Efficacy of high pressure processing in combination with chemical preservatives for the reduction of *Escherichia coli* O157:H7 and *Salmonella* in apple juice and orange juice

By

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ABSTRACT

The effect of pressure on the log reduction of six strains of *E. coli* O157:H7 and five serovars of *Salmonella enterica* were investigated in tryptic soy broth, sterile distilled water and commercially sterile orange and apple juice. Samples were subjected to high hydrostatic pressure (HHP) at 300 and 550 MPa for 2 minutes at 6°C, and then held for 24 hours at 4°C following treatment. *E. coli* O157:H7 strain E009 was the most pressure resistant, having a decrease of only 0.77 $\log_{10}$ CFU/ml directly after pressurization in TSB. *S.* Agona was the most pressure resistant *Salmonella* serovar tested with a decrease of 3.79 $\log_{10}$ CFU/ml in TSB at 550 MPa. The two most pressure resistant cultures were then used in a subsequent study using HHP in conjunction with antimicrobials (dimethyl dicarbonate [DMDC] at 62.5 and 125 ppm, hydrogen peroxide at 150 and 300 ppm, cinnamic acid, potassium salt at 125 and 250 ppm, potassium sorbate [KS] at 500 and 1000 ppm and sodium benzoate [NaB] at 500 and 1000). For both *E. coli* O157:H7 and *Salmonella*, the most effective antimicrobial was DMDC having a 5.79 and 5.96 $\log_{10}$ CFU/ml decrease directly following pressurization, respectively. Other treatments that were significantly different from the samples treated with no antimicrobial were hydrogen peroxide, and NaB at 500 ppm for *E. coli* O157:H7 and a treatment of NaB at 1000 ppm for *S*. Agona. After 24 hours at 4°C, *S.* Agona samples with added antimicrobials had a close to or above 5-$\log_{10}$ CFU/ml reduction. DMDC should be further investigated as an antimicrobial agent that can work in conjunction with HHP.

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Introduction and Justification

In recent years, consumer demand for minimally-processed, "fresh" tasting foods has increased, paving the way for alternative food processing to come to the forefront. High hydrostatic pressure (HHP) has received renewed interest in the past decade, and several products, such as jams and jellies are currently on the market in the United States and abroad that utilize this technology. HHP is a process by which food is subjected to pressures between 100 and 800 MPa. Under this type of pressure, microorganisms that can cause spoilage or illness to the consumer are damaged or destroyed (Hayashi, 1997). High pressure processing has advantages over traditional heat processing in that it does not change the flavor characteristics of the food and retains its nutritional qualities (Butz and Taucher, 2002; Parish,1997a).

Juice products have recently come under new hazard analysis critical control point (HACCP) regulations by the FDA which stipulate that producers of fresh juice products must take steps in their processing that give juices a 5 log decrease of the pertinent pathogen in the associated juice (FDA, 2001a). This regulation comes as a consequence of several documented outbreaks of *Escherichia coli* O157:H7, *Salmonella* and *Cryptosporidium* associated with the consumption of unpasteurized, fresh fruit juices. One of these outbreaks was associated with products from a large processor with state-of-the-art facilities, showing that outbreaks are not just limited to small producers (CDC, 1996,1997). Furthermore, since "pertinent pathogen" is defined as "the most resistant microorganism of public health concern that may occur in the juice" (FDA, 2001), it is important to know the resistance of types and strains of associated microorganisms.

HHP has been considered as a potential processing method that may be used to achieve a 5-log reduction of pathogens in juices. This technology is not feasible for a small juice producer due to its large capital costs; however large juice producers could benefit from using HHP to create a product that is safe to drink yet does not have the flavor changes associated with heat pasteurization.

The current project combines the use of antimicrobials with HHP. This research was undertaken to examine potential combined effects during processing. A combined effect of antimicrobials and HHP may allow reduced energy usage. Including an antimicrobial may cause a combined effect that would lead to a 5-log reduction while decreasing the time and pressure needed for processing.

Objectives

- To determine the pressure resistance of five *Salmonella* serovars and six *Escherichia coli* strains in tryptic soy broth, distilled water and fruit juice.
- To determine combined effects between chemical antimicrobials and HHP
- To determine optimal pressure for producing a 5-log pathogen reduction in fruit juices.

Literature Review

Salmonella

Salmonella spp. are some of the most important pathogens that cause foodborne gastroenteritis. They are commonly found in the intestinal tracts of birds, reptiles, farm animals and humans, yet these carriers may be asymptomatic (Jay, 2000). Salmonellosis can occur when a food contaminated with fecal material is ingested. Common associated foods are meat products, however many cases are caused by cross-contamination by the food-handler (CDC, 2003). Typically around 10^5 cells have to be ingested in order to cause illness, but this number may be less if the food has a high fat content, which may protect cells. Roering et al. (1999) challenged *Salmonella* in simulated gastric fluid with a pH of 1.5. They found no survival after five minutes in the fluid, indicating that a large number of cells needs to be ingested for infection to occur because most of the cells will perish in the stomach before making their way into the intestines where the infection occurs.

Forty-thousand cases of salmonellosis are reported each year, but due to under-reporting, this number does not reflect the true incidence. Though most cases are self-limiting and do not require treatment, approximately 600 persons die each year (CDC, 2003). Onset of the disease usually occurs between 8 and 12 hours after ingestion of the contaminated food. Symptoms include abdominal pain, nausea and watery diarrhea with a short term, low-grade fever. Illness can continue for up to five days (D'Aoust, 1991).

Salmonella spp. have evolved many mechanisms for survival in harsh environments, sometimes enabling them to become more virulent. One such mechanism is known as the acid tolerance response (ATR). It has been shown that log-phase cells grown in nutrient broth at a neutral pH will produce about 50 acid shock proteins when the pH is decreased to 4.0 to 4.5. Of these 50 proteins that are produced, 20 of them are unique to the ATR. Interestingly, the ATR also confers protection to heat, oxidative stress and osmotic stress (Foster and Spector, 1995).

Citrus juice has been erroneously perceived as a low risk food due to its low pH. However, a large outbreak of *Salmonella* in 1995 traced to unpasteurized orange juice set the stage for further research to increase the safety of unpasteurized orange juice (Cook et al, 1998). Parish et al. (1997) found that *S. enterica* serovars Gaminara, Hartford, Rubislaw and Typhimurium could survive in orange juice with a pH of 3.5 for 27 days. *S.* Typhimurium has shown the ability to multiply at pH 4.3 (Foster and Spector, 1995). Because the average pH of

orange juice is typically between 3.6 and 4.3, this research made it clear that orange juice could potentially contain pathogenic *Salmonella* throughout a substantial portion of its shelf life, and perhaps even multiply if the juice were on the higher end of the pH range.

A study by Pao et al. (1998) showed that once a citrus fruit is peeled, it becomes susceptible to the contamination of pathogens that can then multiply on the surface of the peeled orange if it is not properly handled. When inoculated with 4.4 log CFU/g *S.* Typhimurium (ATCC 14028) and *S.* Gaminara (ATCC 8324), the pathogen was able increase by 3.2 to 7.6 log CFU/g after just one day at 24°C. In contrast, populations declined to 3.5 and 2.5 log CFU/g in juice held at 4 and 8°C, showing the need to refrigerate peeled fruit prior to pressing citrus juices.

The survival of *S.* Typhimurium in preservative-free, pasteurized apple juice was tested by Roering et al. (1999). They found that after 21 days of storage at 4 and 10°C, 2.99 and 2.78 log CFU/ml, respectively, remained from an initial concentration of around 7 logs. The results from this show that even after a typical shelf-life of juice, the pathogen is still viable and has the possibility to cause illness.

Escherichia coli O157:H7

E. coli is a Gram-negative bacterium that commonly inhabits the gut of humans and other animals. Over time, many different serotypes of pathogenic *E. coli* have been identified. A particularly troublesome strain, *E. coli* O157:H7 was first identified as a foodborne pathogen in 1982, where it was found to be the causative agent of at least 47 cases of hemorrhagic colitis. The food implicated was ground beef patties from a fast-food restaurant (Wells et al., 1983). Since its identification, it has become recognized as a common cause of foodborne illness, with an estimated 73,000 cases each year (CDC, 2004). The most common carrier of the pathogen is cattle. Ground beef has been implicated in the majority of outbreaks associated with *E. coli* O157:H7. Other foods that have been implicated are fruit juices, cheese curds, and venison jerky (CDC, 1996, 2000; Besser et al., 1993; Keene et al., 1997).

E. coli O157:H7 can have a wide range of effects, from self-limiting diarrhea to death, depending on virulence factors and the age and/or health of the victim. Infections can cause hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP). HC victims suffer from abdominal pain, and either bloody or non-bloody

diarrhea. Fever is typically low, or absent. The duration of HC can be between two and nine days and is usually self-limiting (Meng et al., 2001). HUS has more severe complications, causing microangiopathic hemolytic anemia, thrombocytopenia, and renal failure. HUS typically affects the young, and the mortality rate is about three to five percent (Boyce et al., 1995). Although HUS is not always associated with *E. coli* O157:H7, the bacterium is the leading cause of HUS in the US (Tarr et al., 1997). TTP is similar to HUS, but mainly affects adults and involves malfunctions of the nervous system (Padhye & Doyle, 1992).

Fruit juices have generally been viewed as safe without heat treatment, due to their low pH. However, with several juice outbreaks in the past decade, many studies have been conducted to determine the survival of *E. coli* O157:H7 under different stresses in fruit juice.

Fisher and Golden (1998a) found that ground Golden Delicious and Red Delicious apples could support growth of *E. coli* O157:H7 strains 380-94 and 7927 at 25°C over a period of 5 days. The ability to increase in population was due mostly to the rise in pH of the ground apples. This is of concern because once an apple drops from the tree it can be wounded, potentially causing inoculation of the apple as well as a rise in pH to support further growth of the pathogen. At lower temperatures of 4 and 10°C, about a 1 log CFU/ml decrease over the course of 18 and 12 days, respectively was seen in all apple varieties tested (Golden Delicious, Red Delicious, Rome, and Winesap).

Splittstoesser et al. (1995) determined the D_{52} of *E. coli* O157:H7 in apple juice to be 18 minutes with a z value of 4.8ºC, and that adding sorbic or benzoic acid at a concentration of 1000 mg/L produced a D_{50} of 5.2 minutes and 0.64 minutes, respectively. Sorbic acid was effective in reducing the heat resistance, however benzoic acid was found to have eight times the effect. This study also found no significant difference between the D_{52} of *E. coli* O157:H7 in apple juice when the °brix was adjusted from 11.8 to 16.5. They also found no significant difference in the D_{52} when the pH was 3.6 versus 4.0, but did find a significant increase in the D_{52} when the pH was at 4.4.

Roering et al. (1999) showed the survival of *E. coli* O157:H7 (strain C7927) in apple juice. They found that after 21 days at 4°C, 2.11 log CFU/ml remained of the original 7.27. At 10°C, the survival was very similar having 2.04 log CFU/ml of the original 7.18. In simulated gastric fluid (pH = 1.5) *E. coli* O157:H7 survived at least 2 hours. Given that only a few cells of

E. coli O157:H7 have to be ingested in order to cause illness, the ability to survive in the gut is crucial to its increased virulence (Lin et al., 1996).

Acid tolerance was tested by Miller and Kaspar (1994). This study used both tryptic soy broth (TSB) at varying pH levels and apple juice with and without added preservatives. Two strains of *E. coli* O157:H7 were used, as well as a control *E. coli* strain. After 2 hours at pH 2 in TSB, the control strain was undetectable, while both the O157:H7 strains survived 24 hours with only about a 1-log decrease in numbers. Similar results were found in the cider, where after 5 to 7 days the control strain was undetectable whereas both strains of *E. coli* O157:H7 were still detectable in apple juice after 21 days at 4ºC, both with and without potassium sorbate and sodium benzoate. This study underscores the fact that addition of these commonly used antimicrobials alone is not sufficient to reduce *E. coli* O157:H7 levels.

Stopforth et al. (2004) examined the presence of *E. coli* O157:H7 in apple wounds after washes with water, 5% hydrogen, 5% acetic acid, and 0.2% sodium hypochlorite. No wash step was effective in purging the apple wounds of *E. coli* O157:H7, also showing the need for a post-pressing step to produce a microbiologically safe juice.

Outbreaks

Several outbreaks of foodborne illness have occurred due to the consumption of fresh apple juice or cider and unpasteurized orange juice in the past several decades. Many of these outbreaks have occurred due to a lack of good manufacturing practices (GMPs) and good agricultural practices (GAPs). One of the main routes of contamination of apple juice is the use of dropped apples; in 1992, the CDC took a survey of cider producers at the New England Fruit Growers' meeting and found that 100% of the processors used dropped apples, and only 1/3 of them washed and brushed the apples prior to pressing (Daly, 1997).

In 1975, 229 persons became ill after drinking unpasteurized apple juice in New Jersey. *Salmonella typhimurium* was isolated from 154 patients, six of the thirty employees and from two cider samples. The apple trees from which the fruit came were fertilized with manure and dropped apples were typically collected for cider production (CDC, 1975).

In 1980, prior to the identification of *E. coli* O157:H7 as an important threat to public health, 13 children were hospitalized with HUS after drinking unpasteurized apple juice in Toronto. Two children were comatose, one for three weeks and one for five days. Six of the

patients required dialysis. None of these 13 cases were fatal, however it was later found that a 14th child had fallen ill and died before being hospitalized. The autopsy showed characteristic signs of HUS. Several older family members also drank the cider and had mild cases of abdominal cramps and bloody diarrhea. Microbial analysis of several samples of the juice revealed several fungal species, and several bacterial species including *E. coli*, but at the time it was not identified as the causative agent of the HUS cases (Steele et al., 1982).

In the fall of 1991, 23 persons became ill with diarrhea and four children were hospitalized with HUS in Massachusetts. The implicated food item was apple juice produced from dropped apples from a particular small processor. *E. coli* O157:H7 was isolated from the stool of the patients, and although none of the cider samples obtained were found to have *E. coli* O157:H7, the drinking of apple juice from one certain processor was strongly statistically linked to the illness (Besser et al., 1993).

