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Membrane Damage and Viability Loss of *E. coli* O157:H7 and *Salmonella* spp in Apple Juice Treated with High Hydrostatic Pressure and Thermal Death Time Disks

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Abstract

Differences in membrane damage including leakage of intracellular UV-materials and loss of viability of *Salmonella* spp. and *Escherichia coli* O157:H7 bacteria in apple juice following thermal-death-time (TDT) disk and high hydrostatic pressure treatments were investigated. *Salmonella* spp. and *E. coli* O157H:H7 bacteria were inoculated in apple juice to a final 7.8 log10 CFU/ml and were thermally treated with TDT disks at 25, 35, 45, 50, 55 and 60°C for 4 min or pressurized at 350, 400 and 450 MPa at 25, 35, 45, 50, 55 and 60°C for 20 min. Sublethal injury, leakage of UV- materials and viability loss as a function of membrane damage of these bacterial pathogens was investigated by plating 0.1 ml of treated and untreated samples on non selective Trypticase Soy Agar (TSA) and selective Xylose Lysine Sodium Tetradecylsufate (XLT4) for *Salmonella* and Cefixime Potassium Tellurite Sorbitol-MacConkey (CT-SMACK) agar plates for *E. coli* bacteria with incubation at 36°C for 48 h. Sub-lethal injury occurred in *Salmonella* spp. and *E. coli* populations thermally treated with TDT disk at 55°C and above and at a pressure treatments of 25°C and above. Leakage of intracellular UV-materials and ATP of TDT disk injured cells was lower than the values determined from pressurized cells. Similarly, recovery of TDT injured cells occurred faster than pressurized cells during storage of treated samples at 22°C. The results of this study indicate that pressure treatment of 350 MPa at 35°C for 20 min and thermal treatments of 55 and 60°C and immediate storage of treated samples at 5°C will inhibit recovery and complete inactivation of injured bacteria in apple juice and therefore, will enhance the microbial safety of the treated juice.

Keywords: HHP; TDT-Disks; Bacterial injury; Leakage of intracellular bacterial substances; *Salmonella* spp.*; Escherichia coli;* Apple juice

Introduction

Contamination of juices with pathogenic microorganisms has caused numerous illnesses and some fatalities. From 1923 to 2000, consumption of contaminated fruit juices has been implicated in at least 28 foodborne illness outbreaks [1,2]. Eleven out of 28 (almost 40%) outbreaks were associated with *Salmonella* spp. Eight out of 28 (close to 30%) outbreaks were caused by Enteropathogenic *Escherichia coli* especially *E. coli* O157:H7. Although most bacteria cannot grow at low pH, *E. coli*, *Shigella* and *Salmonella* species can survive for several days or weeks in acidic foods [3-6]. Foodborne outbreaks involving *E. coli* O157:H7 in apple and orange juices [7-10] have raised concerns about the safety of consuming unpasteurized fruit juices. For health reasons and for convenience, consumers are demanding juices that receive no heat or minimal heat treatment. Food manufacturers and distributors are responding to consumers' demand for food products that are safe, fresher and convenient for use [11,12]. In some cases foods may be improperly processed and/or contaminated with spoilage bacteria or human bacterial pathogens [13-16].

Several technologies for nonthermal processing have been commercialized including high-hydrostatic pressure [17-19]. High-hydrostatic pressure (HHP) is a promising alternative to heat pasteurization of foods because of its ability to inactivate enzymes responsible for quality loss [20,21] and pathogenic microorganisms [19]. The effect of HHP on survival of bacterial populations in liquid foods or liquid buffered system has been reported [1,22]. In these reports, inactivation and survival/recovery of *E. coli* populations were discussed but not the details of membrane damage that led to inactivation. Quality attributes of heat sensitive juices should incorporate time-temperature margins necessary for thermal or pressure treatments that can inactivate bacteria in the treated juices. Therefore, the use of a minimum heat treatment process that would ensure food safety is needed. In designing such process treatments, it is important consider materials that may allow even distribution of heat at a short period to minimize bacterial resistant. In this study, thermal inactivation of *E. coli* and *Salmonella* spp. in apple juice using a new aluminum thermal death time (TDT) disk developed at Washington State University (Pullman, WA) [23] was used. The close compact nature of this TDT disk provides near isothermal conditions necessary for the study of thermal death kinetics of microorganisms. Also, the effect of high hydrostatic pressure and TDT disk treatments on membrane damage of *Salmonella* spp. and *E. coli* O157:H7 bacteria in apple juice using scanning electron microscopy were investigated. Finally, we monitored the possibility of HPP and TDT-Disks injured

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Salmonella spp. and *E. coli* O157:H7 to recover in apple juice during storage at 5 or 22°C.

