines (Gabriel *et al*., 2009). Since thermal treatments destroy much of what consumers want in their juice, exploration of other non-thermal processing techniques is essential. One treatment that shows promise is high hydrostatic pressure processing (HPP) (Patterson *et al.*, 2005).

HIGH PRESSURE PROCESSING

THEORY

High Pressure Processing (HPP) is a non-thermal processing treatment that exposes the product to isostatic pressure so that all parts of the product are exposed to equal pressure all at the same time (Patterson, 2005). Rawson *et al.* (2005) defines the parameters of HPP used in the food production industry as using 100-800 MPa with the temperature being from (0 - 100)°C from a few seconds to 20 minutes. HPP can break the hydrophobic and electrostatic bonds of proteins, including enzymes, which can have

activity enhanced or destroyed following these conformational changes (Barba *et al*., 2011). The isostatic pressure does not affect hydrogen or covalent bonds (Patterson, 2005). Low-molecular mass compounds like vitamins, flavor compounds, and color compounds are not affected following treatment (Patterson, 2005; Oey *et al*., 2008). The DNA helix (hydrogen bonds) is typically unharmed during HPP, but replication and transcription are disrupted (Patterson, 2005). HPP can also cause chemical and enzymatic reactions, such as browning to accelerate, slow down, or stop (Butz *et al*., 2003; Oey *et al*., 2008).

HPP can destroy bacteria, yeasts, molds, and viruses (Michiels *et al.,* 2008). Destruction of these organisms helps prevent food spoilage and can reduce pathogens if they are present in the product (Bull *et al*., 2004). Gram-positive bacteria seem to be more resistant to pressure than Gram-negative; and cocci-shaped cells seem to be more resistant than rod-shaped (Patterson, 2005). The food product, which is normally susceptible to bacterial growth, may have chemical changes under different pressure situations that help prevent bacterial growth (Pilavtepe-Çelik, 2012). Bull *et al*. (2004) suggests that a HPP compounded with a slight thermal treatment would completely deactivate all microorganisms while providing a minimally changed product.

ADVANTAGES TO HIGH PRESSURE PROCESSING

High pressure processed food show many benefits. Some of these include retention of flavors, nutrients, colors, reduced need for additives to retain original flavors, reduced need of preservatives, and the process require less energy (Gomes and Ledward, 1996). Many researchers have found HPP juices and whole fruits to be of better quality than similar products that have been thermally treated (Oey *et al.*, 2008). Many of the

quality tests run on juices are judged by their ability to retain their original nutrition content, like ascorbic acid levels. HPP treated juices have been found to have better microbial stability, ascorbic acid retention, and antioxidant retention over a period of 30 days than thermal treatments (Dede *et al*., 2007). For example, HPP led to a better anti-radical scavenging capacity, color, and pH retention of tomato and carrot juices compared to thermally treated (Dede *et al*., 2007). Patterson *et al.* (2012) suggested that HPP may turn on anti-bacterial properties in juices that would otherwise be inactive.

There were no significant changes in the aromatics or brix of melon juice, ascorbic acid in blueberries, or carotenoids, anthocyanins, chlorophyll, and viscosity of most juices as a result of HPP (Barba *et al.*, 2011; Oey *et al.*, 2008; Ma *et al*., 2010; Wolbang *et al*., 2008; Liu *et al.*, 2012). Pigments have been shown to have a great resistance to pressure (Oey *et al.*, 2008) and blueberry juice actually had an increase in phenolic content as shown by a higher extraction of antioxidant components following HPP (Barba *et al.*, 2011). Sometimes there is actually an increase of polyphenol content after HPP treatments due to the pressure breaking up suspended particles in the food system and allowing for these particles to leak out and cause a raise in these antioxidants (Ferrari *et al.*, 2011).

Sensory panel studies have confirmed that HPP low acid juices retain their flavors and freshness better than thermally treated (Pilavtepe- Çelik, 2012). In a study using CW, panelists liked HPP samples of CW more than thermally treated samples of CW initially after treatments (Damar *et al*.; 2009). Thermally treated samples of CW had significantly higher off-flavor sensory scores. A significantly higher number of panelists said they would prefer to purchase the HPP treated CW over the heat-treated (Damar *et*

al., 2009). The study evaluated sensory characteristics and not a reduction in pathogens in the juice.

DISADVANTAGES TO HIGH PRESSURE PROCESSING

High pressure processing has many positive qualities when using it to process food items. However, it does have some shortfalls when using it compared to thermal processing. High pressure processing is very expensive. Equipment can cost upwards of a million dollars, whereas a UHT machine may cost only in the hundreds of thousands. These capital costs alone can dissuade any company from attempting to use this technology.

The HPP equipment is equipped for only batch style of processing rather than a continuous line. This is a problem when it comes to volume production. Companies are trying to put out as much safe product as they can, but if it is taking longer to process, load, and unload each batch, the company will not be able to keep up with a thermal processing unit. Many HPP runs can take upwards of five minutes (Murasaki-Aliberti *et al.*, 2009). To have a realistic chance on the market, a product processed by an alternative processing method should take no longer than five minutes in order to be successful according to Jordan *et al* (2001).

HPP products may also not have as good shelf stability over time compared to thermally treated products (Ferrari *et al*., 2011). One of the reasons for this is because HPP does not always have the ability to inactivate many of the enzymes that cause browning or other degredative effects (Keenan *et al.*, 2012). One of the most notorious enzymes that causes these effects is phenol oxidase (Martinez and Whitaker, 1995).

PHENOL OXIDASE

BROWNING ENZYME

Phenol oxidase (PPO), or 1,2 benzenediol oxidase is an enzyme found in most fruits. It is one of the major enzymes that causes the browning of fruits and vegetables after they are opened up or damaged and exposed to oxygen (Murasaki-Aliberti *et al.*, 2009). The fruits contain o-diphenols, which are oxidized by PPO in the presence of oxygen. This produces o-quinones. The o-quinones self-polymerize with each other. These polymers cause brown pigments to form. These brown pigments are referred to melanins, and are the browning that is seen when fruits are injured (Murasaki-Aliberti *et al.*, 2009; Correa Garcia and Buzaleh, 1994; Weemaes *et al*., 1998; Buckow *et al.*, 2009; Martinez and Whitaker, 1995). PPO is found in most higher plants, some insects, and all mammals (Martinez and Whitaker, 1995; Gomes and Ledward, 1996). PPO is rarely ever found in bacteria or fungi. Most researchers believe it has a protective function and is unlikely to affect metabolism rates within the organism (Martinez and Whitaker, 1995).

PPO is used as a biological indicator of thermal processing due to its resistance to heat (Matsui *et al.*, 2008). PPO from different sources may have different thermal resistance, optimum pH, molecular sizes, and conformations (Gomes and Ledward, 1996). PPO has an optimum pH of around 6.0 - 7.0 at which it is able to function at its highest rate (Correa Garcia and Buzaleh, 1994; Weemaes *et al*., 1998). Among its ability to degrade the food, the PPO also causes other problems in food systems. Since PPO uses polyphenols as its substrate, this leads to an overall drop in antioxidant and polyphenol content in the food system (Keenan *et al.*, 2012). PPO was found to cause severe changes in both color and turbidity in grapes and grape musts being used to create wines (Castellari *et al.*, 1997).

DEACTIVATION OF PHENOL OXIDASE

PPO can be deactivated and controlled by a few different methods employed by various food producers. Thermal processing is the most commonly used process and works well most of the time when thermal processing is allowed (Chutintrasri and Noomhorm, 2006). A decline in pH to levels around 4.0 or below helps to deactivate the PPO in many foods (Palou *et al*., 2000; Martinez and Whitaker, 1995). Lowering dissolved oxygen in food systems may help lower activity (Gomes and Ledward, 1996; Martinez and Whitaker, 1995). Increased salt levels help lower PPO activity (Buckow *et al.*, 2009). Sulphites had been traditionally used to inhibit PPO in wines and various foods. The FDA has banned sulfites from certain food products. Sulphites have been phased out of many products due to customer allergies and overall health issues with them (Castellari *et al.*, 1997; Martinez and Whitaker, 1995). The most effective way to diminish the activity of PPO is to use reducing compounds like ascorbate, bisulfites, and thiol compounds (Martinez and Whitaker, 1995). These compounds are not always approved in food systems, however, so they are not as useful as they seem. High pressure is another method that is used to control PPO (Keenan *et al.*, 2012). Most HPP can reduce PPO levels a little bit, but requires an extremely high amount of pressure for a long time to reduce activity enough (Gomes and Ledward, 1996). HPP has actually been shown to increase PPO activity in some instances (Palou *et al*., 2000). It is believed that HPP caused an increase in hydrophobic interactions and improved hydrogen bonding of certain enzymes, causing an increase in enzyme activity (Buckow *et al.*, 2009).