In 1996, two unrelated outbreaks due to the consumption of apple juice occurred. One outbreak occurred in Connecticut where 12 cases of *E. coli* O157:H7 were identified, seven hospitalizations occurred including three for HUS and one for TTP. The outbreak prompted a recall of cider from a particular processor. The supplier of apples for the implicated product said that some of the apples were "dropped," but that all apples were washed and scrubbed prior to use. A simultaneous outbreak of *Cryptosporidium* occurred in New York with apple juice as the implicated food (CDC, 1997).

Another outbreak occurring in 1996 was more widespread, involving a large juice processor in the Northwest. In this case, 70 people became ill and were identified as having *E. coli* O157:H7 infections: 25 people were hospitalized, 14 children developed HUS and one of those cases was fatal. After finding a juice sample positive for *E. coli* O157:H7, a voluntary recall was issued. A study of the processing facility showed good equipment, procedures and policies, such as sorting, washing and not accepting dropped apples into the facility; however it was also shown that these procedures were not always followed by the employees of the operation or the suppliers. It was concluded that the organism most likely arrived at the plant already on the apples, but sufficient processes to rid the juice of the bacteria were not in place. This outbreak showed that even large production lines with state-of-the art equipment were not sufficient to prevent outbreaks from unpasteurized cider (CDC, 1996; Cody et al., 1999).

In 1995, an outbreak of *Salmonella* Hartford was associated with the consumption of unpasteurized orange juice in a large Florida theme park. Sixty-two cases were confirmed, but an estimated 1240 to 6200 cases may have remained unreported. Upon inspection of the processing facility and orange suppliers, it was determined that the contamination most likely occurred at the processing facility. The facility had cracks and openings where wild life could have entered and contaminated the juice (Cook et al, 1998; Parish, 1997b).

In June of 1999, a *Salmonella* Muenchen outbreak involving unpasteurized orange juice and products made from that orange juice was reported in the Northwest. Nine from Washington became ill, 57 from Oregon, and 66 cases from other states were reported. Samples of the juice itself, blenders used to make smoothies with the juice and a juice dispenser all were positive for *S.* Muenchen. A large recall was issued by the producer (CDC, 1999).

High hydrostatic pressure (HHP)

The first person to experiment with high pressure for application to food was B.H. Hite in 1899. While his first experiments used milk to show a bacterial reduction, in later experiments his group tested fruit juices. Of apple juice samples that had been preserved for 5 years in this manner he said "They were everything that good apples can suggest" and that "a finer non-alcoholic beverage would be hard to imagine" (Hite et al., 1914). Put on a shelf for nearly a century, research into HHP has received renewed interest in food processing technology as an alternative to heat pasteurization.

High pressure processing (HHP) is performed at hydrostatic pressures between 100 and 800 MPa, with temperatures ranging from below freezing to above boiling point, though temperatures and pressures vary with different HHP equipment. Unlike heat processes, the pressure is applied uniformly and is not dependent on shape (FDA, 2000).

HHP Effects on Juice Quality

Parish (1997a) compared fresh, heat pasteurized and high pressure processed orange juice using a variety of tests over the course of 20 weeks. After 20 weeks of storage at 4°C, juice treated at 500 MPa for 90 seconds retained about 70% ascorbic acid content, while traditional heat pasteurized juice retained about 40%. Cloud loss in orange juice is due to the action of the enzyme pectinmethylesterase (PME) that produces a less opaque product that is viewed as

commercially unacceptable. Parish concluded that pressure treatment up to 700 MPa at refrigeration temperature was not sufficient to reduce PME activity to an acceptable level, whereas traditional heat pasteurization was. Sensory analysis was performed on the orange juice after 4, 8, and 16 weeks of storage at either 4 or 8°C. Each sample was scored based on the difference of the sample to fresh squeezed orange juice that had been frozen for reference, with higher scores being the most different. It was found that heat pasteurized juice and juice that was pressurized with mild heat (50 or 60°C) always had higher scores than juices that were pressurized under refrigeration conditions. While there was a significant difference between refrigeration pressurized juice and fresh orange juice, it was always closer than samples that had been exposed to heat.

Nienaber and Shellhammer (2001) conducted a study on commercially feasible pressure processes to see if they could produce a consumer-acceptable product with the technology. Cloud stability (PME activity), ascorbic acid content, and non-enzymatic browning were all investigated. PME was reduced to an acceptable level of below 10% of the original at 800 MPa for 1 minute at 25°C. A longer time of 1 to 2 minutes was needed at 600 MPa. A shelf life study was conducted over the period of 3 months with the initial process at 800 MPa for 1 minute at 20°C. It was found that cloud stability was maintained, ascorbic acid content did not significantly diminish due to the pressure treatment (some loss was seen due to oxygen transfer through the polyethylene terephthalate [PET] bottles), and browning was commercially acceptable in samples that were held at 4, 15 and 26°C. Samples stored at 37°C showed an unacceptable level of browning. The authors conclude that HHP at 800 MPa can produce a commercially acceptable product with a fresh quality without compromising shelf-life. It should be noted that 800 MPa requires processing equipment that is more expensive than most available systems which typically run at a maximum of 600 MPa.

Sanchez-Moreno et al. (2003a) tested the effects of HHP on vitamin C, provitamin A, and carotenoids in orange juice. Samples were taken immediately after pressure treatment and after 10 days of storage at 4°C. Compared to non-treated juice, juice treated at 350 MPa for 2.5 minutes at 30°C showed no significant changes in vitamin C content. Pressures combined at higher temperatures of 40°C and 60°C showed a decrease of 6.89 and 8.08%, respectively. After 10 days of storage, the vitamin C contents had not changed significantly from the original values taken directly after processing. Total carotenoid counts were 31.73% higher in juice treated at

400 MPa for 1 minute at 40°C than the control samples. After 10 days of storage, the total carotenoid levels decreased in treated juice, but did not become lower than the control samples both at the time of treatment, or after 10 days. Provitamin A was calculated based on its precursors, most of which are carotenoids, therefore the behavior of provitamin A was similar to the results for total carotenoid.

In a similar study, Sanchez-Moreno et al. (2003b) looked at the effects of HHP on flavanones and anti-oxidative components in orange juice. It was found that HHP increased the amounts of both narirutin and hesperidin (major flavanones found in orange juice) by 12.12 and 22.12%, respectively in juices treated at 400 MPa for 1 minute at 40°C. After 10 days of storage at 4°C, the increased flavanone levels remained as compared to the control juice. For the radical scavenging components of the juice, there was no significant difference in the levels as compared to the control either just after processing or after 10 days of storage.

Butz et al., (2003) tested the effects of high pressure on vitamin C content, carotenoids, sucrose, and anti-oxidative factors in different fruit and vegetable products. Directly after processing, no significant difference was seen between the treated and untreated samples. In raspberry puree samples that were held at 4°C for 15 and 30 days, a significant decrease in sucrose occurred as compared to traditionally heat pasteurized samples. The group theorized that this was not due directly to the pressure, but rather that the enzyme invertase, which is deactivated under heat pasteurization, was not deactivated during pressure treatment, and therefore converted the sucrose to fructose and glucose. It is possible that this may occur in apple and/or orange juice, and subsequent flavor attributes would have to be examined.

Sancho et al. (1999) looked at the levels of ascorbate, thiamin and pyridoxal in a buffer after a treatment of 600 MPa for 30 minutes at room temperature. They found there was no significant difference between the control and treated samples for thiamin and pyridoxal, but there was a significant 12% decrease in the level of ascorbate from the control after treatment.

Butz and Tauscher (2002) used a mixture of orange, lemon and carrot juice to show the efficacy of high pressure on juice quality over a storage period of 21 days at 4°C. Prior to the storage, there was no significant difference in flavor or odor between the control juice and juice treated at 500 MPa for 5 minutes. After the storage period, the flavor and odor quality of the control juice had decreased significantly, where the treated juice maintained its overall quality according to a trained sensory panel.

The aforementioned studies show that HHP treatment can be comparable to, if not better than, heat treatment in flavor and nutrient retention while maintaining shelf-life and increasing the safety of juice products.

HHP mode of action on cells

HHP has numerous effects on microbial cells. Membrane integrity has been shown to be compromised after HHP treatment. A major source of cell disturbance is the inactivation of enzymes, including those that are responsible for making new proteins (ribosomes). Endonucleases can also inactivate cells by cleaving the DNA, however this process has been found reversible under some circumstances (Chilton, 1997; Smelt, 1998). Microbial inactivation can be affected by many user-manipulated variables, such as hold time, temperature, pressure level, pH of the product and repeated pressure treatments (Smelt, 1998). A complete mechanism by which HHP inactivates organisms has yet to be demonstrated, but the issue is under investigation.

Chilton et al. (1997) performed a series of experiments on pressure treated cells to identify the mechanisms of inactivation. They observed cytoplasmic leakage from the cell as well as leakage into the cell of surrounding fluids. Through electron microscopy they observed a lack of ribosomes after pressurization. A lack of ribosomes in a cell would partially inhibit the ability to repair the cell by halting protein synthesis. It was concluded that repair of the outer membrane could occur spontaneously without enzymes, however repair of inner membranes would require several enzymatic processes. It was shown through gel electrophoresis that DNA inside pressurized cells had been cleaved. Using mutant cells deficient in endonucleases, it was shown that most likely the cleaved DNA was due to enzymatic action. Interestingly, plasmid DNA remained intact.

Tholozan et al. (2000) pressurized *Listeria monocytogenes* for 10 minutes at 20°C and demonstrated a 90% decrease in cellular ATP at 600 MPa. They also found that cellular potassium levels decreased to near zero under the same conditions. The authors attribute the decrease in ATP levels to the inactivation of ATPase by the high pressure. Also performed in this study were scanning electron micrographs. *L. monocytogenes* showed up to a four-fold increase in cellular volume, while *Salmonella* Typhimurium showed none. *S.* Typhimurium did

show dimples and folding of the membrane after pressurization at 400 MPa for 10 minutes at 20°C, but did not show any lysis.

E. coli *pressure resistance*

Numerous research projects have been undertaken to identify pressure resistant strains of various species known to cause food borne illness. Using the most resistant strains to test process times, temperatures and pressures helps to ensure product safety.

Benito et al. (1999) tested several strains of *E. coli*, including 6 strains of *E. coli* O157, in a neutral buffer and found the two most pressure resistant to be strains C9490 and 30-2C4, having a <2 log decrease of stationary phase cells after 30 minutes at 500 MPa. C9490 was also found to be resistant to oxidative stress, acid and osmotic stress. The strain NCTC 12079 was found to be significantly more sensitive, having a 5 log reduction at 500 MPa for 30 minutes. The most sensitive strain tested was non-O157.

Linton et al. (2001) tested 12 strains of *E. coli* in skimmed milk and found the most resistant O157:H7 strain was NCTC 12079, with a 4 log decrease after 15 minutes at 600 MPa and 20ºC. The most resistant strains in the study were serotypes O7:H18 and O124:K72 (B17):H-, which are both pathogenic forms of *E. coli.* At 700 MPa, under the same conditions, no survivors of any strain were left.

Robey et al. (2001) used three of the most pressure resistant *E. coli* O157:H7 strains from the aforementioned studies, strains C9490, 30-2C4, and NCTC 12079. Strain C9490 and 30-2C4 were found to be the most pressure resistant, withstanding 500 MPa for 5 minutes at 20°C with minimal decrease in numbers. NCTC 12079 decreased by 3 to 4 logs under these parameters.

Alpas et al. (2000) worked with *E. coli* O157:H7 933 and 931 to find that at 345 MPa for 10 minutes, 933 was reduced by 2.74 logs at 25°C, while 931 was more sensitive, having been reduced by 4 logs. At higher temperatures (35°C, 45°C, and 50°C), there was greater than 8 log decrease. Acid sensitivity was also investigated and it was found that *E. coli* O157:H7 933 and 931 had an additional log decrease at 25°C by reducing the pH in 0.1% peptone to 4.5 with either citric acid or lactic acid.

Pagan et al. (2001) tested *E. coli* O157:H7 strain C9490 in acidified growth media. Samples were pressurized in buffer, and then diluted in TSBYE adjusted to different pHs. Diluted samples were then held at 37°C for up to 3 hours. No difference from the control was

seen when the samples were pressurized at 100 or 200 MPa. After 3 hours, the samples pressurized at 300 MPa showed a greater than 4 log decrease, while after 1 and 2 hours the 500 and 400 MPa samples showed a greater than 8 log reduction, respectively.

Several studies characterized pathogen destruction in fruit juices as opposed to neutral test media, as in the previously described studies. The food matrix can have differing effects on the pressure resistance and sensitivity of an organism owing to pH differences, water activity, antimicrobial properties of the food, and protective aspects of the food.

Jordan et al. (2001) used *E. coli* O157:H7 C9490 and tested survival in apple (pH 3.5), orange (pH 3.8) and tomato juice (pH 4.1). Strain C9490 decreased by one log in orange juice, at 500 MPa for 5 minutes at approximately 20°C, but was undetectable in apple or tomato juice at this pressure. However, at 450 MPa, C9490 only decreased by about 1 log in all three juices. After 24 hours, the counts had decreased an additional 3 to 4 logs in all juices.

E. coli O157:H7 cocktail of strains SEA 13B88, ATCC 43895, and 932 was tested in apple (pH = 3.7), orange (pH = 3.7), carrot (pH = 6.2) and grapefruit juice (pH = 3.0). Samples were treated at 615 MPa at 15ºC for 1 or 2 minutes. Reduction was the least in apple juice with a 0.02 and 0.41 log decrease at 1 and 2 minutes, respectively. The greatest decrease at 2 minutes was in grapefruit juice with a decrease of 8.34 logs, while the greatest decrease at 1 minute was seen in carrot juice with a decrease of 4.51 logs. Orange juice showed a 1.07 and 2.16 log reduction at 1 and 2 minutes, respectively (Teo et al., 2001).

Four strains of *E. coli* were tested for pressure-resistance by Ogawa et al. (2000). Two strains were O157 (strains CR-3 and CE273) and two were non-O157 (JMC1649 and IFO3301). It was found that the O157 strains were more pressure resistant than the non-O157 strains, having a 1 and 2 log reduction for CR-3 and CE273 respectively, while JMC1649 and IFO3301 had a 5 and 4 log reduction, respectively at 400 MPa for 10 minutes at room temperature.