Materials and Methods

Test strains and preparation of inocula

Bacterial strains used in this study were *Escherichia coli* ATCC 25922 (type strain), O157:H7 strains SEA13B88 and Oklahoma (apple juice cider-related outbreaks); *Salmonella* Stanley H0558 (alfalfa sprout-related outbreak, obtained from Dr. Patricia Griffin, CDC), *Salmonella* Poona RM2350, *Salmonella* Saphra 97A3312 (cantaloupe-related outbreaks, obtained from Ms. Sharon Abbott and Dr. Michael Janda, CA Dept. of Health Services). Cell culture was maintained on tryptic soy agar (TSA) at 4°C. Prior to use, the cells were inoculated by loop in tryptic soy broth (TSB: Remel, Inc., Lenexa, KS) and then incubated at 37°C for 16-18 h with shaking. A 0.1 ml cell aliquot was transferred to 100 ml of TSB and incubated at 37°C for 24 h. The overnight cell suspensions were centrifuged at 3,000 *g* for 10min at 5°C. The cell pellets were washed with equal volume (100 mL) of sterile phosphate buffered saline (PBS, pH 7.2) solution. *Escherichia coli* O157:H7 strains and *Salmonella* spp. was combined separately and used as the inoculum (10^9 CFU/mL) for this study.

Sample preparation

Escherichia coli O157:H7 strains and *Salmonella* spp. inoculum stated above was mixed in apple juice concentrate (1.30 L) purchased from a local store. A serial dilution was prepared and aliquot (0.1 mL) of the diluted samples was plated in duplicate on Tryptic Soy Agar (TSA) for aerobic mesophilic bacteria, Xylose Lysine Sodium Tetradecylsufate (XLT4) for *Salmonella* and Cefixime Potassium Tellurite Sorbitol-MacConkey (CT-SMACK) agar plates for *E. coli* O157:H7 (BBL/Difco) with incubation at 36°C for 24 h to determine the initial number of colony forming unit (CFU) in the apple juice before treatment.

HHP treatment of apple juice

Three 25 mL sterile test tubes containing 9 ml apple juice were inoculated with cell suspensions (1mL) of *Salmonella* spp. and *E. coli* O157:H7 to give a final concentration of 4.5×10^8 and 6.2×10^8 CFU/mL of *Salmonella* spp. and *E. coli* O157:H7, respectively. All samples were vortexed, and 2 mL of each were dispensed into individual sterile plastic bags (5.0 by 8.0 cm) (W. R. Grace & Co., Cedar Rapids, IA) and sealed using a Doughboy heat-sealer (Doughboy Packaging Machinery, Inc., New Richmond, WI). Each plastic bag was double-bagged (7.0 by 10.0 cm), sealed to prevent leakage, and then kept at 4°C, prior to pressurization (<1 h). All samples were placed inside a batch hydrostatic pressurization unit (Model 2 L, AVURE Technology, Kent, WA, USA) chamber filled with deionized water. The HHP pressure was applied at 300, 350 and 400MPa for up to 20 min at set temperatures of 25, 35, 45, 55 and 60°C. Temperature increase at 300-350 MPa and 400 MPa pressure treatments averaged approximately 7 and 9°C, respectively. The pressure increased during treatments averaging 240 MPa/min, the come-up time was 45 sec, and the come-down time was 6 sec. All samples were removed immediately after pressurization and placed in an ice bath to cool. Survival, injury and viability loss for *Salmonella* spp. and *E. coli* O157:H7 were determined by plating on differential agar (selective and non selective) plates described below. All tests were performed in duplicate within 1 h of HHP treatment.