HIGH PRESSURE PROCESSING ON PHENOL OXIDASE

PPO enzymes can vary drastically in how they react to different treatments. Thermal resistance to the PPO in CW was much higher than the resistance in PPO from another fruit (Murasaki-Aliberti *et al.*, 2009). In a study by Matsui *et al.*, the PPO in CW was found to be more thermally resistant than PPO found in mushrooms or grapes, but less thermally resistant than PPO found in apples. PPO in mushrooms was found to completely inactivate after a treatment with HPP and this inactivation was irreversible. The same treatment with potato PPO allowed for some PPO activity to be retained (Gomes and Ledward, 1996). The HPP is believed to have inactivated these enzymes by inducing conformational changes in the protein structure, such as the breaking of many α-helix structures (Gomes and Ledward, 1996; Keenan *et al.*, 2012). Gomes and Ledward found differences in PPO activity with the same HPP treatments between mushrooms, apples, and potatoes. The protein structure alterations caused by the HPP were observed to be mostly reversible if treated with less than 200 MPa. Changes in protein structure were shown when eggs were treated with HPP. The HPP treated egg's ovalbumin did not have the same structure as its untreated form (Hayakawa *et al.*, 1992). The slow renaturation process produced conformational drift and the food system showed hysteresis (Gomes and Ledward, 1996). HPP may also help with lowering the amount of dissolved oxygen in the product (Keenan *et al.*, 2012). Another finding was that when the pressure treatments increased in pressure, the less active the PPO. Studies have shown HPP treatments with higher pressures or longer times showed a greater decrease in PPO activity (Keenan *et al.*, 2012; Castellari *et al.*, 1997; Chutintrasri and Noomhorm, 2006; Rawson *et al.*, 2011). Buckow *et al.* (2009) believes that in order to lower PPO

activity in foods, the PPO from different food sources must be studied rather than thinking PPO from every product all react the same to every treatment.

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Survival and Growth of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in Coconut Water

ABSTRACT

Juices have been implicated in numerous foodborne microbial outbreaks over the last couple decades. Coconut water (CW) is a juice that is gaining popularity in the United States, with some estimates indicating that sales of CW have increased over 300% in recent years. CW has yet to be implicated in a foodborne outbreak but parameters of the juice (nutrients, minerals, high pH, and high A_w) would likely aid in survival and growth of foodborne pathogens if contamination were to occur. This study examines the survival and growth of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in CW. CW and trypic soy broth (TSB, control) were inoculated with each pathogen separately to achieve an initial inoculum level of approximately 2-$\log_{10}$ CFU/ml, and incubated for 24 hours at 37°C. Inoculated CW and TSB were sampled for each pathogen at 0, 3, 4, 5, 6, 7, 8, 12, and 24 hours. All three pathogens reached a level of at least 8-$\log_{10}$ CFU/ml in CW after 24 hours. *L. monocytogenes* showed a lag in initial growth, but eventually grew to similar levels as *S.* Typhimurium and *E. coli*. Growth of the pathogens in CW was significantly less (about 1-$\log_{10}$ CFU/ml less), on average, than the growth in TSB. This study showed that select foodborne pathogens were able to grow in CW under these conditions.

INTRODUCTION

A reported 21 US outbreaks associated with juices occurred between 1995 and 2005 (Vojdani *et al*., 2008). Some of these outbreaks have been caused by *Escherichia coli* O157:H7, *Salmonella enterica spp.*, and *Listeria monocytogenes* (Vojdani *et al*., 2008). Fresh fruits and vegetables can become contaminated with foodborne pathogens during growth, harvest, and even in the processing plant. After being extracted from the fruits or vegetables, the juices are processed, which is most often done thermally (Rawson *et al.*, 2011). Juice processors must follow the FDA's Juice HACCP regulation and achieve a 5-$\log_{10}$ CFU/ml reduction in the pathogen pertinent to each specific juice (FDA, 2001).

Relatively new to the US markets, coconut water (CW) is gaining popularity. The industry has seen a growth rate of over 300% and sales of over 30 million dollars a year in recent years (Burkitt, 2009). CW is classified as a juice and its producers must follow the juice HACCP guidelines implemented by the FDA (FDA, 2001). CW is typically processed thermally, through Ultra-High Temperature - >100°C for up to 5 seconds (UHT) and then is often acidified with citric acid to achieve a pH below 4.6 and sweetened (Damar *et al.*, 2009; Gabriel *et al*., 2009). It is known to be a healthy drink with B and C vitamins, minerals, and is excellent for rehydrating after a taxing workout (Prades *et al.*, 2012).

CW has a pH of 4.2-6.0 and A_w of .995, (Walter *et al.*, 2009). Due to its nutrient composition, CW could make an excellent growth medium for foodborne pathogens. CW is currently used in laboratory settings to help encourage plant tissue culturing and is known as a good growth-promoting component for plant cells (Yong *et al*., 2009). The

CW while inside the coconut is thought to be sterile (Walter *et al.*, 2009). However, once the coconut is cracked and the juice is extracted, contamination could occur during processing. To our knowledge no known outbreaks have been linked to CW, and no studies have investigated and quantified the survival and/or growth of these foodborne pathogens in CW.

The objective of this study was to determine if CW would support the growth of three foodborne pathogens (*E. coli* O157:H7, *Salmonella spp*., and *Listeria monocytogenes*).

MATERIALS AND METHODS

INOCULUM PREPARATION

Escherichia coli O157:H7 Cider (strain isolated from cider outbreak and obtained from Dr. Larry Beuchat, University of Georgia); *Salmonella* Typhimurium (ATCC 14028); and *Listeria monocytogenes* Scott A (strain isolated from milk outbreak and obtained from Dr. Larry Beuchat, University of Georgia) were used in this study. Cultures were stored at -80°C in a 20% Glycerol solution prior to use. To activate, cultures were obtained from the -80°C freezer and transferred to 10mL Tryptic Soy Broth (TSB; Difco, Detroit, MI), or 10ml TSB with a 0.6% Yeast Extract (*L. monocytogenes* ; TSBYE; Difco, Detroit, MI) and incubated for 24h at 37°C. Following incubation, one loopful of each culture was transferred to fresh TSB and TSBYE (10 ml) and two successive 24 hr transfers were completed.

Activated cultures of each pathogen, *E. coli*, *S.* Typhimurium and *L. monocytogenes* were streaked onto selective media: Sorbitol MacConkey Agar (SMAC), Xylose lysine deoxycholate agar (XLD), and Modified Oxford Agar (MOX), respectively (all from Difco, Detroit, MI). Presumptively positive colonies from each medium were then confirmed using biochemical testing. Colonies from the MOX plates were then used to inoculate an API Listeria Test Kit (bioMérieux, Inc., Durham, NC). Colonies from SMAC and XLD plates were used to inoculate an API 20E Test Kit (bioMérieux, Inc., Durham, NC). Colonies from SMAC and XLD were also used for a RIM *E. coli* O157:H7 Latex Agglutination Kit (Remel, Lanexa, KS) and *Salmonella* Latex Test (Oxoid Ltd, Hants, UK), respectively. Following confirmation procedures, cultures were kept stored at 4°C prior to each experiment.

COCONUT WATER PREPARATION

Coconuts (Florida Coconuts, Davie, FL) were freshly picked and shipped to Virginia. They were stored at 4°C for no more than two weeks before use. The coconuts were sprayed with a 70% w/v aqueous ethanol solution until completely covered by the ethanol in a biological safety cabinet (Type A/B3, NuAire, Plymouth, MN) and allowed to dry for two minutes. A hole was bored into the center of the stem scar of the coconut with a sterile metal coconut opener (Florida Coconuts, Davie, FL). The CW was drained into a sterile beaker and poured into a Stericup® 0.45 μm 250ml filter unit (EMD Millipore, Darmstadt, Germany) and vacuum filtered. Portions (99ml) of CW were then pipetted into 12 sterilized glass 100 ml dilution bottles each and stored no longer than an hour until use.