Linton et al. (1999) found that a >6 log decrease in *E. coli* O157:H7 NCTC 12079 occurred at a pressure of 550 MPa for 5 min at 20ºC in orange juice with a pH adjusted to 3.4, 3.6, 3.9, and 4.5, but not 5.0. A 6 log reduction was seen at pH 5.0 when the temperature was increased to 30°C at 550 MPa. Three different pressures were used in this study (400 MPa, 500 MPa and 550 MPa) with the same trend of decreased numbers at a decreased pH values seen at each pressure.

Garcia-Graells et al. (1998) used a pressure resistant *E. coli* mutant to test its survival in orange juice and apple juice. It was found that at 300 MPa a 1.1 log reduction occurred after 15 minutes at 20°C in apple juice (pH = 3.3), and a 0.8 log reduction occurred in orange juice (pH = 3.8). At 400 MPa under the same conditions a greater than 4.7 log reduction was seen in apple juice while only a 1.5 log reduction was seen in orange juice.

Differences between studies can be attributed to several different factors such as strain difference, time, temperature and pressure differences, as well as differences in the pressure unit used. Pressure units can differ in how long it takes to reach the desired pressure, as well as how the pressure release occurs, which may have an impact on microbial destruction.

Salmonella *pressure resistance*

Salmonella spp. are typically more pressure sensitive than *E. coli* O157:H7 strains. Alpas et al. (2000) used *S.* Enteritidis FDA and *S.* Typhimurium E21274 VL and tested strains at different pressure and temperature combinations in 0.1% peptone buffer at a neutral pH. They found a 4.92 and 5.51 log decrease in numbers at 345 MPa for 10 minutes at 25°C in *S.* Enteritidis and *S.* Typhimurium, respectively. At higher temperatures (35, 45, 50°C), there was no recovery with a decrease greater than 8 logs for both species. This was true for samples run for 5 minutes and 10 minutes. At the lowest pressure tested, 207 MPa, it was found that *S.* Enteritidis and *S.* Typhimurium decreased by 1.04 and 1.60 logs at 25°C for 10 minutes. Alpas et al.(2000) also did studies on the addition of citric and lactic acid to the buffer to reduce the pH to 4.5, 5.5 and 6.5. At pH 4.5, after being pressurized for 5 minutes at 345 MPa at 25°C, *S.* Typhimurium was below the limit of detection, having a greater than 8 log reduction. Under these same conditions, *S.* Enteritidis had a 5.67 and 5.96 log reduction in citric acid and lactic acid respectively.

Ponce et al. (1999) tested *S.* Enteritidis in liquid whole eggs. Two pressures were tested, 350 and 450 MPa, and a wide range of temperatures were tested (50, 20, 2 and -15°C). They tested 5 different time combinations: 5, 10, and 15 minutes, as well as 5min+5min and 5min+5min+5min, where pressure was administered consecutively, but in separate runs of the machine. It seems that eggs may confer protection to the bacteria during pressurization in conjunction with heat. After 5 minutes at 50°C at 450 MPa, *S.* Enteritidis was still detectable with a 5.9 log decrease. While this is a substantial reduction, the results do not agree with other

studies done on *Salmonella* strains in different foods or buffers. As the temperature decreased, the effectiveness of the pressure on inactivating the pathogen decreased as well. At 450 MPa for 5 minutes, the log reduction was 5.9, 4.04, 3.19 and 1.53 at 50, 20, 2, and –15°C, respectively. The consecutive 5 minute runs were more effective at the higher of the two temperatures. At 50°C and 350 MPa, there was a 3.34 and 4.71 log decrease after 10 and 15 minutes, respectively. At both 5+5 and 5+5+5 minutes, the count was below the limit of detection, having an overall reduction of greater than 7 logs. At 20°C and 450 MPa, there was a 4.31 and 5.09 log reduction at 10 and 15 minutes, respectively. At 5min+5min and 5min+5min+5min, the counts were also below the limit of detection. In contrast, at the lower two temperatures, the greatest difference seen was at 450 MPa at -15°C. When pressurized for 15 minutes, the log decrease was 3.61, while at 5min+5min+5min the log decrease was 2.17.

Alpas and Bozoglu (2000) tested the recovery of *S.* Enteritidis FDA and *S.* Typhi E21274 in orange juice after being pressurized at 345 MPa for 5 minutes at 50°C. As was evidenced by a previously published study, there was no growth directly following pressurization, with a greater than 8 log decrease noted. Three different holding conditions were used after pressurization: 24 hours at 4°C, 24 hours at 37°C and 48 hours at 37°C. Neither strain of *Salmonella* showed recovery after being stored under any of these conditions.

Tholozan et al. (2000) found *S.* Typhimurium strain Mutton (ATCC 13311) to have a greater than 8 log decrease at 400 MPa after 10 minutes at 20°C. However, at 325 MPa the log decrease was about 3.

Teo et al. (2001) used serovars Agona, Hartford H0610, Typhimurium, Muenchen and Enteritidis and found a 4.95, 3.92, 4.97, 5.07 and 8.62 log decrease in numbers at 615 MPa for 2 minutes at 15ºC in apple juice (pH = 3.7), respectively. The same study was done using orange juice (pH = 3.7), and all but *S.* Typhimurium had a greater than 8 log increase. In orange juice, *S.* Typhimurium had a 6.91 log decrease after 2 minutes at 615 MPa and 15°C. In grapefruit juice (pH = 3.0) after 1 minute at 615 MPa and 15°C, all strains but *S.* Typhimurium showed a greater than 8 log decrease. *S.* Typhimurium showed a 3.55 log decrease under these conditions. After 2 minutes at 615 MPa at 15°C, all strains showed a greater than an 8 log decrease.

Metrick et al. (1989) compared the pressure resistance of a heat-resistant (*S.* Senftenberg 77W) and heat-senistive (*S.* Typhimurium ATCC7136) strain of *Salmonella* in a neutral buffer

and chicken baby food. It was found that the heat-resistant *Salmonella* strain was more pressure sensitive, having a 4 log decrease after 10 minutes at 340 MPa (no pressurization temperature stated). The chicken baby food also seemed to confer some protection to the *Salmonella*, as there was only a 3 log decrease under the same conditions. In contrast, the heat sensitive strain had only a 2 log decrease in both the mediums under identical conditions.

In contrast to many published studies, Igura et al. (2003) found that inactivation of *S.* Typhimurium IFO 13245 was greater at 0 and 5°C than at 10°C. At 200 MPa with no hold time at 0°C, there was a 7 log decrease. Under the same conditions at 10°C, there was a 5 log reduction. After 40 minutes of pressure at 200 MPa, there was a complete reduction regardless of temperature. The trend of greater inactivation at lower temperatures was seen at lower pressure levels (50, 100 and 150 MPa) as well. Possible explanations for the discrepancy between studies could be the pressure treatments used, most of the studies reported here have used a greater than 200 MPa pressure treatment.

HHP in conjunction with antimicrobials

The hurdle strategy is a good way to increase the safety of foods. The principle is to add several different antimicrobial effects to the food, thereby decreasing the chances of the target organism growing or working in tandem to kill any microorganism present. The goal is to have a greater total effect than the individual effects combined. Common hurdles used are pH extremes, low water activity, heat and chemical antimicrobials. The following studies highlight that HHP in conjunction with many different antimicrobials can have enhanced effects on pathogens. This avenue should be further explored so that HHP times, temperature and pressure levels can be decreased to produce a more economically advantageous process.

Aymerich et al. (2005) tested the combined use of nisin (44.85 ppm) and HHP (400 MPa for 10 min at 17°C) on sliced ham. They inoculated separate ham samples with *L. monocytogenes* and a cocktail of *S. enterica* serotypes London CTC1003, Schwarzengrund CTC1015, and Derby CTC1022. Directly after treatment, *Salmonella* levels were significantly reduced. After 3 months at either 1°C or 6°C, they found the average level of *Salmonella* to be 4 MPN/g, and 50% of those averaged samples were not positive for *Salmonella* at all. A synergistic effect between HHP, 1.8% potassium lactate and 1°C was seen in the samples inoculated with *L. monocytogenes.*

Masschalck et al. (2000) used pressure resistant strains of *E. coli* to test the effectiveness of high pressure combined with either lysozyme or nisin. They found that for every pressure tested (200, 300, 400, 500 and 600 MPa) the log decrease in bacterial counts was always greater with either antimicrobial. Of particular interest was nisin in conjunction with low pressure (200 MPa). A very small log decrease (0.1 and 0.2 log CFU/ml), depending on strain, was seen with just pressure alone, however with the addition of 100 IU/ml nisin the decrease ranged from 1.3 to 2.1. It should be noted that at this concentration, nisin does not have a significant effect without pressure.

Ogawa et al. (2000) tested the effectiveness of adding allyl isothiocyanate (AIT) after pressure treatment (400 MPa for 10 minutes at 20°C) on *E. coli* both O157 and non-O157. They found that even the smallest amount of AIT added (20 μg/ml) after pressurization inhibited growth of non-O157 strains in heart infusion broth. In contrast, both O157 strains needed greater than 80 μg/ml AIT after pressurization to inhibit growth.

A combined effect has been observed when the lactoperoxidase system have been used in conjunction with HHP. It has been shown that the species *Listeria innocua*, *Enterococcus faecalis* and *Lactobacillus plantarum* all have a greater inactivation, showing at least a 6 log reduction at pressures between 400-450 MPa at 20°C. (Garcia-Graells et al., 2003).

Karatzas et al. (2001) investigated the use of carvacrol and high pressure on inactivating *Listeria monocytogenes* Scott A. They found carvacrol (3 mmol/l) and a 20 minute pressure treatment at 150 MPa alone each gave less than a 1 log reduction in numbers, while combined they gave nearly a 3 log reduction.

Antimicrobials

Sodium Benzoate

Benzoic acid is an antimicrobial organic acid that is naturally found in cranberries, prunes, plums, cinnamon, cloves and most berries (Foegeding and Busta, 1991). It is one of the oldest chemical preservatives and was the first antimicrobial compounds approved by the FDA for use in foods (Chipley, 1993). Benzoic acid is an inexpensive antimicrobial; however its use has decreased in favor of other organic acids that do not affect the flavor of foods (Luck and Jager, 1997). It is still widely used in foods that can mask its flavor such as salads, pickles, and fruit juices (Foegeding and Busta, 1991). Its anti-fungal properties are greater than its anti-

bacterial properties, however, it has been shown to be effective against common foodborne illness bacteria, such as *E. coli*, *Listeria monocytogenes* and *Bacillus cereus* (Davidson et al., 2002). It is commonly used in the sodium salt form due to its increased solubility (Foegeding and Busta, 1991). Benzoic acid is generally recognized as safe (GRAS) in concentrations up to 0.1% in food items in the United States, but many other countries have a higher maximum allowable use (Davidson et al., 2002). In fruit juices, it is typically applied in the concentration of 0.05 to 0.2%, with concentrations over 0.1% only used outside of the United States (Luck and Jager, 1997).

For microbial inactivation to be effective, benzoic acid ($pK_a = 4.1$) must be in the non-charged, undissociated form so it can enter through the cell membrane. The mechanism for microbial inactivation is largely unknown, but several studies have suggested various methods (Davidson et al., 2002). One proposed mechanism of inactivation is the decreased permeability of the cell membrane, and disruption of the transport systems. This can lead to cell death by starvation because nutrients cannot pass through. It has also been suggested that once inside the cell, the undissociated molecule will ionize, and thus decrease the intracellular pH level, interfering with various cellular activities. One of the most studied areas of inactivation is enzyme inhibition. It has been shown that enzymes of the citric acid cycle are affected by benzoic acid, as well as those in the oxidative phosphorylation pathways (Chipley, 1993).

Studies of the uptake of sodium benzoate into cells have shown that above 60°C, uptake is reduced, perhaps due to the denaturation of proteins involved in transport across the membrane (Davidson et al., 2002). Because pressure processing is typically done at lower temperatures than 60°C, this decrease in uptake may not occur.

Fisher and Golden (1998b) tested a cocktail of two strains of *E. coli* O157:H7 (strain 380-94 and strain 7927) in apple juice. At 4°C, a 0.045% addition of sodium benzoate reduced *E. coli* O157:H7 to an undetectable level (>7 log decrease) after 9 days of storage. They also showed a >7 log decrease of *E. coli* O157:H7 after 12 days of storage at 10ºC. At 25°C, *E. coli* levels were reduced to undetectable after just one day of storage.

Miller and Kaspar (1994) showed a 2% and 57% viability loss of *E. coli* O157:H7 strain 43895 in two separate lots of apple juice with a 0.1% sodium benzoate concentration. The loss was measured after 21 days at 4°C. It should be noted that even with a 57% decrease, about 10^4 cells still remained after 21 days.

In contrast to the results by Miller and Kaspar, Zhao et al. (1993) showed no *E. coli* O157:H7 strain 7927 survivors were detectable after 7 days in 5 of 6 lots of apple juice with 0.1% sodium benzoate. The authors also recommend to cider producers the use of sodium benzoate to improve the safety of their juices.

Potassium Sorbate

Potassium sorbate has been used as a food-preserving agent since 1945, mostly targeting fungal growth. Sorbic acid is typically used in foods in the potassium salt form because of its enhanced solubility. It is currently used in cider in a concentration of 0.05 to 0.1% (Davidson et al., 2002). As with benzoic acid, sorbic acid ($pK_a = 4.75$) is more effective at lower pH levels because more will exist in the undissociated form, allowing entry into the cell (Feogeding and Busta, 1991).

Conflicting data about mechanisms of microbial inhibition have made it difficult to piece together an exact mechanism. Most likely this is due to the varying conditions studies are performed under and mechanisms vary depending on the organism, food and processes it has been through. One of the most well documented reactions is that of sorbates with the thiol group on sulfhydryl enzymes. These enzymes include fumarase, aspartase, succinic dehydrogenase and alcohol dehydrogenase. Another proposed mechanism of action is inhibition of the dehydrogenase enzymes in the fatty acid oxidation pathway. This fatty acid accumulation halts metabolism in fungi (Davidson et al., 2002). It has also been shown that sorbates inhibit the transport of amino acids across the cytoplamic membrane. The depletion of ATP in a cell has been shown. Once the undissociated form enters the cell, it will then dissociate and the cation build-up will cause the cell to deplete its energy resources attempting to correct the imbalance (Sofos, 1989). Because microbial inactivation has been shown to be selective for certain species and food stuffs, it is important to verify the process using the targeted organism prior to commercial use (Sofos and Busta, 1993).