Thermal inactivation of bacteria

The inside and outside temperatures of the TDT disk were monitored and recorded using a Fluke 54 II thermometer (Everett, WA) for all experiments. Before each use, the TDT disks were dipped in sanitizer (Coverage Plus, E. R. Squibb & Sons, Inc., St. Louis, MO) for 2 min according to the manufacturer's instructions, rinsed five times with sterile water, and then air dried. Aliquot (1 mL) of apple juice inoculated with *E. coli* or *S.* spp. were added to each TDT disk. The disks were hermetically sealed and then submerged completely in a water bath (Isotemp, Fisher Scientific, Pittsburgh, PA) with temperatures set at 25, 35, 45, 55 or 60 ± 0.1°C. The samples were pulled from the water bath at 4 min. Survival, injury and inactivation of treated *E. coli* or *Salmonella* spp. in apple juice were determined by plating on a range of agar plates mentioned below. The controls were *E. coli* and *Salmonella* spp. populations in apple juice inside the TDT disks placed in water bath maintained at room temperature (~22 ± 1°C) for the same amount of time.

Determination of injured bacteria and viability loss

To determine the initial and final bacterial population in apple juice, 0.1 mL of HHP and TDT disk treated samples were plated in duplicate on TSA, CT-SMAC) and (XLT4) described above. Viability loss and sub-lethal injury resulting from the pressure treatments were determined using differential plating methods on non-selective (TSA) vs. selective (CT-SMAC, XLT4) agar plates. Samples treated at different pressures (300 to 400 MPa) and temperatures (25, 35, 45, 55 and 60°C) for 20 min were plated 141 (0.1 mL) on agar plates as stated above and the percent injury was calculated using the following formula:

[1 – (colonies on selective agar/counts on nonselective)] ×100. (F-1)

The number of colony forming unit (CFU/mL) on nonselective and selective agar media was used to calculate bacterial inactivation or the viability loss which is defined as the differences in log CFU/mL of bacteria between control and the treated samples [23]. Bacterial cells from untreated inoculated apple juice were used as positive controls for each experiment. All agar plates were incubated at 37°C for 48 h.

Scanning electron microscopy (SEM)

Aliquots (50 µL) of cellular suspensions in treated apple juice were deposited on 12 mm diameter glass coverslips. After ~30 sec, the coverslips were gently immersed into 2 mL volumes of 2.5% glutaraldehyde-0.1M sodium imidazole buffer solution (pH 7.0) in a multi-well plate, sealed and stored for further processing. For SEM preparation, the cover slips were washed with buffer solution, dehydrated by exchange with graded ethanol solutions (50%, 80% and absolute) and critical point dried from liquid CO_2. The dry cover slips with cells were glued to aluminum specimen stubs and coated with a thin layer of gold by DC sputtering. Digital images of cells were collected using a Quanta 200 FEG scanning electron microscope (FEI Co., Inc., Hillsboro, OR) operated in the secondary electron imaging mode at an instrumental magnification of 25,000X to visualize topographical details of cell surfaces.

Leakage of intracellular Ultra Violet (UV)-absorbing materials from bacteria

Leakage of intracellular bacterial substances as a function of membrane damage by the pressures used for this study was quantified by measuring released adenosine tryphosphate (ATP), protein and nucleic acid materials spectrophotometrically (DUR 530, Beckman Coulter, Fullerton, CA) at 280 nm, respectively [24-28].

Bioluminescence ATP assay

The extracellular bacterial ATP content of apple juice inoculated with *Salmonella* spp. and *E. coli* O157:H7 bacteria was determined immediately before and after HHP and DTD disk treatments using an ATP bioluminescent assay kit (Turner Design, Sunnyvale, CA). Portions (0.1 ml) of HHP treated and untreated samples were mixed with 0.1mL luciferin-luciferase (Sigma, St Louis, MO), and the light signal (RLU) was measured using a TD-20/20 (DL Ready) luminometer (Turner Design) after a 3 sec delay time and a 14 sec integration time. Assays of standard amounts of purified ATP were used to calculate ATP levels, and ATP concentrations in samples were expressed as log10 femtogram (fg)/mL. Possible inhibition of the luciferase reaction by any residues from the apple juice was corrected for by addition of known amounts of ATP standard to the reaction vial followed by addition of the luciferase enzyme [29].

Data analysis

All experiments were done in triplicate, and samples obtained at each sampling time were analyzed in duplicate. Data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System Program (SAS Institute, Cary, NC, USA). The SAS program was used to determine significant differences in injury, inactivation, viability loss, and leakage of ATP and intracellular UV-absorbing substances from the treated bacteria. Significant differences ($p<0.05$) between mean values of number of cells and ATP concentrations were determined by the Bonferroni LSD method [30].