GROWTH CURVE

Two 10 ml test tubes of each 24h culture were spun at 6000 rpm for 10 minutes. Supernatant was discarded and cells were re-suspended in 10 ml of sterile peptone water (SPW). Cells were washed twice more and finally re-suspended in SPW. The cultures were then serially diluted in SPW. One ml of each diluted culture was then added to three different flasks containing 99ml of sterile CW to achieve approximately 2-$\log_{10}$ CFU/ml concentration in each flask. Each flask was sampled and plated onto trypic soy agar at 0, 3, 4, 5, 6, 7, 8, 12, and 24 hours. This experiment was run in triplicate (n=9). As a positive control, the process was repeated using TSB/TSBYE as the growth medium, and measured at hours 0, 4, 8, 12, and 24 hrs. As a negative control, filter sterilized, uninoculated CW was enumerated on TSB to ensure that there was no background growth on TSB to interfere with the plate counts.

STATISTICAL ANALYSIS

The growth of *E. coli*, *S.* Typhimurium, and *L. monocytogenes* in both CW and TSB/TSBYE was performed in triplicate on three different days. Each hourly plate count on TSA/TSAYE was averaged. Error bars were created using $\alpha = 0.05$ and significant differences were analyzed with ANOVA and Tukey-Kramer HSD method on JMP Pro 10 (SAS Institute Inc, Cary, NC).

RESULTS

CONFRIMATION OF BACTERIAL CULTURES:

Growth on the Sorbitol MacConkey agar plates indicated a positive result (colorless colony growth) for the presence of *E. coli* O157:H7. Results for the RIM *E. coli* latex agglutination test resulted in positive agglutinations for the presence of the O157 antigen, and the H7 antigen. Growth on the XLD agar plates indicated a positive result (black colonies indicating H_2S production) for the presence of *Salmonella*. Results for the *Salmonella* Latex Tests showed a positive agglutination for the presence of *Salmonella*. Results for the API 20E test was positive for the presence of *Salmonella enterica* species. Growth on the Modified Oxford agar plates indicated a positive result (media became black with colony growth) for the presence of *Listeria monocytogenes*. Results for the API Listeria test showed the presences of *Listeria monocytogenes* as well.

GROWTH IN CW:

Throughout the experiment, the negative control plates (undiluted CW plated on TSB) had no apparent bacterial growth. The pH of the coconut water ranged from 5.5 to 6.1.

The starting populations of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* in CW were between 2.1 and 2.2-$\log_{10}$ CFU/ml, and the starting populations in TSB were 2.4, 2.3 and 2.1-$\log_{10}$ CFU/ml, respectively. After 12 hours, the populations of *E. coli* O157:H7 and S. Typhimurium in CW were significantly less than the population in the TSB ($p \leq 0.01$), and remained significantly less than the population in TSB after 24 hours ($p \leq 0.01$;Figures 1,2).

L. monocytogenes had a growth curve that showed a lower bacterial count at the early hours of growth in CW, but after 12 hours, the population of *L. monocytogenes* in CW was not significantly different than the population in TSB ($p= 0.065$). However, after the full 24 hours of growth, the *Listeria monocytogenes* population in CW was significantly less than the growth in TSB ($p= 0.045$) (Figure 3). After 12 hours of growth in CW, the population of *L.monocytogenes* was significantly less than the populations of *E. coli* and *S.* Typhimurium ($p \leq 0.01$), but was not significantly different after 24 hours of growth ($p=0.60$; Figures 1,2,3).

Significantly higher populations in *E. coli*, *S.* Typhimurium, and *L. monocytogenes* were all recovered in the nutrient broths of TSB or TSBYE after 24 hours. The highest population growth in both CW and TSB after 24 hours was reached by *E. coli* O157:H7.

DISCUSSION

Untreated coconut water was a good medium for *L. monocytogenes*, *S.* Typhimurium, and *E. coli* O157:H7 to grow in. Various constituents such as proteins, lipids, carbohydrates and a pH of over 5.5 make CW an acceptable medium for bacteria to grow (Narataruksa *et al.,* 2010). This was not unexpected because CW is used as an ingredient in growth media for plant tissue cultures (Yong *et al.*, 2009). The abundance of vitamins and minerals was expected help growth of bacteria as well (Narataruksa *et al.,* 2010; Yong *et al*., 2009). The final 24 hour populations of all three species in CW were significantly less than the same species in TSB, but still high (> 10^8 CFU/ml after 24 hours).

L. monocytogenes had a greater initial lag time in the first four hours of growth when compared to *E. coli* and *S.* Typhimurium in both media (TSBYE and CW). *L. monocytogenes* is a psychrotrophic bacterium (Ray and Bhunia, 2008). After 12 hours, the growth of the *L. monocytogenes* was still significantly less than the other two cultures' bacterial load. After a full 24 hours of growth, all three species had reached their stationary phase in growth.

The pH of the coconut water ranged from 5.5 to 6.1. These conditions are above the range all three bacteria are able to grow well in (> pH 4.5). The shell around the coconuts may be one reason that there have been no foodborne outbreaks linked to coconuts (Mandal *et al.*, 2009; Jackson *et al*., 2004). The sheer thickness of the shell makes it extremely unlikely for bacteria just to penetrate into the coconut. Recent discoveries into antifungal properties of the coconut fibers (Wang and Ng, 2005) and antimicrobial peptides in the coconut pulp against human pathogens (Mandal *et al.*, 2009)

may help explain why no outbreaks have occurred. The inside of the coconut is filled with vitamins, minerals, carbohydrates, and proteins, which make the coconut an excellent growth source for bacteria, but the thick outer shell may help prevent internalization.

Coconut water is a fast-growing commodity on the consumer market (Burkitt, 2009). This study shows CW has the ability to become a dangerous vehicle for outbreak if it is handled improperly or exposed to pathogens at the wrong time. Because of its vulnerability to host foodborne pathogens, the FDA's regulations for a mandatory 5-$\log_{10}$ CFU/ml reduction for juices are necessary for the production of CW.

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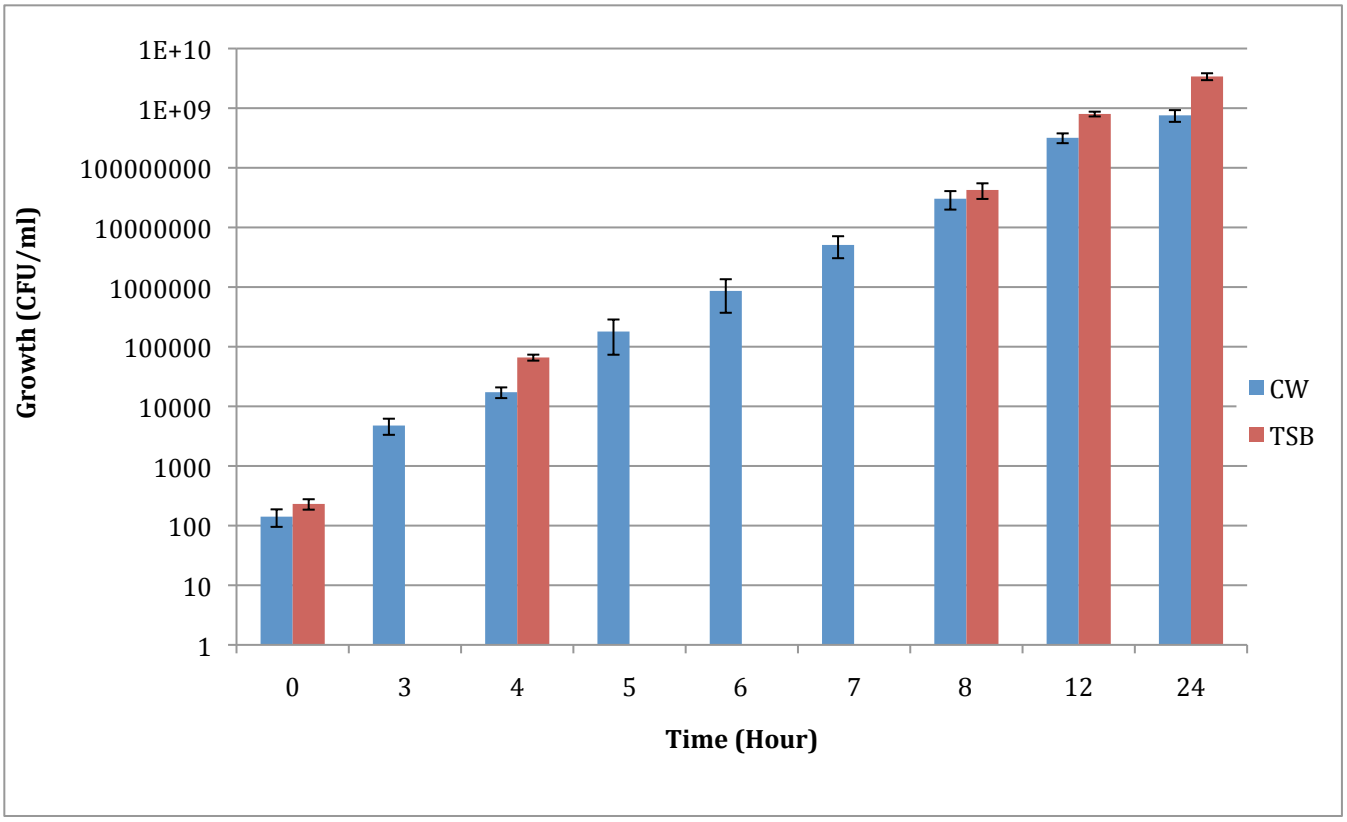

Figure 1. Growth *E. coli* O157:H7 in Coconut Water and Tryptic Soy Broth for 24 hours at 37°C.