Miller and Kaspar (1994) showed a 10% and 11% loss in viable *E. coli* O157:H7 strain 43895 cells in two lots of apple juice with 0.1% potassium sorbate over a 21-day storage period at 4°C. In cider with 0.05% potassium sorbate, the percent lost was 7. Though statistical differences were not reported for those sets of data, the juices with no antimicrobial yielded a 2%

and 9% loss in two separate batches, meaning that potassium sorbate was only slightly more effective than no preservative in reducing the numbers of *E. coli* O157:H7.

Zhao et al. (1993) tested *E. coli* O157:H7 strain 7927 in apple juice containing 0.1% potassium sorbate. They found that there was no significant difference between the control lots and the lots with potassium sorbate. Further, *E. coli* O157:H7 was still detectable after 20 days at 8°C in 4 of 6 lots of juice with 0.1% potassium sorbate.

In cider with a pH of 4.1, it was shown that adding a 0.1% solution of sorbic acid and holding for 6 hours at 35°C produced a 5 log reduction in *E. coli* O157:H7 (cocktail of strains ATCC 43895, C7927 and USDA-FSIS-380-94) and *Salmonella* Typhimurium DT104 (cocktail of strains DT104b, U302, DT104) (Uljas and Ingham, 1999). This study also looked at the effects of freeze-thawing in combination with sorbic acid and found that 12 hours at 25°C with freeze-thaw and 4 hours with freeze-thaw also produced a 5 log decrease in both pathogens.

Hydrogen Peroxide

Hydrogen peroxide rapidly degrades into oxygen and water in the presence of organic material. Microbial inactivation in the presence of hydrogen peroxide is rapid, owing to hydrogen peroxide's strong, non-preferential oxidizing nature (Block, 1991). It is used less frequently today than in the past due to undesirable effects on the food product, such as oxidation, bleaching, and most of all, loss of nutrients. It is still used for certain types of cheese-making at a concentration of 0.05% (Luck and Jager, 1997). Hydrogen peroxide holds GRAS status and is approved for use by the FDA for cheese, production of modified whey and for preparation of thermophile-free starch. The FDA also stipulates that residual hydrogen peroxide must be removed from the product during processing (CFR, 2004a).

Hydrogen peroxide has been studied at by Sapers and Simmons (1998) as a rinse for fresh fruits, vegetables and mushrooms. A 5% hydrogen peroxide solution was more effective in delaying spoilage than a 200 ppm chlorine wash on zucchini. Similar results were seen with other vegetables, fruits and mushrooms.

Venkitanarayanan et al. (1999) found that a 0.1% hydrogen peroxide had minimal effects on the reduction of *E. coli* O157:H7 after 30 minutes at 8°C, 20 minutes at 22°C and 10 minutes at 40°C in 0.1% peptone. Further, a 5 log reduction was not found after 30 minutes at 40°C (this was the most heat stress applied to the samples in this study). However, the addition of 1.0%

lactic acid enhanced the antimicrobial effect of both agents. This shows the potential hydrogen peroxide has for synergistic effects with other antimicrobial factors.

Dimethyl Dicarbonate (DMDC)

DMDC has a fruity aroma and is only slightly soluble in an aqueous solution (Davidson et al., 2002). It is unstable in an aqueous solution and hydrolyzes to small amounts of methanol and CO_2, natural components of fruit juice in small quantities. It is approved for use in wines in amounts up to 200 ppm, ready-to-drink tea in up to 250 ppm, and carbonated beverages up to 250 ppm. Historically it has been used as a "inhibitor of yeast" but recently the FDA has changed the wording to "microbial control agent" after the Bayer Corporation petitioned, showing evidence that DMDC is effective against *E. coli* O157:H7 (FDA, 2001b). In April of 2005, the LANXESS Corporation, formerly part of the Bayer Corporation, announced the FDA had approved the use of DMDC in flavored water and carbonated juice beverages containing up to 100% juice. The LANXESS Corporation markets Velcorin, the trade name for DMDC (LANXESS, 2005).

The microbial inactivation mechanism of DMDC has mostly been studied in yeasts. It is believed that inactivation of the microorganisms is closely associated with deactivation of enzymes. This is partially due to the fact that dicarbonates will readily react with imidazoles, amines, or thiol groups, all normal constituents of enzymes. Another source of inactivation may be through conformational changes or blocking of active sites that in turn will not allow the substrates to come in contact with the enzyme. Because enzymes are crucial to most cellular activities, the deactivation of one or more key enzymes can lead to cell death (Ough, 1993).

A study done by Bizri and Wahem (1994) found that DMDC (250 ppm) with or without the addition of citric acid was the most effective in controlling both fungi and bacteria when compared to citric acid alone and citric acid combined with both sodium benzoate and potassium sorbate (0.15% combined). This study was performed in tomato juice over the course of 56 days. However, DMDC also showed the most decrease in the amounts of ascorbic acid, total amino acid, fructose, glucose lycopene, and β-carotene, which are essential to the quality of the juice. It was also shown that there was a significant difference in color from both heat-treated and the control juice.

Fisher and Golden (1998b) tested a cocktail of two strains of *E. coli* O157:H7 (strain 380-94 and strain 7927) in apple juice. Three different storage temperatures were used (4, 10 and 25°C) and three different antimicrobial agents were tested (DMDC, sodium bisulfite and sodium benzoate). At 4°C, juice samples treated with 0.025% DMDC were found to have a 3.1 log decrease after 3 days. At 6 days *E. coli* was undetectable at the 1 log CFU/ml level, having a greater than 6 log reduction. At 10°C, *E. coli* was still detectable after 9 days in juice treated with DMDC, with a 3.8 log reduction. At 25°C survival was undetectable after 3 days.

Cinnamic Acid

The antimicrobial characteristics of cinnamon have been known and tested for some time, but it was only recently that specific derivatives, such as cinnamon aldehyde and cinnamic acid, were identified as main antimicrobial components (Davidson and Naidu, 2000). Cinnamic acid is also found in cranberries, prunes and cloves (Anslow et al., 2000). Cinnamic acid has been used as a flavoring agent in drinks, marinades, chewing gum and other foods, and it has GRAS status with the FDA (CFR, 2004b; Cirigliano et al., 2000). Several patents have been issued to Lipton for the use of cinnamic acid in ready-to-drink tea beverages as an alternative to high amounts of either benzoic acid or sorbic acid. Cinnamic acid is favored due to the undesirable flavors sorbic acid and benzoic acid can impart to delicately flavored tea beverages (Blyth et al., 2004; Anslow et al., 2000; Cirigliano et al., 2000). The most recently proposed combination for preservation of tea is 1 to 175 ppm cinnamic acid, 10 to 200 ppm of either sorbic acid or benzoic acid, and 1 to 100 ppm of another essential oil (Blyth et al., 2004). *Trans*-cinnamic acid is the most common naturally occurring conformation, and has therefore been the compound used for incorporation into the teas. Cinnamic acid is not readily soluble in an aqueous solution, and therefore other methods of incorporation must be employed, such as spraying a carrier powder, using a soluble salt form or dissolving in an organic solvent (Anslow et al., 2000).

The microbial inactivation mechanism of cinnamic acid is thought to be similar to other organic acids. The pK_a of cinnamic acid is between 4.37 and 4.44, and is most effective in the undissociated form. Once cinnamic acid is in the cell, it can dissociate and decrease the internal pH of the cell. The double bond found on the side chain is highly reactive and can cause damage inside the cell. Some organisms that have shown resistance to other organic acids may not be

resistant to cinnamic acid because of the reactivity of the double bond (Cirigliano, 2000). It has also been noted that cinnamic acid will prevent microorganisms from using the amino acids phenylalanine and tyrosine (Anslow, 2000).

Cinnamic acid has been shown to be a good inhibitor of yeast and molds at a low pH level (3.0). Without the cinnamic acid, beverages at this pH would still be susceptible to yeast and mold growth, but with the addition of 100 ppm, no growth is reported. However, at higher pH levels, this inhibition is not seen (Anslow et al., 2000). This relationship between pH and cinnamic acid makes it a good candidate for use in orange juice and apple juice where the average pH level for both juices typically falls below 4.0.

Dorantes et al. (2000) tested several extracts of serrano chili peppers, one of them being cinnamic acid, and found that of the four pathogenic bacteria strains tested, *S.* Typhimurium (ATCC 13311) was the least susceptible. The most susceptible was *B. cereus* with an 8.0 mm zone of inhibition, while *S.* Typhimurium only had a 2 mm zone.

Said et al., (2004) tested the effects of cinnamic acid on the growth of the fungus *Neurospora crassa*. They found that while other benzene compounds such as coumaric, catechol, salicylic, caffeic or ferulic acid did not have a significant impact on the growth of *N. crassa*, cinnamic acid reduced colony growth by 94%.

Bahk et al. (1990) used a growth media with 0.5% cinnamon to study the lag period, maximum growth and generation time of *Listeria monocytogenes* strain V7 at 4°C and 35°C. Cinnamon was effective at decreasing maximum growth and increasing generation time and lag period at both 4°C and 35°C. Maximum growth was around 2 logs below the control at both temperatures. At 35°C generation time was substantially increased to 1.6 hours, while the control was about 0.4 hours. It should be noted that this study used cinnamon as the spice, rather than any refined derivatives.

E. coli O157:H7 (ATCC 43888) showed a 52% growth inhibition when grown at 37°C for 12 hours in media containing 0.4 mL/L of cinnamic aldehyde. *Salmonella eneritidis* (ATCC13076) showed an 87% growth inhibition under the same conditions (Kwon et al., 2003).

Smith-Palmer (1998) showed the bacteriostatic concentration of cinnamon against both *E. coli* strain 8007 and *S.* Enteritidis strain 4444 to be 0.05%. The bacteriocidal concentration was found to be twice the bacteriostatic concentration for both *E. coli* and *S.* Enteritidis.

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High pressure resistance variation in *Escherichia coli* O157:H7 strains and *Salmonella* serovars in tryptic soy broth, distilled water and fruit juice

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ABSTRACT

The effect of high pressure on the log reduction of six strains of *Escherichia coli* O157:H7 (E0019, E009, E994, Cider, F4546, and H1730) and five serovars of *Salmonella enterica* (Agona, Baildon, Gaminara, Michigan and Typhimurium) was investigated in tryptic soy broth, sterile distilled water and commercially sterile orange (for *Salmonella*) and apple (for *E. coli*) juice. Samples were inoculated with approximately 10^6 CFU/ml and subjected to high hydrostatic pressure treatment at 300 and 550 MPa for 2 minutes at 6°C. Samples were plated onto TSA directly after pressurization and after being held for 24 hours at 4°C. At 300 MPa, little effect was seen on *E. coli* O157:H7, with the largest decrease (0.53 $\log_{10}$ CFU/ml) seen directly after pressurization. The *Salmonella* serovars varied in resistance showing reductions between 0.26 and 3.95 $\log_{10}$ CFU/ml. At 550 MPa, *E. coli* O157:H7 strains had a range of decrease from 0.28 to 4.39 $\log_{10}$ CFU/ml, while most *Salmonella* populations decreased beyond the detection limit, having a greater than 5-log decrease just after pressurization. The most resistant *E. coli* strain was E009, having a decrease of 0.77 log CFU/ml at 550 MPa in TSB directly after pressurization, while *S.* Agona was the most resistant *Salmonella* serovars tested having a decrease of 3.79 $\log_{10}$ CFU/ml at 550 MPa in TSB. Bacterial populations in fruit juices showed larger decreases than populations in TSB and distilled water with the exception of *Salmonella* serovars in orange juice at 300 MPa, whose populations showed minimal decrease. *E. coli* O157:H7 cultures held for 24 hours at 4°C following treatment at 550 MPa showed a significant log decrease compared to directly after treatment, while *Salmonella* serovars did not show this significant decrease ($P \leq 0.05$). All *Salmonella* serovars tested in orange juice treated at 550 MPa for 2 minutes at 6°C and held for 24 hours showed a greater than 5-log decrease, while *E. coli* O157:H7 strains require a higher pressure, temperature, longer pressurization, or chemical additive to achieve a 5-log decrease.

INTRODUCTION

The first studies on microbial inactivation by high hydrostatic pressure (HHP) occurred in the late 1890s (Hite, 1899), however the industry has only taken interest in the technology in the past several decades. HHP is a good alternative to heat pasteurization with studies showing that HHP does not have adverse affects on vitamins and other nutrient components while allowing retention of product flavor quality (Butz et al., 2003; Butz and Tauscher, 2002; Nienaber and Shellhammer, 2001; Parish, 1997; Sanchez-Moreno et al., 2003; Sancho et al., 1999). High pressure processing (HHP) is performed at hydrostatic pressures between 100 and 800 MPa, with temperatures ranging from below freezing to above boiling point and unlike traditional heat processes, the pressure is applied uniformly and is not dependent on shape (FDA, 2000). In the United States HHP is currently being used on items such as guacamole, salsa, fruit smoothies, and oysters (FDA, 2000); however some of the first HPP-treated products brought to any market were fruit products in the form of jams and jellies in Japan (Hayashi, 1997).

Fresh fruit juices have in the past been erroneously perceived as a safe product, and were pasteurized for the purpose of shelf-life rather than safety of the product. Several outbreaks of *Escherichia coli* O157:H7 and *Salmonella* associated with the consumption of fresh apple (Besser et al., 1993; CDC, 1997; CDC 1996; Cody et al., 1999) and orange juice (Cook et al., 1998; Parish, 1997; CDC 1999) in the 1990s prompted the FDA to mandate that all juice processors must have a hazard analysis critical control point (HACCP) plan that results in a 99.999% reduction of the pertinent pathogen in the juice being processed (FDA, 2001). HHP has been recognized as a potential processing technique that will accomplish the reduction without compromising the fresh quality of the juice (Parish, 1997; Butz and Tauscher, 2002).

Escherichia coli O157:H7 is an enteric pathogen with a low infectious does that usually causes enterohemoragic colitis, but has the potential to cause hemolytic uremic syndrome in young children and the immunocompromised (Boyce et al., 1995). *Salmonella* is a well-recognized enteric pathogen that usually infects with a self-limiting illness, but can lead to complications in the young, old and immunocompromised (CDC, 2003).