Results and Discussion

Survival of *E. coli* O157:H7 and *Salmonella* spp. after treatments

Survival of *E. coli* O157:H7 and *Salmonella* spp. population in apple juice treated with high hydrostatic pressure processing (HHP) for 20 min are shown in table 1. The initial populations of *E. coli* O157:H7 and *Salmonella* spp. determined in apple juice after inoculation averaged 7.8 and 7.7 log, respectively. Pressure (400 MPa) treatment at 25°C for 20 min led to the least surviving populations of *E. coli* O157:H7 and *Salmonella* spp. in treated apple juice. This trend was followed by 350 MPa and 300 MPa treatments, respectively at all temperatures tested. The populations of *E. coli* O157:H7 and *Salmonella* spp. surviving the HHP treatments was high at 25°C at the pressures tested. Increase in treatment temperatures decreased the total population densities of surviving *E. coli* O157:H7 and *Salmonella* spp. and at 50°C and 55°C; the populations were below detection (< 2 CFU/ml) at 400 MPa and 300-350 MPa, respectively. An average 5 log reductions for both pathogens was achieved at 350 MPa treatments at 35°C for 20 min. Therefore, this pressure-temperature-time was adopted and used for the thermal death time (TDT) disk study.

The effect of TDT disk treatment temperatures was investigated and the result is shown in figure 1. The results of TDT disk treatments on survival of *E. coli* O157:H7 and *Salmonella* spp. in apple juice were not significantly ($p<0.05$) different at ≤ 45°C. TDT disks treatment at 50°C and above decreased the populations of *E. coli* O157:H7 and *Salmonella* spp. bacteria and at 60°C, the surviving populations for both pathogens averaged 4 log. Viability loss for *E. coli* O157:H7 and *Salmonella* spp. treated at higher temperatures (≥ 50°C) were significantly ($p<0.05$) different that values determined in sample treated below 40°C.

Percent injured cells and SEM observation

Percent populations of injured bacteria among the surviving *E. coli* O157:H7 and *Salmonella* spp. bacteria in apple juice treated at 350MPa and TDT disk treatments were determined (Figure 2). For the HHP treatments, the injured populations for *E. coli* O157:H7 and *Salmonella* spp. bacteria in apple juice increased during treatment at 25°C to 50°C. The extent of injury as measured by the populations of injured bacteria increased with an increase in treatment temperature. The injured populations of *E. coli* O157:H7 and *Salmonella* spp. bacteria estimated at 25°C averaged 42% and 44%, respectively. At 55°C treatment, the injured populations for both pathogens declined to an average of 60% suggesting that this temperature and pressure combination caused more death than injury to the bacterial populations. A similar increase of injured bacteria was observed for the TDT disk treatments. There were no significant ($p<0.05$) different on injured populations of bacteria treated up to 45°C. The highest populations of injured *E. coli* O157:H7 and *Salmonella* spp. bacteria in apple juice (~80%) were determined at 60°C and unlike the HPP treatment; these populations did not decline in TDT disk treated apple juice.

Results of the scanning electron microscopy (SEM) observation of the bacterial cell surface structure are shown in figure 3. The surfaces of the control (A) bacterial cell membrane at room temperature were

Bacteria	Temperature (°C)	300 MPa	350 MPa	400 MPa
Salmonella spp.	Control	7.7 ± 0.26	7.7 ± 0.26	7.7 ± 0.26
	25	4.3 ± 0.14	3.4 ± 0.16	2.0 ± 0.12
	35	2.1 ± 0.12	1.6 ± 0.12	0.8 ± 0.10
	45	1.0 ± 0.12	ND	ND
	50	ND	ND	ND
	55	ND*	ND*	ND*
	60	ND	ND	ND
E. coli O157:H7	Control	7.8 ± 0.26	7.8 ± 0.26	7.7 ± 0.26
	25	5.6 ± 0.14	4.2 ± 0.16	2.4 ± 0.12
	35	3.0 ± 0.12	2.1 ± 0.12	1.2 ± 0.12
	45	1.4 ± 0.12	ND	ND
	50	ND	ND	ND
	55	ND*	ND*	ND*
	60	ND	ND	ND

[a]Values represent mean ± SD of three experiments with duplicate determinations.
ND* = no colony forming unit (CFU) determined on the agar plates.
Survivors (Log10 CFU/ml)[a]

Table 1: Survival of *Salmonella* spp. and *E. coli* O157:H7 in apple juice treated with high hydrostatic pressure for 25 min.