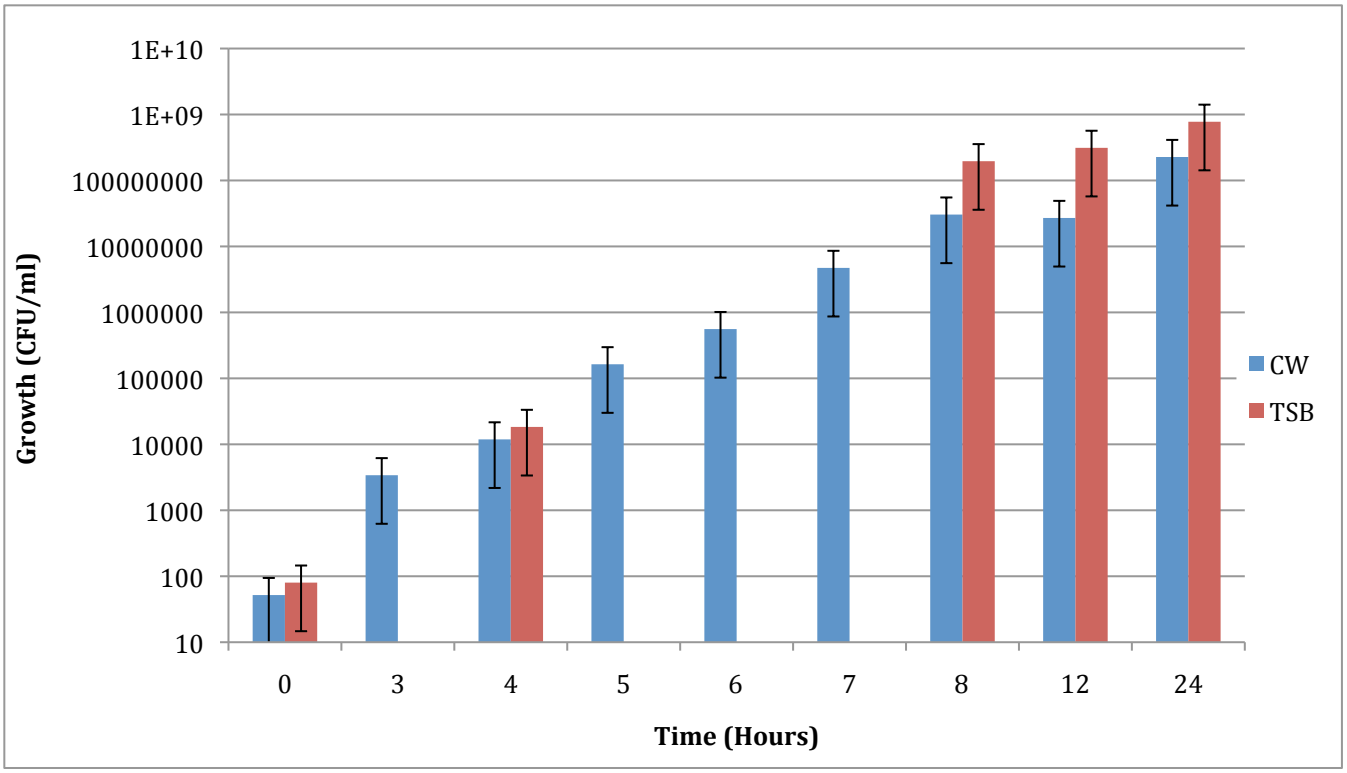

Figure 2. Growth of *Salmonella enterica* Typhimurium in Coconut Water and Tryptic Soy Broth for 24 hours at 37°C.

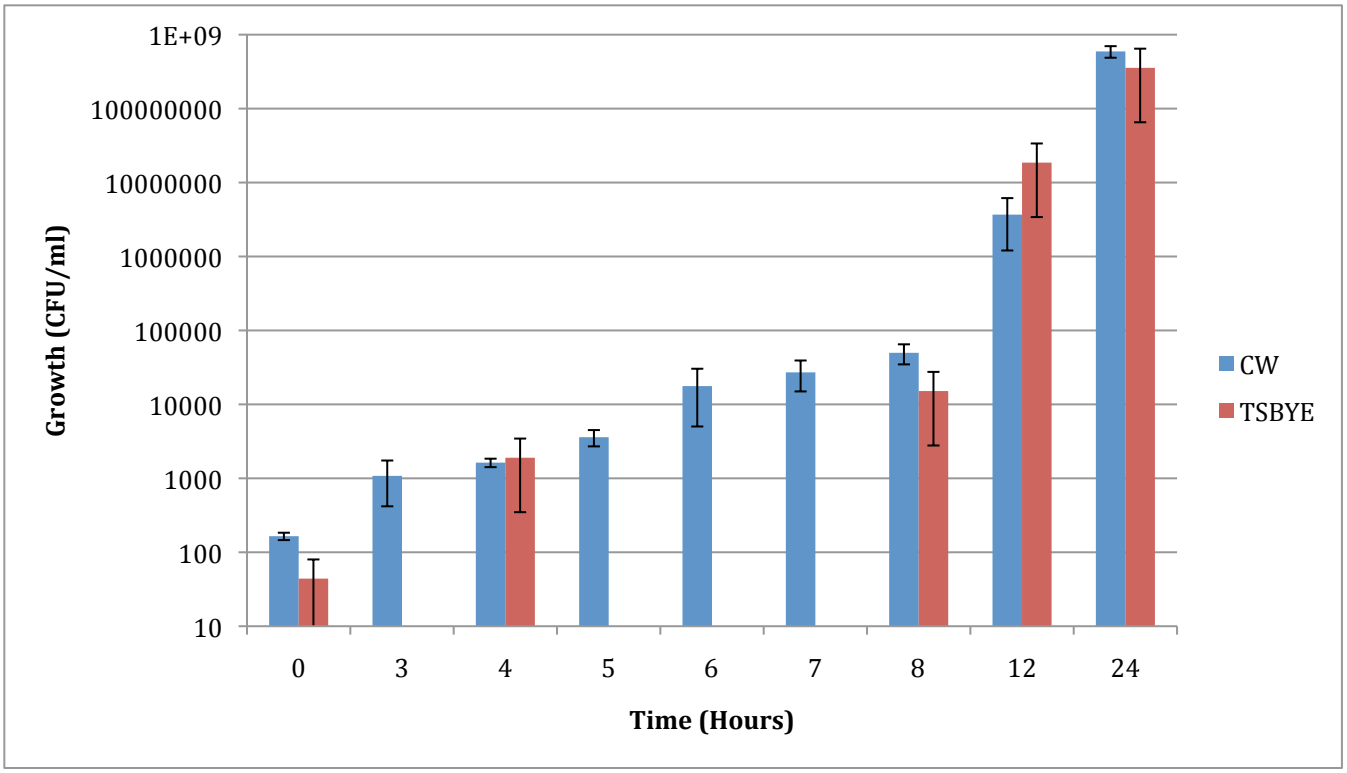

Figure 3. Growth curve of *Listeria monocytogenes* in Coconut Water and Tryptic Soy Broth with 0.6% Yeast Extract for 24 hours at 37°C.