Benito et al. showed that *E. coli* O157:H7 strain C9490, a clinical isolate from the Jack-In-The-Box outbreak associated with hamburger, was significantly more pressure resistant that four other strains tested in phosphate-buffered saline (Benito et al., 1999). In the current study, a

beef isolate from the same outbreak, strain E009 was compared against five other *E. coli* O157:H7 strains from outbreaks that occurred in the United States. Five serovars of *Salmonella* from outbreaks in the United States were compared as well. This study was undertaken because it is important to identify the most pressure resistant strain of a species for use in subsequent studies, particularly in a targeted food item. Therefore, the current study compares the pressure resistance of eleven test cultures in three mediums: tryptic soy broth (TSB), distilled water, apple juice for (*E. coli* O157:H7 strains) and orange juice for (*Salmonella* strains).

MATERIALS AND METHODS

Cultures and culture maintenance. The following *E. coli* O157:H7 strains were obtained from Dr. Larry Beuchat (University of Georgia, Griffin): H1730, lettuce-associated outbreak; E0019, beef isolate; F4546, alfalfa sprout associated-outbreak; 994, salami isolate; Cider, apple juice isolate. Dr. Beuchat also provided the following *Salmonella* serovars: *S.* Agona, alfalfa-associated outbreak; *S.* Baildon, lettuce/tomato-associated outbreak; *S.* Gaminara, orange juice isolate; and *S.* Michigan, cantaloupe-associated outbreak. *E. coli* O157:H7 strain E009 (beef isolate) was provided by Dr. M. Doyle (University of Georgia, Griffin). *S.* Typhimurium ATCC 14028 was also used. Cultures were kept at -60°C in a 50% glycerol solution until use. Cultures were revived in Tryptic Soy Broth (TSB, Difco, Franklin Lakes, NJ) at 37°C for 24 hours and maintained on Tryptic Soy Agar (TSA, Difco, Franklin Lakes, NJ) slants kept at 4°C.

Preparation of inoculum. Cultures underwent two successive 24-hour transfers from stock slant to 10 ml TSB at 37°C before use in test media. Cultures used for experimentation were inoculated into 10 ml TSB and incubated at 37°C for 18 hours prior to use. Cells were harvested by centrifugation at 1225 x *g* for 10 minutes. Cells were resuspended in 10 ml 0.1% peptone water (PW) for a concentration of approximately 10^8 CFU/ml. Inoculas were kept at 4°C no longer than 5 hours prior to use.

Inoculation and treatment. Prepared TSB and distilled water were dispensed into glass bottles in 99 ml portions and autoclaved (121°C) for 25 minutes. Shelf-stable unfiltered apple

juice (pH = 3.7; °brix = 11.7) and orange juice (pH = 3.9; °brix = 12.1) without any added preservatives were purchased at a local grocery store in Blacksburg, Virginia. The juices were aseptically dispensed into sterile glass bottles in 99 ml portions. All test media was brought to 4°C prior to use. Test media were inoculated with 1 ml of inoculum. Each media was dispensed into four separate 76.2 μm nylon/polyethylene heat sealable bags (7.5 x 15 cm; Cryovac, Duncan, SC) in 5 ml portions. Bags were vacuum sealed to 70 mbar (Multivac, model A300/16, Kansas City, MO) and double bagged (3 bags total) with disinfectant in the outer bag to ensure no leakage of viable cells into the pressure chamber. Samples were kept on ice prior to pressurization. Two bags from each sample were pressure treated, while two served as controls. Samples were pressure treated in a 35 L pressure vessel with an internal diameter of 19 cm and a height of 1.22 meters (Quintus, model QFP 35L-600; Flow International, Kent, Washington, USA). The pressure medium was water pre-chilled in a separate tank that flowed directly into the chamber. Samples were run at 6°C ± 2 for 2 minutes. The adiabatic rise was approximately 3°C for every 100 MPa.

Enumeration of viable *E. coli* O157:H7 and *Salmonella*. One control and one treated sample from each strain/media combination was plated directly after pressurization, and one control and one treated sample were plated after 24 hours at 4°C. Pressurized and control samples were serially diluted in PW and 0.1 ml was plated in duplicate onto prepared TSA plates using the spread plate technique. Plates were incubated at 37°C for 48 hours and plates containing between 25 and 250 colonies were enumerated. The limit of detection was 10 CFU/ml. Log differences were calculated by averaging the duplicated plates, taking the log of each count and subtracting the treated from the control samples.

Statistical analysis. This experiment was performed in three replications. A split-split plot design was used to model the data. Log differences between control and treated samples were analyzed with the mixed procedure in SAS version 9.1 (SAS Institute, Cary, NC). The mean separation technique was the difference of least squared means with Tukey's adjustment. A P-value of 0.05 was used.

RESULTS

***E. coli* O157:H7 strains at 300 MPa (Table 1).** Pressure treatment at 300 MPa for 2 minutes at 6°C had little effect on the reduction of *E. coli* O157:H7 in TSB and distilled water. None of the strains showed a reduction greater than 0.5 $\log_{10}$ CFU/ml. In addition, no strains showed a significant decrease between 0 hour and 24 hours after pressure treatment ($P \leq 0.05$). In contrast all cultures showed a significantly higher decrease at 0 hours in apple juice when compared to water and except for F4546, all cultures treated in apple juice showed a significant decrease after 24 hours ($P \leq 0.05$). The most sensitive strain at this pressure was strain Cider, having a 0.53 and 1.58 $\log_{10}$ CFU/ml reduction at 0 hour and 24 hours in apple juice, respectively. Overall, populations reductions in TSB and water were not different, while population reductions in apple juice were significantly greater than reductions in both TSB and water ($P \leq 0.05$).

***E. coli* O157:H7 strains at 550 MPa (Table 2).** Pressure treatment at 550 MPa for 2 minutes at 6°C had a much greater effect on *E. coli* O157:H7 strains than did pressure treatment at 300 MPa. In apple juice at zero hours, the strain with the smallest mean reduction was E009 with a 1.25 $\log_{10}$ CFU/ml reduction, although this mean was not significantly different than the means for strains E0019, E994 and F4546 ($P \geq 0.05$). *E. coli* O157:H7 strain Cider was again the most sensitive to pressurization, having a 4.39 $\log_{10}$ reduction in apple juice at zero hours. Excluding media differences and hold time differences, the most pressure resistant was strain E009 which was significantly different from all but strain F4546 at $P \leq 0.05$, but was significantly different from that strain at $P \leq 0.10$. Excluding strain differences, there was no significant decrease in values between zero hours and 24 hours in TSB, however there was a significant decrease between zero and 24 hours in water and apple juice ($P \leq 0.05$). Overall there was not a significant difference between TSB and distilled water, while apple juice produced population reductions that were significantly greater than both TSB and water ($P \leq 0.05$).

***Salmonella* at 300 MPa (Table 3).** *Salmonella* strains varied widely in their response to 300 MPa for 2 minutes at 6°C in the various media. In TSB at zero hours, *S.* Agona had a significantly lower decrease than any other serovar, with a 0.53 $\log_{10}$ reduction ($P \leq 0.05$). There was no difference between strains seen in water or orange juice at this pressure ($P \geq 0.05$). The

most sensitive strain was *S.* Michigan with a decrease of 3.95 $\log_{10}$ CFU/ml in TSB at zero hours. For all media, there was no significant difference between zero hours and 24 hours ($P \leq 0.05$). Serovars in orange juice showed a significantly lower decrease in population numbers than both TSB and distilled water ($P \leq 0.05$). The average decrease at zero hours in TSB and water was 2.35 and 2.00, respectively, while the average $\log_{10}$ decrease in orange juice at zero hours was 0.42.

***Salmonella* at 550 MPa (Table 4).** A pressure treatment of 550 MPa for 2 minutes at 6°C was very effective in reducing the populations of *Salmonella* serovars in various media. *S.* Agona again had the arithmetically lowest mean reduction in TSB at zero hours, having a 3.79 $\log_{10}$ CFU/ml reduction; however this was not significantly different than the reduction seen with *S.* Baildon or *S.* Typhimurium ($P \geq 0.05$). After 24 hours in both water and orange juice, populations were undetectable (i.e., below 1 $\log_{10}$ CFU/ml), although, statistically there was no difference between zero hours and 24 hours in any of the media ($P \leq 0.05$). The most sensitive strain again was *S.* Michigan, with a 5.37 $\log_{10}$ reduction in TSB at zero hours, although this serovar was not significantly different from *S.* Gaminara or *S.* Baildon ($P \geq 0.05$). Overall, the mean reductions were not significantly different between water and orange juice, but the log decreases were significantly lower in TBS than either of the other two media.

DISCUSSION

***E. coli* O157:H7 Pressure studies.** In the current study the most pressure resistant strain of *E. coli* O157:H7 was the beef isolate from the Jack-In-The-Box outbreak strain E009. A clinical isolate from this outbreak, C9490 has been used for several pressure-challenge experiments, following the discovery of its pressure-resistance (Benito et al., 1999; Robey et al., 2001; Pagan et al., 2001). The results of the current study showed that at 550 MPa for 2 minutes at 6°C, strain E009 had a 0.77, 0.28, and 1.25 $\log_{10}$ CFU/ml decrease in TSB, distilled water and apple juice, respectively. These results concur with several experiments that have used strain C9490 (Benito et al., 1999; Robey et al., 2001; Pagan et al., 2001). In contrast to the current study and other studies cited, Jordan et al. (Jordan et al., 2001) used strain C9490 to test resistance in apple juice. They found that at 500 MPa for 5 minutes at 20°C, the population numbers had decreased

to beyond the detection limit, showing a greater than 5 $\log_{10}$ CFU/ml decrease. Variables in the two experiments were not the same, which could account for some differences. The most pressure sensitive strain tested was strain Cider, having a 4.39 $\log_{10}$ CFU/ml decrease in apple juice directly after a pressure treatment at 550 MPa for 2 minutes at 6°C; however, this strain is not as sensitive as several non-O157 strains that have been tested by others (Pagan et al., 2001; Linton et al., 2001; Robey et al, 2001).

The differences between 0 hour and 24 hours was not significant at the lower pressure level of 300 MPa. This could be attributed to the fact that this pressure was not very effective against *E. coli* O157:H7. At 550 MPa, significant decreases occurred between the initial sampling after the samples were held at 4°C for 24 hours in both distilled water and apple juice. The average additional decrease in apple juice was 2.02 $\log_{10}$ CFU/ml, while the decrease in water was 0.7 $\log_{10}$ CFU/ml. There was not a significant difference in TSB, perhaps because of its neutral environment that does not inflict any added stress to the cells. It is likely that holding the samples longer would increase the reduction; however it has been shown that the greatest reduction during a hold-time will occur within 24 hours (Jordan et al., 2001; Linton et al., 1999; Garcia-Graells et al, 1998).

Cultures pressurized in apple juice consistently showed a higher reduction than cultures pressurized in either TSB or distilled water at both 300 and 550 MPa. These results are consistent with similar studies conducted in fruit juices and in acidified buffers or growth media (Alpas et al., 2000; Pagan et al., 2001; Teo et al., 2001; Linton et al., 1999; Garcia-Graells, 1998). Acid resistant strains have been shown to be more resistant to pressurization; however a low pH still causes some stress to the cell (Benito et al., 1999). With the added stress of pressurization, the cells may perish, or be sublethally injured. Population reductions between distilled water and TSB were not significantly different. This shows that the osmotic stress that water places on the cells is not an important factor when dealing with pressurization.

***Salmonella* pressure studies.** Little significant difference was seen between serovars of *Salmonella* in orange juice and in distilled water at both 300 and 550 MPa. At 550 MPa, significant differences could not be established because many of the samples tested were below the limit of detection. In TSB, the most pressure resistant strain was *S.* Agona, having a 0.53 and 3.79 $\log_{10}$ decrease at 300 and 550 MPa immediately after pressurization, respectively. At 550

MPa, however, this decrease was not significantly different than the log decreases seen in *S.* Baildon and *S.* Typhimurium. Strain differences has been tested by Teo et al. (2001) and found that *S.* Typhimurium was the most pressure resistant at 615 MPa for 2 minutes at 15°C in apple juice, while *S.* Agona was the second most resistant serovar tested.

Statistically there was no difference between samples taken directly after pressurization and samples that were held for 24 hours at 4°C. This could show that the mechanism by which *Salmonella* is deactivated occurs during pressurization, rather than having an effect over time, as was seen with the *E. coli*. A study by Alpas and Bozoglu (2000) showed that *S.* Enteritidis and *S.* Typhi were undetectable after being pressurized at 345 for 5 minutes at 50°C, and after being held at 4°C for 24 hours, there was no recovery seen. The results presented in the current study are in agreement with those found by Alpas and Bozoglu (2000), having no recovery seen after 24 hours in samples where populations were below the limit of detection just after pressurization.

At 300 MPa, the differences between TSB and distilled water were not significant, while the differences between orange juice and both TSB and distilled water were significant. The average reduction immediately after pressurization was 2.38, 2.28 and 0.42 $\log_{10}$ CFU/ml in TSB, distilled water and orange juice, respectively. These results do not agree with published studies, which show that a more acidic environments cause a greater log reduction (Alpas et al., 2000; Pagan et al., 2001; Teo et al., 2001; Linton et al., 1999; Garcia-Graells, 1998), however those studies either used a higher pressure or a higher temperature, so the combined effect of low pressure and low temperature on *Salmonella* in orange juice has not been studied. A possible explanation for these unexpected results is that once the cells are placed in orange juice, a subsequent acid tolerance response mechanism may confer protection to pressurization. This seems unlikely due to the cold temperature at which the experiment was run, however, the relationship between acid tolerance and pressure resistance has been shown in *E. coli* O157:H7 by Benito et al. (Benito et al., 1999). In samples treated at 550 MPa, significant differences were seen between population reductions in TSB and both distilled water and orange juice. Distilled water and orange juice had higher population reductions: the phenomenon of orange juice conferring protection was not seen at the higher pressurization level. The results at this higher pressure are consistent with other studies using similar acidic conditions (Alpas et al., 2000; Alpas and Bozoglu, 2000; Teo et al., 2001).