High Hydrostatic Pressure Processing Reduces Foodborne Pathogens in Coconut Water

ABSTRACT

Juices have been implicated in numerous foodborne outbreaks over the last several decades. Coconut water (CW) is a juice that is gaining popularity in the United States, and while it has not been implicated in foodborne outbreaks, it contains nutrients, minerals, and has conditions (pH of 4.2-6 and high A_w) that could support bacterial growth. CW is classified as a juice and must receive a process verified to achieve a 5-$\log_{10}$ CFU/ml reduction in foodborne pathogens. Thermal processing is the most common method for achieving this 5-$\log_{10}$ CFU/ml reduction; however, this method changes flavors, nutrient contents and colors of CW. This study examines the ability of high pressure processing to reduce bacterial populations of *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* in CW after treatments of 400, 500, and 600 MPa for 120 seconds at 4°C. Untreated and treated samples were enumerated on Tryptic Soy Agar (*L. monocytogenes* on agar supplemented with 0.6% yeast extract). Uninoculated samples of CW were tested for D-glucose, D-fructose, sucrose, and phenol oxidase levels before and after treatments. All three pathogens were reduced over 6-$\log_{10}$ CFU/ml after treatments of 500 and 600 MPa. There were no significant changes in D-glucose, D-fructose, sucrose, and phenol oxidase after any treatments. The study confirmed that the treatments of 500 and 600 MPa for 120 seconds was enough to reach the FDA's mandatory 5-$\log_{10}$ CFU/ml reduction in these pathogens. However, the failure of HPP to reduce phenol oxidase could shorten shelf life of the product.

INTRODUCTION

Numerous outbreaks have been associated with juices over the last several decades. Between 1995 and 2005, 21 outbreaks in the U.S. were linked to juices (Vojdani *et al*., 2008). Some of these outbreaks were caused by *Escherichia coli* O157:H7, *Salmonella enterica spp.*, and *Listeria monocytogenes* (Vojdani *et al*., 2008). Juice processors must follow the Juice HACCP rule and fresh juice must be processed using a method to achieve a 5-$\log_{10}$ CFU/ml reduction in the pertinent pathogen for each specific juice (FDA, 2001). This 5-$\log_{10}$ reduction is typically achieved using thermal processing (FDA, 2001; Rawson *et al.*, 2011). Coconut water (CW), a new product gaining popularity, is classified as a juice and typically receives a thermal process prior to sale (Damar *et al.*, 2009).

However, many problems arise from the thermal processing. These problems include sensory degredation and the loss of nutrients (Wolbang *et al.*, 2008). The most well known problem with thermal processing is that is causes the loss of ascorbic acid and changes color in many products (Ma *et al*., 2010; Patterson *et al.*, 2012). When the consumer demands a product that tastes similar to the fresh product, an alternative method must be used if thermal processing is causing negative sensory changes. One of these alternative processing methods being used more and more in the market is High Pressure Processing (HPP) (Patterson, 2005). High pressure processing uses isostatic pressure to reduce or eliminate pathogens that could cause an outbreak in the product (Patterson, 2005). This method uses between 100-800 MPa of pressure, typically at temperatures below 40°C. The exposure of the food to the pressure can last anywhere from a few seconds up to 20 minutes (Rawson *et al*., 2011). This process should reduce

the pathogens without causing the detrimental effects on nutrient or sensorial content that thermal processing does (Oey *et al.*, 2008).

The objectives of this experiment was to determine whether HPP would significantly reduce *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* in CW and ultimately to determine whether the process successfully achieves the 5-$\log_{10}$ CFU/ml reduction as required by the FDA (FDA, 2001). Additionally, the effect of HPP on nutrient levels (D-glucose, D-fructose, sucrose, and phenol oxidase) in the CW was evaluated.

MATERIALS AND METHODS

INOCULUM PREPARATION

Escherichia coli O157:H7 Cider (strain isolated from cider outbreak and obtained from Dr. Larry Beuchat, University of Georgia) *Salmonella* Typhimurium (ATCC 14028), and *Listeria monocytogenes* Scott A (strain isolated from milk outbreak and obtained from Dr. Larry Beuchat, University of Georgia) were used in this experiment. Cultures were stored at -80°C in a 20% Glycerol solution until use. To activate, cultures were obtained from -80°C storage and transferred to 10mL Tryptic Soy Broth (TSB; Difco, Detroit, MI), or 10ml TSB with a 0.6% Yeast Extract (*L. monocytogenes* ; TSBYE; Difco, Detroit, MI) and incubated for 24h at 37°C. Following incubation, one loopful of each culture was transferred to new 10ml TSB and TSBYE and successive transfers were done twice more.

Activated cultures of each pathogen, *E. coli*, *S.* Typhimurium and *L. monocytogenes* were streaked onto selective media: Sorbitol MacConkey Agar (SMAC), Xylose lysine deoxycholate agar XLD Agar, and Modified Oxford Agar (MOX), respectively (all from Difco, Detroit, MI). Presumptively positive colonies from each medium were then confirmed using biochemical testing. Colonies from the MOX plates were then used for an API Listeria Test Kit (bioMérieux, Inc., Durham, NC). Colonies from SMAC and XLD plates were used for an API 20E Test Kit (bioMérieux, Inc., Durham, NC). Colonies from SMAC and XLD were also used for a RIM *E. coli* O157:H7 Latex Agglutination Kit (Remel, Lanexa, KS) and Salmonella Latex Test (Oxoid Ltd, Hants, UK), respectively. Following confirmation procedures, cultures were kept stored at 4°C prior to each experiment.

COCONUT WATER PREPARATION

Coconuts (Florida Coconuts, Davie, FL) were freshly picked and shipped to Virginia. They were stored at 4°C for no more than two weeks before use. The coconuts were sprayed with a 70% w/v aqueous ethanol solution until completely covered by the ethanol in a biological safety cabinet (Type A/B3, NuAire, Plymouth, MN) and allowed to dry for two minutes. A hole was bored into the center of the stem scar of the coconut with a sterile metal coconut opener (Florida Coconuts, Davie, FL). The CW was drained into a sterile beaker and poured into a Stericup® 0.45 μm 250ml filter unit (EMD Millipore, Darmstadt, Germany) and vacuum filtered. Portions (99ml) of CW were then pipetted into 12 sterilized glass 100 ml dilution bottles each and stored no longer than an hour until use.

HIGH PRESSURE MICROBRIAL PREPARATION

Activated, 24h cultures of each pathogen were centrifuged at 6000 rpm for 10 minutes. The supernatant was discarded and cells were resuspended in 10ml of sterile peptone water (SPW). Cells were washed twice more and finally resuspended in SPW. One ml of each culture was added to three different sterile flasks, to result in each flask containing 99 ml of sterile CW. At the start of each replication, three flasks contained approximately 7-$\log_{10}$ CFU/ml of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* inoculum respectively (9 flasks); and three were uninoculated (total = 12 flasks). From each flask, 20 ml was pipetted into three separate 5”X7” Prime Source® 4-Mil Vacuum Pouches (Prime Source International LLC, Westerville, OH) (n=9). Three extra bags were filled (one for each bacteria) from one of their respective flasks. All 39 bags were vacuum sealed with a 70-mbar vacuum (Multivac, model A300/16, Kansas

City, MO), and then double bagged with another 5"X7" Prime Source® 4-Mil Vacuum Pouches. Then double-bagged samples were placed in a third, 8"x12" vacuum pouch containing ~2ml of quaternary ammonium compound.

HIGH PRESSURE TREATMENT

The three bags from each flask/bacterium combination (27 bags) were treated at 400, 500 or 600 MPa for 120 seconds in a 35L pressure chamber (Quintus QFP 35L-600, Flow International, Kent, WA). Three of the nine bags for each bacterium were treated at 400 MPa, three at 500 MPa, and three at 600 MPa. Uninoculated bags were plated without being treated at any press. The three bags inoculated, but not treated, were opened and plated for original counts to serve as a positive control, and the uninoculated bags were plated and counted to serve as a negative control. All samples were kept on ice before and after treatment for no longer than one hour. This process was run in triplicate, on separate days, with separate coconuts (n=9).

Microbiological analysis:

All treated and untreated bags were opened and serially diluted in SPW. Some of the bags were plated without dilution by pipetting 0.25ml on four plates to get a 10^0 dilution. Each bag was plated in duplicate on TSA/TSAYE and plates, incubated in 37°C for 24 hours and then counted. Plates within the range of 25-250 colonies per plate were counted.

PHENOL OXIDASE TESTING

To assess CW enzymes, bags were filled with uninoculated CW similar to the filling process used for microbiological experiments and triple bagged. Bags were treated with either 400, 500, or 600 MPa for 120 seconds. The bags were opened and

0.6ml from each bag was placed into three wells each on a 96 well plate. 0.4ml of a 0.03M catechol solution (Sigma-Aldrich, St. Louis, MO) was added to the wells and the absorbance was read on an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT) every minute for eight minutes at 480nm. This process was done in triplicate.