Conclusions. The current study shows that pressurization at 550 MPa for 2 minutes at 6°C is not a sufficient process for achieving a 5-log reduction in *E. coli* O157:H7 in apple juice. This process was effective in reducing four of the five serovars of *Salmonella* to an acceptable level as stipulated by juice HACCP directly after pressurization, and with a hold time of 24 hours at 4°C, all serovars showed a greater than 5 $\log_{10}$ reduction. To achieve a 5-log reduction of *E. coli* O157:H7 strains in apple juice, several methods could be employed: the addition of a chemical antimicrobial, increasing the process time, temperature or pressure. Increasing the process temperature and pressure may not be commercially feasible, as processes should be optimized to have the shortest allowable process time, and pressure vessels that run above 600 MPa are more costly than those that run from 100 to 600 MPa.

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Table 1. *Population reductions of six* E. coli *O157:H7 strains pressurized at 300 MPa for 2 min at 6°C and stored for 24 hours at 4°C in TSB, distilled water and shelf-stable apple juice containing no added preservatives*

	Log_{10} CFU/ml reduction[a]					
	TSB		Distilled Water		Apple juice	
Strain	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
E0019	0.15 AB (6.73)[b]	0.26 B (6.64)	0.07 AB (6.78)	0.05 A (6.74)	0.13 A (6.74)	0.63 A (6.74)
E009	0.00 A (6.80)	-0.10 A (6.65)	0.01 AB (6.84)	0.05 A (6.74)	0.21 AB (6.86)	0.66 A (6.80)
E994	0.07 A (6.78)	0.07 AB (6.67)	-0.04 A (6.72)	0.04 A (6.69)	0.12 A (6.75)	0.44 A (6.74)
Cider	0.42 B (6.91)	-0.13 A (6.45)	0.30 B (6.95)	0.36 B (6.93)	0.53 C (6.75)	1.58 C (6.46)
F4546	0.07 A (6.66)	0.23 B (6.70)	0.04 AB (6.75)	0.11 AB (6.73)	0.23 ABC (6.71)	0.53 A (6.65)
H1730	0.27 AB (6.78)	0.29 B (6.56)	0.10 AB (6.81)	0.27 AB (6.87)	0.44 BC (6.80)	1.27 B (6.69)

[a] Mean value, N=3. Values in the same column with the same letter are not significantly different ($P \leq 0.05$)

[b] Values in parentheses represent the populations (log_{10} CFU/ml) of control samples

Table 2. *Population reductions of six* E. coli *O157:H7 strains pressurized at 550 MPa for 2 min at 6°C and stored for 24 hours at 4°C in TSB, distilled water and shelf-stable apple juice containing no added preservatives*

	Log_{10} CFU/ml reduction[a]					
	TSB		Distilled Water		Apple juice	
Strain	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
E0019	1.30 AB (6.79)[b]	1.73 AB (6.69)	0.37 A (6.80)	1.53 A (6.82)	1.61 A (6.89)	4.02 AB (6.78)
E009	0.77 A (6.82)	1.77 AB (6.62)	0.28 A (6.84)	0.54 A (6.80)	1.25 A (6.85)	3.24 A (6.80)
E994	1.05 AB (6.80)	1.44 A (6.66)	0.57 A (6.82)	1.49 A (6.78)	1.81 A (6.83)	4.28 BC (6.76)
Cider	2.80 C (6.80)	2.59 B (6.40)	3.18 C (6.86)	2.98 B (6.86)	4.39 C (6.74)	>5.57[c] (6.57)
F4546	1.25 AB (6.84)	1.65 AB (6.67)	0.68 AB (6.82)	1.37 A (6.78)	1.90 AB (6.81)	3.34 ABC (6.78)
H1730	1.98 BC (6.56)	1.93 AB (6.36)	1.63 B (7.24)	3.01 B (6.87)	2.77 BC (6.88)	5.23 C (6.81)

[a] Mean value, N=3. Values in the same column with the same letter are not significantly different ($P \leq 0.05$)

[b] Values in parentheses represent the populations (log_{10} CFU/ml) of control samples

[c] Difference between control population and limit of detection (1 log_{10} CFU/ml)

Table 3. *Population reductions of five* Salmonella *serovars pressurized at 300 MPa for 2 min at 6°C and stored for 24 hours at 4°C in TSB, distilled water and shelf-stable orange juice containing no added preservatives*

	Log_{10} CFU/ml reduction[a]					
	TSB		Distilled Water		Orange Juice	
Serovar	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Agona	0.53 A (6.94)[b]	0.55 A (6.70)	2.07 A (6.94)	2.20 A (6.80)	0.26 A (6.89)	0.61 A (6.75)
Baildon	2.40 B (6.89)	2.00 BC (6.68)	2.39 A (6.88)	2.78 A (6.54)	0.36 A (6.90)	0.41 A (6.48)
Gaminara	2.00 B (6.56)	1.07 AB (6.07)	1.72 A (6.87)	2.28 A (6.82)	0.62 A (6.66)	0.83 A (6.79)
Michigan	3.95 C (6.87)	4.06 D (6.93)	1.78 A (7.00)	1.67 A (7.00)	0.54 A (7.09)	0.75 A (6.87)
Typhimurium	3.04 BC (6.79)	2.48 C (6.76)	2.52 A (6.87)	2.26 A (6.87)	0.32 A (6.82)	0.86 A (6.78)

[a] Mean value, N=3. Values in the same column with the same letter are not significantly different ($P \leq 0.05$)

[b] Values in parentheses represent the populations (log_{10} CFU/ml) of control samples

Table 4. *Population reductions of five* Salmonella *serovars pressurized at 550 MPa for 2 min at 6°C and stored for 24 hours at 4°C in TSB, distilled water and shelf-stable orange juice containing no added preservatives*

	Log_{10} CFU/ml reduction[a]					
	TSB		Distilled Water		Orange Juice	
Serovar	0 hr	24 hr	0 hr	24 hr	0 hr	24 hr
Agona	3.79 A (6.95)[b]	3.29 A (6.81)	>6.02[c] AB (7.02)	>5.99 (6.99)	5.28 A (6.89)	>5.89 (6.89)
Baildon	4.63 ABC (6.92)	3.95 AB (6.85)	>5.95 AB (6.95)	>5.95 (6.95)	>5.82 A (6.82)	>5.80 (6.80)
Gaminara	5.21 BC (6.67)	4.70 BC (6.59)	5.06 A (6.77)	>5.93 (6.93)	4.88 A (6.57)	>5.70 (6.70)
Michigan	5.37 C (6.99)	5.45 C (6.95)	>6.05 B (7.05)	>5.91 (6.91)	>5.80 A (6.80)	>5.97 (6.97)
Typhimurium	4.33 AB (6.97)	4.04 AB (6.93)	>5.96 AB (6.96)	>5.90 (6.90)	5.62 A (6.80)	>5.78 (6.78)

[a] Mean value, N=3. Values in the same column with the same letter are not significantly different ($P \leq 0.05$)

[b] Values in parentheses represent the untreated samples

[c] Difference between control population and limit of detection (1 log_{10} CFU/ml)

Efficacy of high pressure processing in combination with chemical preservatives for the reduction of *Escherichia coli* and *Salmonella* in apple juice and orange juice

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ABSTRACT

The effect of high hydrostatic pressure (HHP) in conjunction with chemical antimicrobials (dimethyl dicarbonate [DMDC] at 62.5 and 125 ppm, hydrogen peroxide at 150 and 300 ppm, cinnamic acid at 125 and 250 ppm, potassium sorbate [KS] at 500 and 1000 ppm and sodium benzoate [NaB] at 500 and 1000) on *E. coli* O157:H7 strain E009 and *Salmonella enterica* serovar Agona was investigated in apple juice and orange juice, respectively. Samples were inoculated with approximately 10^6 CFU/ml juice and subjected to pressures of 550 MPa (*E. coli* O157:H7 samples) and 400 MPa (*S.* Agona samples) for 2 minutes at 6°C. Samples were held for 24 hours at 4°C following treatment. For the *E. coli* O157:H7 samples, the most effective treatment directly after pressurization was DMDC at 125 ppm, which caused 0.81 log reduction without HHP treatment, and with pressure treatment caused an additional 4.98 log reduction. Other treatments that were significantly different from the sample with no added antimicrobial were DMDC at 62.5 ppm, hydrogen peroxide at 300 ppm and NaB at 500 ppm with a total log reduction of 4.97, 5.79, and 3.91, respectively. After 24 hours at 4°C, none of the samples, including the sample with no antimicrobials showed growth. In samples with *Salmonella*, the most effective treatment was DMDC at a level of 62.5 ppm, with a log decrease of 5.96 directly following pressure treatment. NaB at 1000 ppm was also significantly different from the sample containing no antimicrobials with a log decrease of 3.26. After 24 hours at 4°C, all samples with added antimicrobials had a close to or above 5-log total reduction in *S.* Agona. DMDC should be further investigated as an antimicrobial agent that can work in conjunction with HHP.

INTRODUCTION

High hydrostatic pressure (HHP) is a processing technique by which pressures between 100 and 800 MPa are applied to a food to increase its safety and shelf-life. HHP is a good alternative to heat pasteurization with studies showing that HHP does not have adverse affects on vitamins and other nutrient components while allowing retention of product flavor quality (Butz et al., 2003; Butz and Tauscher, 2002; Nienaber and Shellhammer, 2001; Parish, 1997; Sanchez-Moreno et al., 2003; Sancho et al., 1999). In the United States HHP is currently being used on items such as guacamole, salsa, fruit smoothies, and oysters (FDA, 2000)

Fresh fruit juices have in the past been erroneously perceived as a safe product, and were pasteurized for the purpose of shelf-life rather than safety of the product. Several outbreaks of *Escherichia coli* O157:H7 and *Salmonella* associated with the consumption of fresh apple (Besser et al., 1993; CDC, 1997; CDC 1996; Cody et al., 1999) and orange juice (Cook et al., 1998; Parish, 1997; CDC 1999) in the 1990s prompted the FDA to mandate that all juice processors must have a hazard analysis critical control point (HACCP) plan that results in a 99.999% reduction of the pertinent pathogen in the juice being processed (FDA, 2001). HHP has been recognized as a potential processing technique that will accomplish the reduction without compromising the fresh quality of the juice (Parish, 1997; Butz and Tauscher, 2002).

Escherichia coli O157:H7 is an enteric pathogen with a low infectious dose that usually causes enterohemoragic colitis, but has the potential to cause hemolytic uremic syndrome in young children and the immunocompromised (Boyce et al., 1995). *Salmonella* is a well-recognized enteric pathogen that usually infects with a self-limiting illness, but can lead to complications in the young, old and immunocompromised (CDC, 2003).

To decrease processing time and pressure to save on energy costs, the use of various antimicrobials in conjunction with HHP has been investigated (Ogawa et al., 2000; Karatzas et al., 2001; Alpas and Bozoglu, 2000; Garcia-Graells et al., 2003; Masschalck et al, 2000). The current study uses potassium sorbate and sodium benzoate, hydrogen peroxide, dimethyl dicarbonate (DMDC), and cinnamic acid. Potassium sorbate and sodium benzoate are two antimicrobial agents that have long been in use, are currently used in fruit juices as preservatives, and have been shown to be effective in reducing populations of *E. coli* O157:H7 and *Salmonella*, although benzoates have been shown to be more effective than sorbates (Fisher and Golden,

1998; Miller and Kaspar, 1994; Zhao et al., 1993; Uljas and Ingham, 1999). Hydrogen peroxide has been used more in the past than it has today due to bleaching effects and adverse effects on vitamins (Luck and Jager, 1997), however it has been shown to be effective against spoilage microorganisms (Sapers and Simmons, 1998) and *E. coli* O157:H7 in conjunction with acid (Venkitanarayanan et al., 1999). In the current study, hydrogen peroxide was not used in orange juice due to its bleaching effects. DMDC has been widely used in the wine industry as a yeast inhibitor, and recently the food industry has been looking into the use of it as an antibacterial additive (LANXESS, 2005; Fisher and Golden, 1998). Cinnamic acid is an antimicrobial compound found in cinnamon, prunes and cloves. Historically it has been used as a flavor additive, but its use as an antimicrobial is being studied (Cirigliano et al., 2000; Blyth et al., 2004; Dorantes et al., 2000). Studies using cinnamic acid have typically used the *trans* isomer, however due to solubility problems, a potassium salt was used in the current study which was only available in the *cis* form.

This study assessed the effectiveness of these antimicrobials DMDC at 62.5 and 125 ppm, hydrogen peroxide at 150 and 300 ppm, cinnamic acid at 125 and 250 ppm, potassium sorbate [KS] at 500 and 1000 ppm and sodium benzoate [NaB] at 500 and 1000) in conjunction with HHP on *E. coli* O157:H7 strain E009 and *S.* Agona in apple juice and orange juice, respectively. Both of these cultures have been shown to be pressure resistant (Whitney, 2005).

MATERIALS AND METHODS

Cultures and culture maintenance. *E. coli* O157:H7 strain E009 (beef isolate) was provided by Dr. M. Doyle (University of Georgia, Griffin). *S.* Agona (alfalfa-associated outbreak) was provided by Dr. Larry Beuchat (University of Georgia, Griffin). Cultures were kept at -60°C in a 50% glycerol solution until use. Cultures were revived in Tryptic Soy Broth (TSB, Difco, Franklin Lakes, NJ) at 37°C for 24 hours and maintained on Tryptic Soy Agar (TSA, Difco, Franklin Lakes, NJ) slants kept at 4°C.

Preparation of inoculum. Cultures underwent two successive 24-hour transfers from stock slant to 10 ml TSB at 37°C before use in test media. Cultures were inoculated into 10 ml TSB and incubated at 37°C for 18 hours prior to use. Cells were harvested by centrifugation at 1225 x

g for 10 minutes. Cells were resuspended in 10 ml 0.1% peptone for a concentration of approximately 10^8 CFU/ml. Inoculums were kept at 4°C not longer than 5 hours prior to use.