CARBOHYDRATE TESTING

Bags were filled with uninoculated CW similar to the filling process used for microbiological experiments. The bags were filled and triple bagged in the same process. Bags were treated with either 400, 500, or 600 MPa for 120 seconds. All samples were then tested in the sucrose/D-glucose/D-fructose test kit (R-biopharm AG, Darmstadt, Germany). Samples, in triplicate, from each of the bags were put into one well each. First, .20 ml of solution 1 was added to each well and incubated at 37°C for 5 minutes. Then, 1 ml of solution 2 was added to each well and allowed to sit for three minutes at room temperature. Readings were taken at this time. Next 0.020 ml of solution 3 was added to each well and allowed to sit for 10 minutes at room temperature. The second readings were taken at this time. Finally 0.020 ml of solution 4 was added to each well and allowed to sit for 10 minutes. The third readings were taken at this time. Readings were taken with the Epoch Microplate Spectrophotometer at 340 nm.

STATISTICAL ANALYSIS

All log reductions from the HPP treatments were recorded and plotted on Microsoft Office Excel 2008 (Microsoft, Redmond, WA). Significant differences were analyzed with ANOVAs and means were separated using Tukey-Kramer HSD method on JMP Pro 10 (SAS Institute Inc, Cary, NC). All phenol oxidase test results and

carbohydrate test kit results were analyzed with ANOVA and means were separated using Tukey-Kramer HSD method on JMP Pro 10.

RESULTS

All plates from uninoculated bags had no growth. The plates from inoculated but untreated plates resulted in counts of $1.84*10^7$, $1.29*10^7$, and $1.79*10^7$ CFU/ml for *E. coli, S.* Typhimurium and *L. monocytogenes*, respectively. The results of the high pressure treatments revealed that there were significant differences in log reduction of *L. monocytogenes* ($p= 0.006$), *S.* Typhimurium ($p= 0.004$), and *E. coli* O157:H7 ($p= 0.01$) between 400 MPa and 500 MPa for 120s (Figure 1).

The *L. monocytogenes* treated with 400 MPa had the lowest reduction in bacterial population (3.2-$\log_{10}$ CFU/ml). There was a significantly greater reduction in all pathogens when processed at pressures 500 and 600 MPa compared to 400 MPa ($p\leq 0.001$; Figure 1). All pathogens tested had at least a 6.1-$\log_{10}$ CFU/ml reduction in the 500 and 600 MPa for 120-second treatments. There were no significant differences ($\alpha = 0.05$) between *E. coli*, *S.* Typhimurium, and *L. monocytogenes*' log reduction value with 500 MPa and 600 MPa treatments ($p= 0.42$; $p= 0.62$; $p=0.44$; Figure 1).

D-Glucose levels in untreated, 400 MPa, 500 MPa, and 600 MPa for 120s were not significantly different ($P=0.49$) (Figure 2). D-Fructose levels in untreated, 400 MPa, 500 MPa, and 600 MPa for 120s were not significantly different ($P=0.051$) (Figure 3). Sucrose levels in untreated, 400 MPa, 500 MPa, and 600 MPa for 120s were not significantly different ($p=0.32$) (Figure 4). None of the major sugars found in CW had significant differences between any of the pressure treatments compared to the untreated CW.

Phenol oxidase levels untreated, 400 MPa, 500 MPa, and 600 MPa for 120s were not significantly different ($p=0.38$) (Figure 5).

DISCUSSION

The 400 MPa treatments had the lowest log reduction, and the 500 MPa and the 600 MPa treatments had roughly the same log reduction for all of the bacteria. Different pressures can have different effects on the same species of bacteria. This difference in reduction between pressures has been found in other studies (Keenan *et al.*, 2012; Rawson *et al.*, 2011). The higher log reductions, with the higher pressures was seen in experiments by Jordan *et al*. (2001) as well. Most of the 500 MPa and 600 MPa treatments made the bacterial populations starting at a level of 7-$\log_{10}$ CFU/ml fall below the detectable limit. No visible changes were seen with the color of the CW following any of the treatments.

The FDA juice guidelines state that a 5-$\log_{10}$ CFU/ml reduction must be achieved for a process to be approved (FDA, 2001). This means that none of the treatments at 400 MPa for 120s would be approved for treatment of CW. Both 500 MPa and 600 MPa for 120s achieved at least 6-$\log_{10}$ CFU/ml reductions for all bacterial species tested. These processes would pass under the FDA juice production guidelines. Not all of the pathogens had the same reductions. This is because bacteria have different levels of resistance to pressure (Doyle and Beuchat, 2007). This experiment suggests that this strain of *Listeria* is more resistant to high pressure than the serovars of *S.* Typhimuruim and *E. coli* O157:H7 used in this experiment because it saw the lowest reduction in bacterial load. This follows the trend that Gram-positive bacteria tend to be more piezotolerant than Gram-negative bacteria (Michiels *et al*., 2008).

D-glucose, D-fructose, and sucrose levels found in the original CW did not change after any of the pressure treatments. Since HPP is not known to affect covalent

bonds, like glycosidic bonds (Patterson, 2005), it does not affect carbohydrate structure or function. If the structure or functions were uninterrupted, the levels of carbohydrates were not expected to differ. This was consistent in these experiments.

Phenol oxidase (PPO) levels in the CW did not significantly change after any of the treatments with the high pressure. Normally, this would be an undesired effect because the PPO enzyme causes browning within the coconut, as well as many other fruits (Weemaes *et al*., 1998). Most juice processing is done to alter or destroy these enzymes and prevent them from causing browning within the juice (Murasaki-Aliberti *et al.*, 2009). The untreated CW did not contain much of this enzyme to begin with. This could be because as the coconut matures, there is a higher level of total solids (Jackson *et al*., 2004). These total solids include ions and proteins. Since PPO is an enzyme, this could be when the PPO levels rise significantly. Since the coconuts used were not turbid, the total solids could have still been extremely low. The coconuts were less than nine months old, which is when turbidity is still supposed to be low (Jackson *et al*., 2004).

Overall, HPP is an appropriate method for achieving the mandatory 5-$\log_{10}$ reduction required by the FDA. Since different juices require different pressures to achieve this 5-$\log_{10}$ CFU/ml reduction, research is needed for each juice and its changes in formula. When deciding between two pressures to use, the higher pressure would always be recommended. No further damage of flavor, tastes, or colors happens at a higher pressure (Keenan *et al.*, 2012), but there seems to be a higher reduction in both pathogens and activity of enzymes (Gomes and Ledward, 1996). Another reduction method, (such as acidification or a minimal thermal treatment) along with HPP, is recommended if producer is trying to achieve an extended shelf life of CW.

FUTURE DIRECTIONS

For future research, there needs to be more testing in between the large gaps of pressures. There needs to be a test of 425, 450, and 475 MPa when trying to achieve the desired log reduction. This will save time and money if the machine can do the same log reductions and not have to get all the way up to 500 MPa and just use 450 MPa. More time selections need to be tested. This study focused on changing the pressures. Could a producer use 600 MPa for only 60s and still get a 5-$\log_{10}$ CFU/ml reduction? There needs to be more studies about these varying pressures and times along with some added antimicrobials, slight heat, or changes in pH. More tests need to be done with some other families of bacteria. There could be tests done on *Staphylococcus* or *Camphylobacter* species. More juices need to be tested as well to see if these log reductions are seen with just CW or if they generally work for most juices. Testing should also be considered for viruses like Hepatitis A. There is a lot of research to be done in HPP, and benefits could be had for both the producers and the consumers when it comes to new HPP products coming out on the market.