Inoculation and treatment. Shelf-stable unfiltered apple juice (pH = 3.7; °brix = 11.7) and orange juice (pH = 3.9; °brix = 12.1) without any added preservatives were purchased at a local grocery store in Blacksburg, Virginia. The juices were aseptically dispensed into sterile glass bottles in 99 ml portions. Juice was brought to 4°C prior to use. The following chemicals were added to make the appropriate concentration in each bottle of juice: *cis*-cinnamic acid potassium salt (125 and 250 ppm), (Sigma-Aldrich, St. Louis, MO), dimethyl dicarbonate (62.5 and 125 ppm) (DMDC, Sigma-Aldrich, St. Louis, MO), 30% hydrogen peroxide (150 and 300 ppm) (Fisher, Hampton, NH), potassium sorbate (500 and 1000 ppm) (Fisher, Hampton, NH), and sodium benzoate (500 and 1000 ppm) (Fisher, Hampton, NH). Juice bottles with added antimicrobial were inoculated with 1 ml of inoculum. Apple juice was inoculated with *E. coli* O157:H7 strain E009 and orange juice was inoculated with *S.* Agona. Each inoculated juice sample was dispensed into four separate 76.2 μm nylon/polyethylene heat sealable bags (15 x 20 cm; Cryovac, Duncan, SC) in 20 ml portions. Bags were vacuum sealed to 70 mbar (Multivac, model A300/16, Kansas City, MO) and double bagged (3 bags total) with disinfectant in the outer bag to ensure no leakage of viable cells into the pressure chamber. Samples were kept on ice prior to pressurization. The time between inoculation of the samples and pressure treatment was not longer than 1 hour. Two bags from each sample were pressure treated, while two served as controls. Samples were pressure treated in a 35 L pressure vessel with an internal diameter of 19 cm and a height of 1.22 meters (Quintus, model QFP 35L-600; Flow International, Kent, Washington, USA). The pressure medium was water, pre-chilled in a separate tank that flowed directly into the chamber. *E. coli* O157:H7 samples were run at 550 MPa and *Salmonella* samples at 400 MPa for 2 minutes at 6°C ± 2. The adiabatic rise was approximately 3°C for every 100 MPa.

Enumeration of viable *E. coli* O157:H7 and *Salmonella*. One non-pressure treated and one pressure treated portion from each sample were plated directly after pressurization, and one non-pressure treated and one pressure treated portion were plated after holding for 24 hours at 4°C. Pressurized and non-pressure treated samples were serially diluted in buffered peptone water

(BPW) and 1 ml was plated in duplicate onto aerobic plate count Petrifilm (3M, St. Paul, MN). Selective plating was not used to allow for maximum recovery of cells. Plates were incubated at 37°C for 48 hours and plates containing between 20 and 200 colonies were enumerated. The limit of detection was 1 $\log_{10}$ CFU/ml because samples were not plated at the 10^0 concentration due to the sample acidity. Log differences were calculated by averaging the duplicate plates, taking the log of each count and subtracting the treated from the control samples.

Statistical analysis. This experiment was performed in three replications. A split-split plot design was used to model the data. Log differences between control and treated samples were analyzed the mixed procedure of SAS version 9.1 (SAS Institute, Cary, NC). The mean separation technique was the difference of least squared means with Tukey's adjustment. A P-value of 0.05 was used.

RESULTS & DISCUSSION

Effects of HHP in conjunction with antimicrobials on *E. coli* O157:H7 in apple juice (Table 1). There were four antimicrobial/level combinations that, in combination with HHP, produced a significantly higher reduction than the control sample that contained no added antimicrobial: DMDC at both 62.5 and 125 ppm, hydrogen peroxide at 300 ppm, and NaB at 500 ppm. DMDC has been shown to be effective in apple juice over a period of several days against *E. coli* O157:H7, decreasing counts by about 3 $\log_{10}$ CFU/ml (Fisher and Golden, 1998). The results of this experiment show an immediate decrease of about 1 $\log_{10}$ CFU/ml at the level of 125 ppm without pressure treatment (data not shown). Hydrogen peroxide also caused an immediate significant decrease in population counts when used at a concentration of 300 ppm ($P \leq 0.05$). No other antimicrobials showed a significant decrease directly following the addition of the antimicrobial ($P \leq 0.05$). DMDC and hydrogen peroxide degrade fairly rapidly in a food matrix, and therefore in order to be effective must exert their antimicrobial properties soon after addition to the food.

KS and NaB were both more effective at reducing population numbers when used at a concentration of 500 ppm. Log_{10} reductions were 2.47 and 3.88 in KS and NaB at concentrations of 500 ppm, respectively. Both of these values were statistically different from

the log reductions when the antimicrobial was used at a concentration of 1000 ppm ($P \leq 0.05$). Past studies using these two antimicrobials have typically used one concentration, and found that sodium benzoate was more effective than potassium sorbate at reducing *E. coli* O157:H7 populations over a period of time (Zhao et al., 1993; Fisher and Golden, 1998; Uljas and Ingham, 1999). The reasons for this observed effect are unclear.

When held for 24 hours at 4°C, all samples, including the sample with no added antimicrobial were undetectable at the 1 log cfu/ml level (data not shown). Due to this fact, no comparisons between different antimicrobials can be made. This reduction shows that a hold time of 24 hours at 4°C following pressurization at 550 MPa may be sufficient to cause a 5-log reduction of *E. coli* O157:H7 populations in apple juice, but this process should be tested using several recovery techniques.

Combined effects of chemical antimicrobials and HHP on *E. coli* (Figure 1). Combined effects were seen with DMDC at both concentrations, hydrogen peroxide at 300 ppm and NaB at 500 ppm. The effects of the antimicrobial by itself and the HHP by itself were added and compared to the effect of the two combined. The combined effect was most pronounced in samples treated with DMDC; the combined effect was about two times greater than the additive effects of the individuals, having a 4.97 and 5.79 log decrease at concentrations of 62.5 ppm and 125 ppm, respectively. NaB at 500 ppm also shows about twice as much log decrease, with the overall $\log_{10}$ decrease being 3.91. Hydrogen peroxide showed some combined effect at 150 ppm, with about a log increase over the additive effects of the individual treatments. Hydrogen peroxide at the concentration of 300 ppm had a large effect on the population reduction without the HHP, and with HHP, had a 5.71 $\log_{10}$ decrease. Studies have shown that HHP disrupts the membrane (Chilton et al., 1997) and may allow for the entry of the antimicrobials into the cells at a faster rate than would occur without HHP. Because DMDC and hydrogen peroxide act quickly, these antimicrobial effects can be seen directly after pressurization. As previously stated, NaB has been shown to be effective against *E. coli* O157:H7 over a period of several days.

Effects of antimicrobials in conjunction with HHP on *Salmonella* serovar Agona in orange juice (Table 2). Only two antimicrobial-level combinations had a significantly higher log reduction than the sample containing no antimicrobial just after pressurization. While KS at a concentration of 500 ppm had a lower reduction than the sample with no antimicrobial, it was

not significantly different. The two antimicrobial-level combinations that were significantly different were DMDC at 62.5 ppm and NaB at 1000 ppm with a greater than 5.74 and 3.03 $\log_{10}$ reduction in populations, respectively. The effects of DMDC against *Salmonella* are not well known, and at the low concentration of 62.5 ppm, there was no significant decrease in population counts without the pressure treatment, even after 24 hours ($P \leq 0.05$). Only the lower level of DMDC was tested because at this level with pressurization, the population decreased beyond the limit of detection.

There was no significant difference in log decrease between the two levels used for cinnamic acid and the two levels for NaB. KS had a significantly larger population reduction when used at 1000 ppm than at 500 ppm. KS has been shown to be effective against *Salmonella*, having a 5-log decrease in apple juice with 0.1% KS after 6 hours at 35°C (Uljas and Ingham, 1999).

After 24 hours at 4°C, non-pressure treated samples were not significantly different from one another regardless of the antimicrobial added ($P \leq 0.05$). Pressure treated samples with antimicrobials all had a significantly higher reduction than the sample with no added antimicrobial. Of the samples treated with an antimicrobial, all but KS at 1000 ppm (4.84 $\log_{10}$ CFU/ml reduction) exhibited a greater than 5-log reduction. Differences between non-pressure-treated samples at 0 hour and 24 hours were not significant, showing that none of the antimicrobial-level treatments used had an effect on populations of *Salmonella* after 24 hours at 4°C without pressure treatment ($P \leq 0.05$).

Directly after pressurization, the two antimicrobial-level combinations that caused a combined effect were DMDC at 62.5 ppm and NaB at 1000 ppm, having a 2.19 and 2.2 $\log_{10}$ reduction when used separately, and having a >5.96 and 3.26 $\log_{10}$ reduction when the antimicrobial and HHP are used in conjunction, respectively. After 24 hours at 4°C, all antimicrobial-level combinations had a pronounced combined effect, all having around a two or greater log difference between the antimicrobial and pressure used alone and the antimicrobial and pressure used in conjunction (Figure 2). HHP may cause membrane permeability (Chilton et al., 1997), allowing the antimicrobials to enter the cells and cause destruction. DMDC acts in a rapid manner, and this may explain why its effects were seen directly after pressurization. The organic acids take time to disrupt the pH and cause enzyme inhibition, and their effects were only seen after a 24 hour hold time.

Conclusion. The use of HHP in conjunction with chemical antimicrobials is an effective way of reducing populations of both *Salmonella* and *E. coli* O157:H7 in orange and apple juice, respectively. For all antimicrobials tested, a greater than 5 log reduction was seen in orange juice inoculated with *Salmonella* following a pressurization at 400 MPa for 2 minutes at 6°C and a hold time of 24 hours at 4°C. The antimicrobial most effective at reducing populations without the hold time was DMDC, having reductions of *E. coli* O157:H7 and *Salmonella* near 5-logs at concentrations of 125 and 62.5 ppm, respectively. Further studies should optimize the amounts of antimicrobials, time and pressures needed to incur a 5-log reduction of *E. coli* O157:H7 and *Salmonella* in apple juice and orange juice, respectively.

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Table 1. *Population reductions of* E. coli *O157:H7 strain E009 in shelf-stable apple juice treated with chemical antimicrobials and pressure treated at 550 MPa for 2 minutes at 6°C*

		Log_{10} CFU/ml reduction[a]
Antimicrobial	Level (ppm)	Population reduction
Cinnamic Acid	125	1.24 (6.81)[b] AB
Cinnamic Acid	250	1.03 (6.77) A
DMDC	62.5	4.73 (6.55) D
DMDC	125	>4.98[c] (5.98) D
HP	150	2.72 (6.73) CD
HP	300	>3.71 (4.71) D
K sorbate	500	2.47 (6.78) BCD
K sorbate	1000	0.87 (7.21) A
Na benzoate	500	3.88 (6.76) D
Na benzoate	1000	1.36 (7.22) AB
No Antimicrobial	n/a	1.92 (6.79) ABC

[a] Mean value, N = 3. Values in the same column with the same letter are not significantly different ($P \leq 0.05$)

[b] Values in parentheses represent populations (log_{10} CFU/ml) of samples treated with the antimicrobial, but not pressure treated

[c] Difference between control population and limit of detection (1 log_{10} CFU/ml)

Table 2. *Population reductions of* Salmonella enterica *serovar Agona in shelf-stable orange juice treated with chemical antimicrobials and pressure treated at 400 MPa for 2 minutes at 6°C then stored for 24 hours at 4°C.*

		Log CFU/ml reduction[a]	
Antimicrobial	Level (ppm)	0 hour	24 hours
Cinnamic Acid	125	2.50 (6.70)[b] BC	5.11 (6.68) B
Cinnamic Acid	250	2.84 (6.77) BC	5.31 (6.63) B
DMDC	62.5	>5.74[c] (6.74) D	>5.52 (6.52) B
K sorbate	500	1.49 (6.75) A	4.84 (6.70) B
K sorbate	1000	2.64 (7.08) BC	5.59 (6.65) B
Na benzoate	500	2.24 (6.75) ABC	5.17 (6.39) B
Na benzoate	1000	3.03 (6.73) C	>5.28 (6.28) B
No Antimicrobial	n/a	1.97 (6.96) AB	2.86 (6.77) A

[a] Mean value, N = 3. Values in the same column with the same letter are not significantly different ($P \leq 0.05$)

[b] Values in parentheses represent populations ($\log_{10}$ CFU/ml) of samples treated with the antimicrobial, but not pressure treated

[c] Difference between control population and limit of detection (1 $\log_{10}$ CFU/ml)

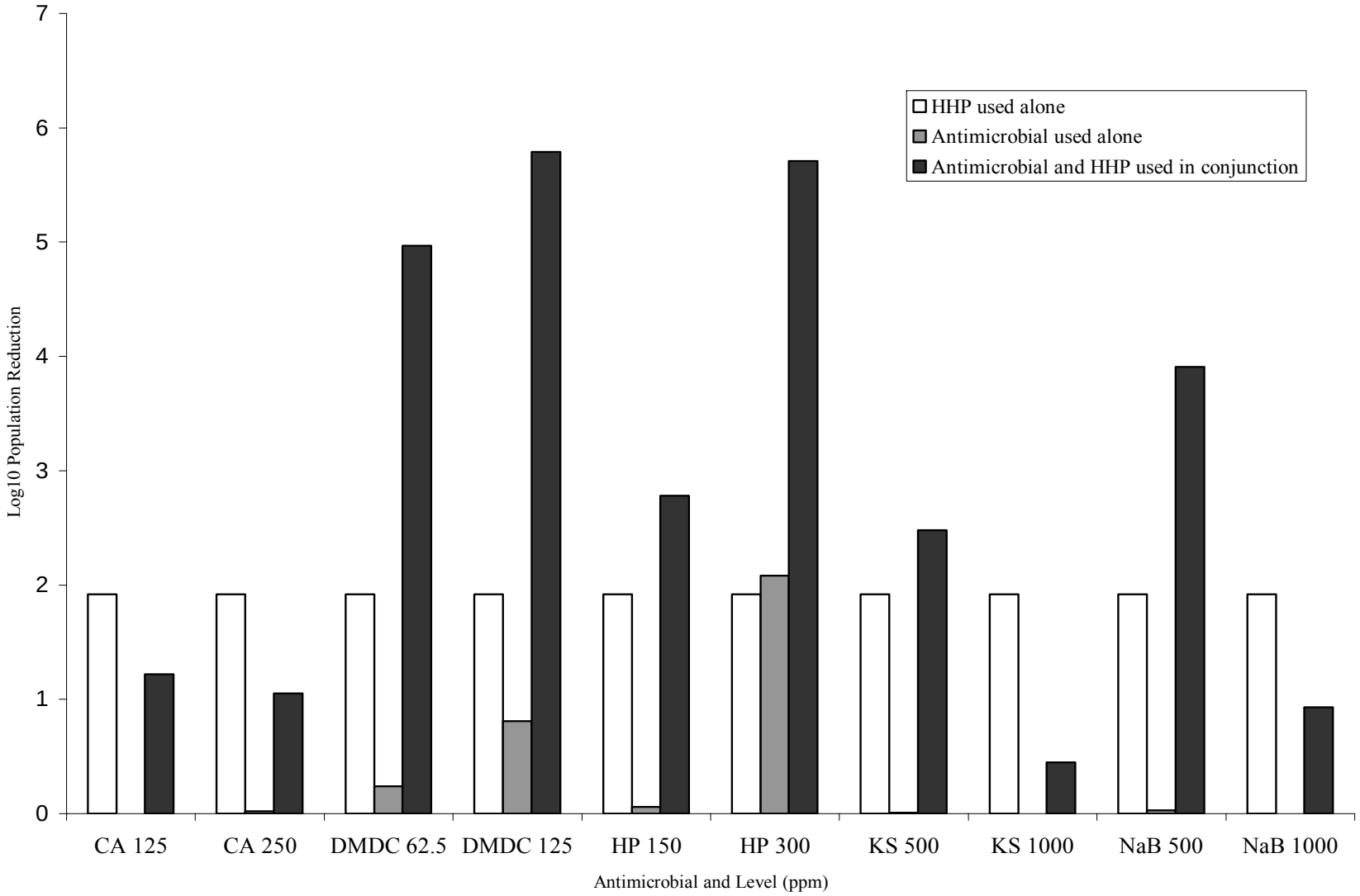


Figure 1. Combined effect of pressure treatment at 550 MPa for 2 minutes at 6°C in conjunction with antimicrobials on *E. coli* O157:H7 strain E009 in shelf-stable apple juice