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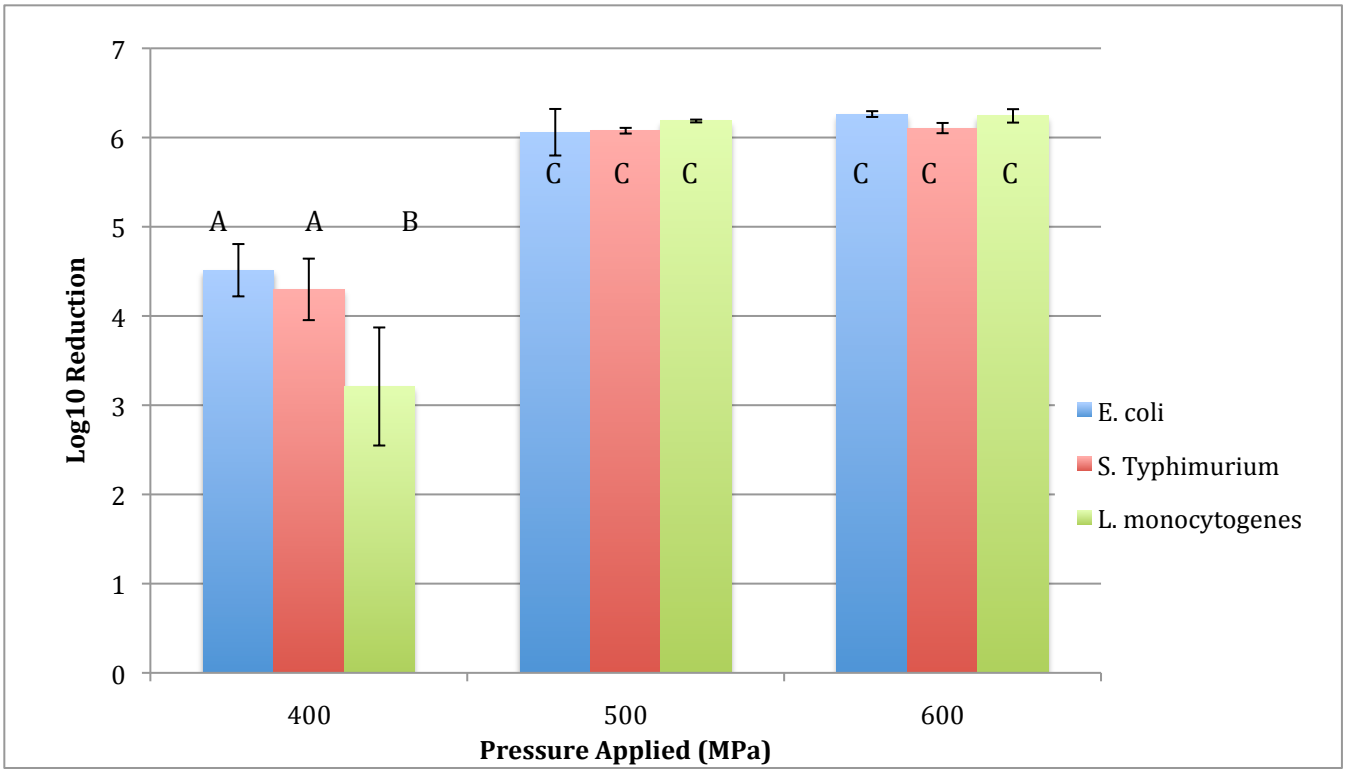

Figure 1. Average $\log_{10}$ reduction of *E. coli* O157:H7 Cider, *Salmonella* Typhimurium, and *Listeria monocytogenes* in Coconut Water at 400, 500, and 600 MPa for 120 seconds at starting temperature of 4°C.

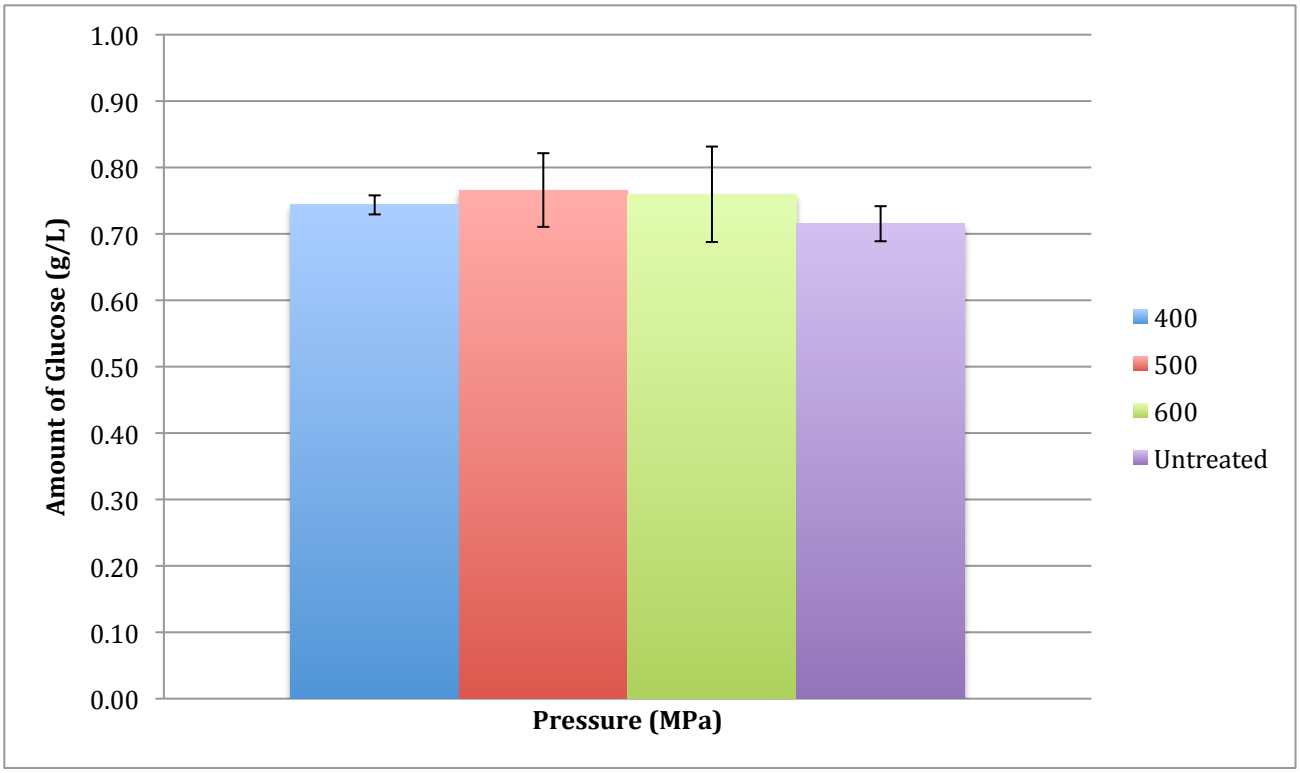

Figure 2. Level of D-glucose in untreated Coconut Water and after High Pressure treatments of 400, 500, and 600 MPa for 120 seconds starting at 4°C.

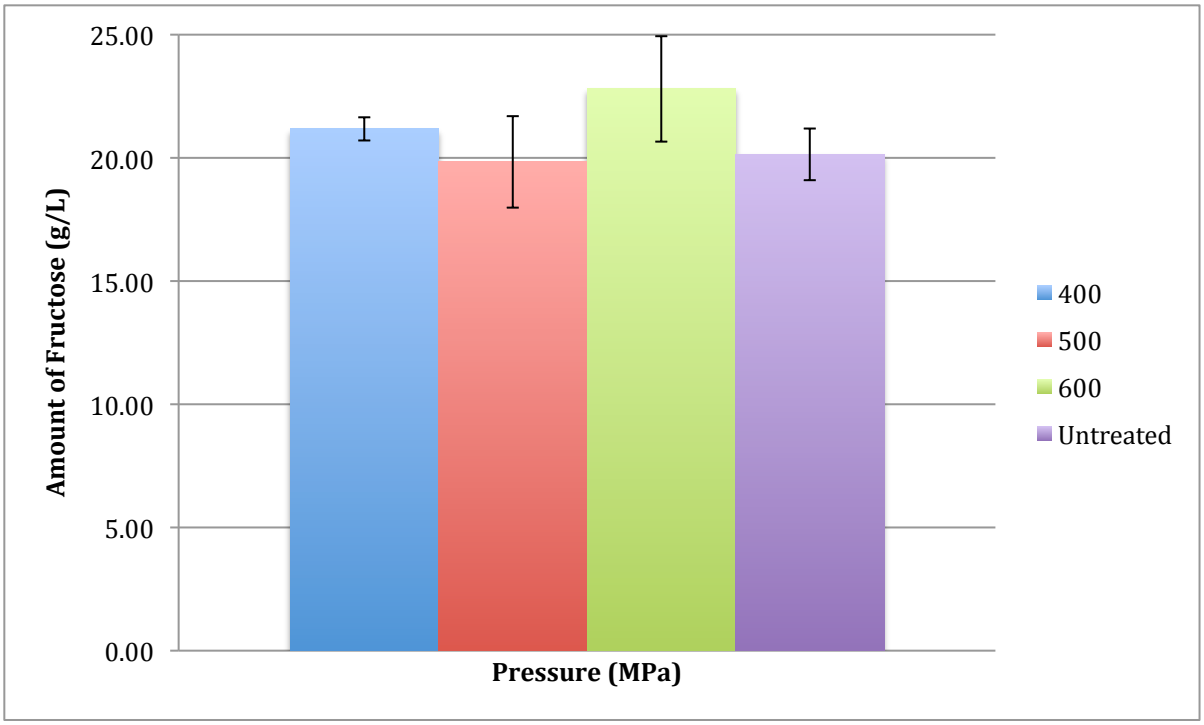

Figure 3. Level of D-fructose in untreated Coconut Water and after High Pressure treatments of 400, 500, and 600 MPa for 120 seconds starting at 4°C.