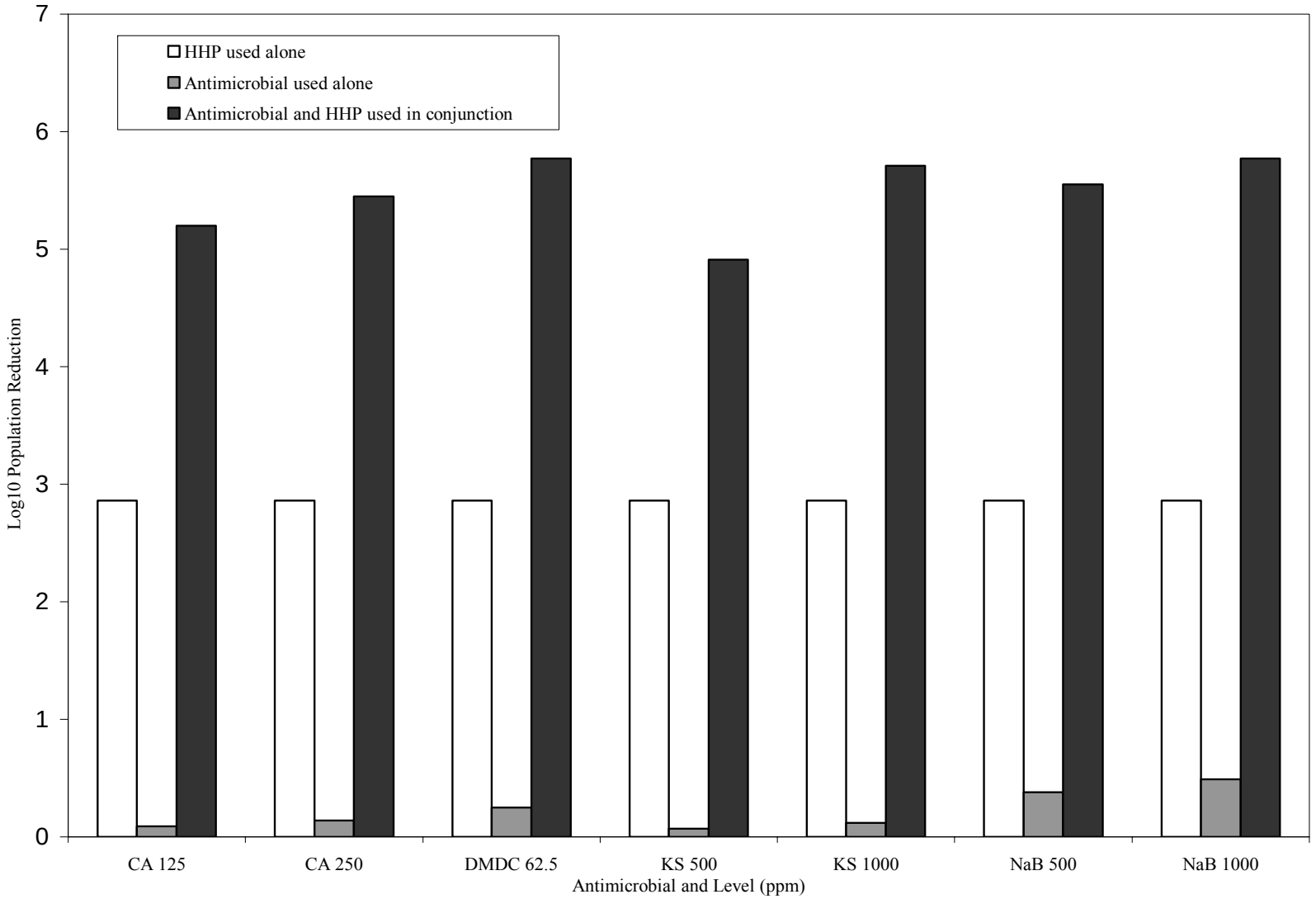


Figure 2. Combined effect of pressure treatment at 400 MPa for 2 minutes at 6°C in conjunction with antimicrobials on *Salmonella enterica* serovar Agona after storage at 4°C for 24 hours in shelf-stable orange juice

SUMMARY

Fresh, unpasteurized fruit juices have in the past been perceived as safe due to their low pH. However, in the 1990s several outbreaks associated with unpasteurized juices were documented. The suspect organisms were *Salmonella* spp., *E. coli* O157:H7 and *Cryptosporidium*. These outbreaks prompted the FDA to implement the juice HACCP regulation, stipulating that juices must undergo a process by which the pertinent pathogens are reduced by 99.999% or 5 logs.

In recent decades, HHP has received renewed interest as an alternative to heat pasteurization which can cause reduction in vitamins and other nutrients as well as having associated flavor changes. HHP has been shown to retain the nutritional quality of the foods as well as preserving its "fresh" quality.

The objectives of this study were the following: to determine the pressure resistance of five *Salmonella* serovars and six *E. coli* O157:H7 strains in tryptic soy broth, distilled water and fruit juice, to determine combined effects between chemical antimicrobials and high pressure processing, and to determine optimal pressure for producing a 5-log pathogen reduction in fruit juices.

The first objective was met and it was found that *E. coli* O157:H7 strain E009 was the most pressure resistant, having a log decrease of 0.77 $\log_{10}$ CFU/ml directly after pressurization in TSB. *S.* Agona was the most pressure resistant serovar tested with a log decrease of 3.79 $\log_{10}$ CFU/ml in TSB at 550 MPa.

Combined effects were seen in *E. coli* O157:H7 samples directly after pressure treatment treated with DMDC at both 62.5 and 125 ppm, hydrogen peroxide at 300 ppm and in NaB at 500. Combined effects was undeterminable after 24 hours at 4°C, due to the samples with no antimicrobial added dropping below the limit of detection of 1 $\log_{10}$ CFU/ml. Samples inoculated with *Salmonella* showed synergistic effects directly after pressure treatment with DMDC at 62.5 ppm and with NaB at 1000 ppm. After 24 hours at 4°C, synergistic effects were seen in all samples with an added antimicrobial, having close to or above a 5-log decrease in population numbers.

As for the third objective, a pressure treatment of 550 MPa for 2 minutes at 6°C, followed by a hold time of 24 hours at 4°C caused a 5-log reduction of *Salmonella* in orange juice. These parameters did not cause a 5-log reduction in all the *E. coli* O157:H7 strains tested,

and therefore the optimum pressure treatment for a 5-log reduction is higher for the low temperature used. Other studies have shown that increasing the temperature of either the pressure treatment or the hold time will increase the effectiveness of the HHP on *E. coli* O157:H7.

DMDC was found to be highly effective against both *Salmonella* and *E. coli* O157:H7 in conjunction with HHP. Research is needed into the effects of this antimicrobial on the constituents of the juices, such as vitamins and flavor attributes. More research is also needed in order to optimize the amount of antimicrobial used, the pressure, time and temperature of processing to minimize processing costs.

Appendix A – High pressure equipment specifications

Quintus Food Press QFP 35L-600
7XS-6000 Intensifier Pump

Operating temperature: 40-95°F (4-35°C) (excluding adiabatic temperature rise)
Temperature control accuracy: ±4.5°F (±2.5°C)

Process pressure range: 14,500 – 87,000 psi (100 – 600 MPa)

Cycle time: approximately 5 minutes at 87,000 psi (excluding hold time and loading/unloading)

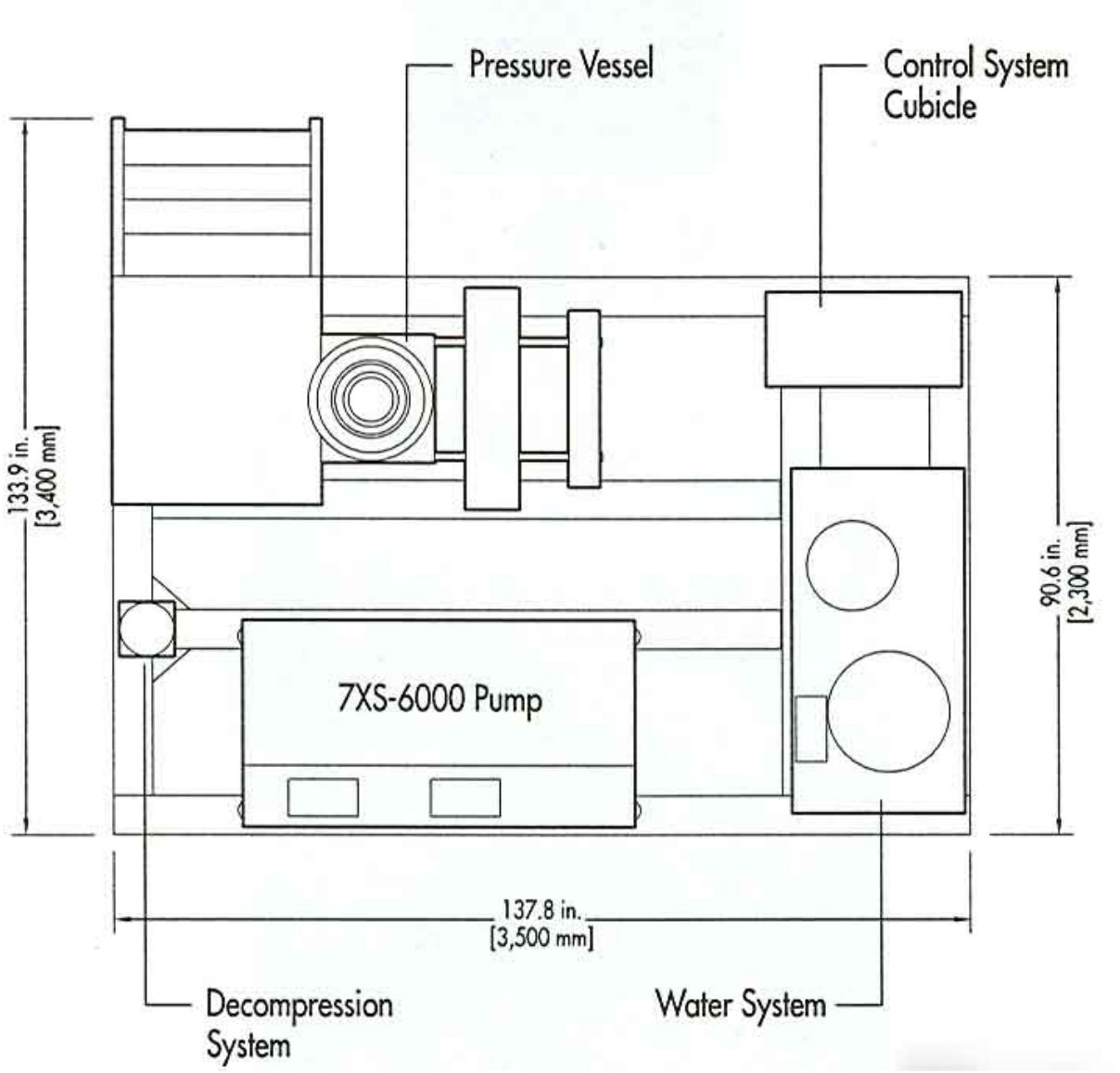
Maximum hold time: 15 minutes

Process medium: water

Overall dimensions:
Maximum height (pressure vessel) 11.5 ft (3.5 m)
Height to hook (for loading and unloading baskets) – 13.0 ft (3.9 m)
Total press weight – 17,600 lbs (8,000 kg)
Pressure vessel volume – 9.25 gal (35 L)
Internal diameter – 7.5 in (190 mm)
Internal height – 48.0 in (1,220 mm)

Basket dimensions
Regular basket
Internal diameter – 6 ¾ in
Height – 46 in
Liner and basket
Internal diameter – 5 ¾ in
Height – 45 in

Appendix B – Diagram of high pressure apparatus



VITA

Brooke Meredith (Hettenhouser) Whitney was born in South Carolina and raised in Vienna, Virginia where she attended James Madison High School. After graduating high school, she began as a biology major at Virginia Tech. She soon added biochemistry as a second major with a minor in chemistry. In her freshman and sophomore year, she was privileged to do undergraduate research in the biochemistry department. In her junior and senior year, she was able to continue undergraduate research in the plant pathology, physiology and weed science department. She received her bachelor's degree with honors in 2003 and began work on her master's degree at Virginia Tech in food science and technology. She will be continuing her education at North Carolina State University in the fall of 2005.