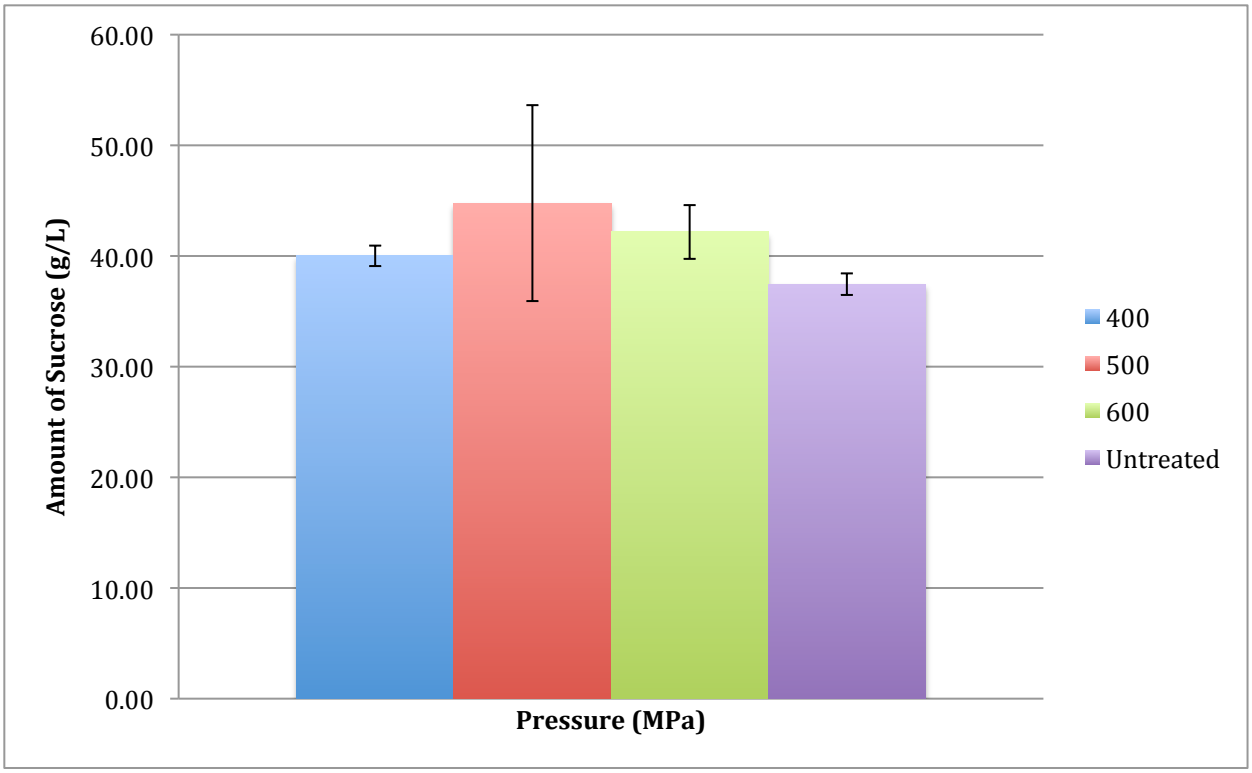

Figure 4. Level of Sucrose in untreated Coconut Water and after High Pressure treatments of 400, 500, and 600 MPa for 120 seconds starting at 4°C.

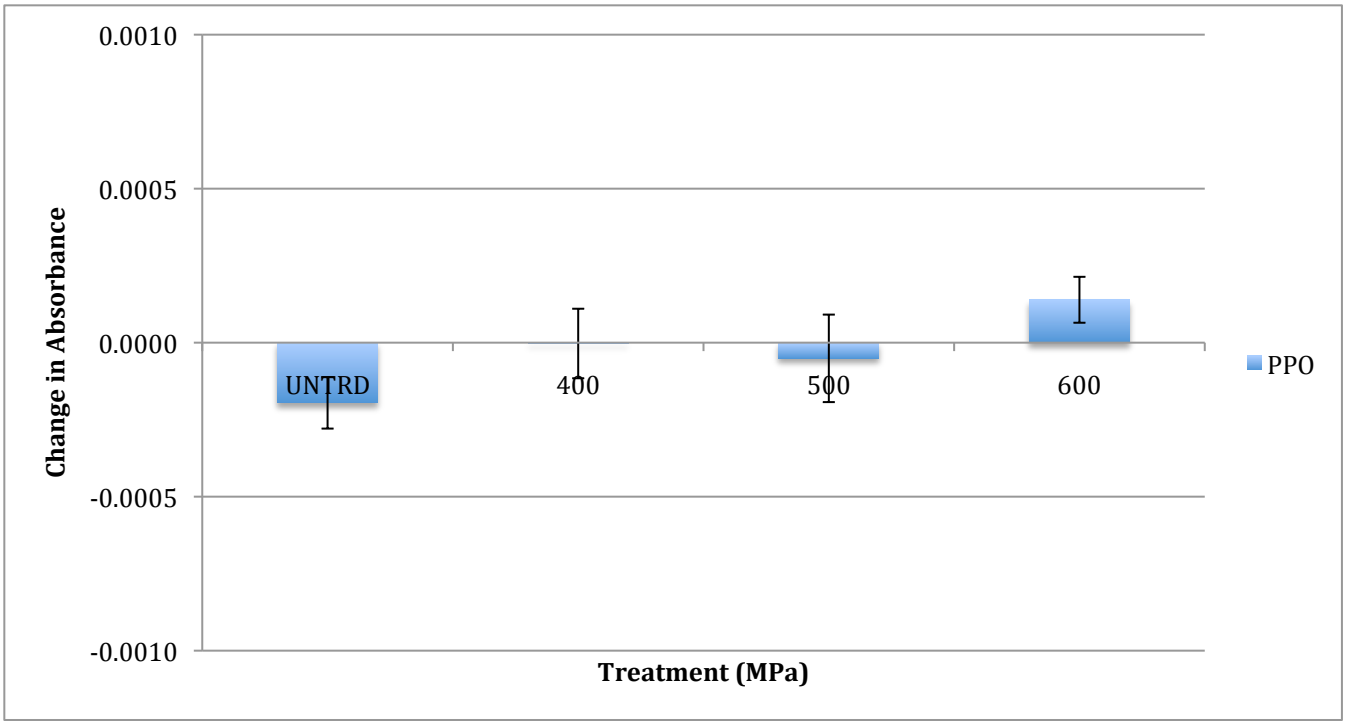

Figure 5. Change in phenol oxidase levels in Coconut water after treatments of 400, 500, or 600 MPa or untreated. Absorbancies were read at 480nm.
*UNTRD is Untreated Sample

APPENDIX A:

BIOSCREEN

Initially, preliminary studies were done using the Bioscreen to evaluate the growth of each pathogen (*E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes*) in coconut water (CW). This experiment was done to see at which hours the bacteria species began to grow, which hour they began their log-phase of growth, and when they reached their stationary phase. These time frames were also used to estimate the hours at which the researcher would take plating samples for the growth curve done by hand. The data obtained from the Bioscreen was not quantitative, unlike the growth curve done by hand, which was desired for the results section.

BIOSCREEN METHODS

Two 10 ml test tubes of each 24h culture were spun at 6000 rpm for 10 minutes. Supernatant was discarded and cells were re-suspended in 10 ml of sterile peptone water (SPW). Cells were washed twice more and finally re-suspended in SPW. The cultures were then serially diluted in SPW. The final serial dilution was completed with either CW or TSB/TSBYE to achieve a final inoculum level of approximately 2-$\log_{10}$ CFU/ml. A portion (200µl) of each culture/broth combination was pipeted into 10 honeycomb wells each. Thirty wells (10 each) were filled with uninocluated TSB, TSBYE, or CW. The honeycomb plate was inserted into a Bioscreen Growth Curve Machine (Growth Curves USA, Piscattaway, NJ) for 24h at 37°C. The optical density was taken every 15 minutes using the wideband spectrum (450-580 nm). This process was run in duplicate (n= 20).

The Bioscreen tests were run to gather preliminary data in order to get an estimation of how to design the growth curve experiments. The data from the Bioscreen runs helped gather an understanding of what hours the data needed to be gathered at for the growth curve as well.