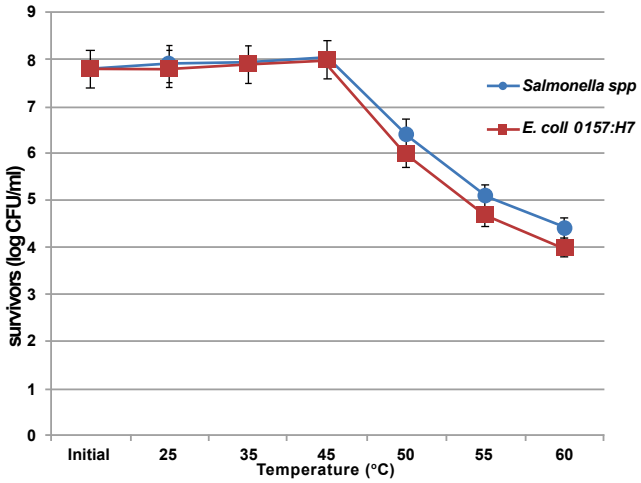

Figure 1: Survival of Escherichia coli O157H:H7 and Salmonella spp. in TDT disk treated apple juice for 25 min. Values are means of three determinations ± standard deviation.

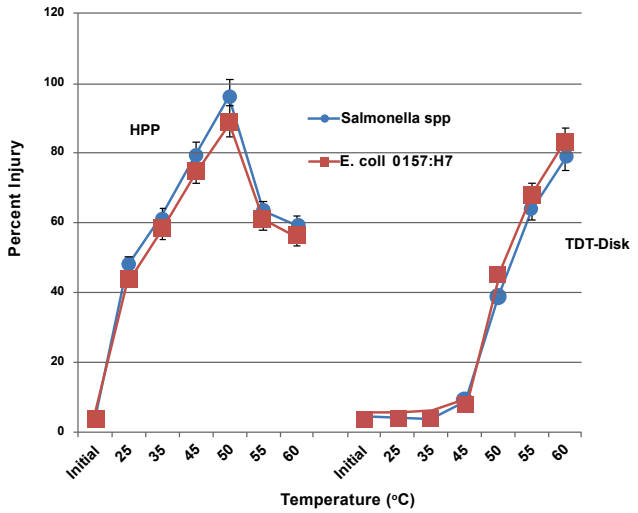

Figure 2: Percent injured population of Salmonella spp. and E. coli O157:H7 in apple juice pressurized at 350 MPa and thermal death time disks treatments at 25, 35, 45, 50, 55 and 60°C for 25 min. Values are means of three determinations ± standard deviation.

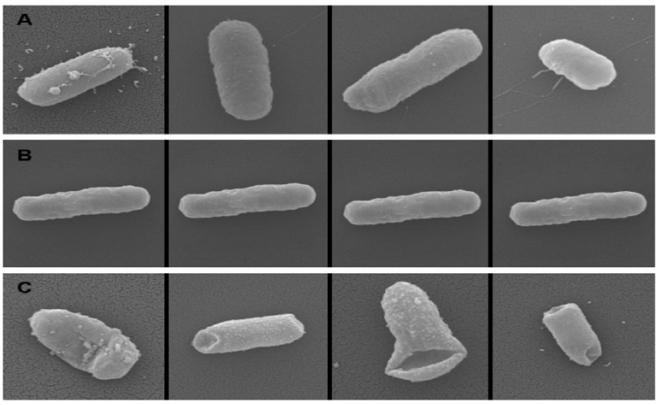

Figure 3: Scanning electron microscopy observation of Salmonella spp. and E. coli O157:H7 bacteria in apple juice (A), thermal death time disks treated apple juice (50°C, B) and apple juice pressurized at 350 MPa (35°C) for 25 min. Values are means of three determinations ± standard deviation.

smooth and cylindrical, showing profiles of the short, narrow rods with a uniform contour, smooth sides and regular semi-circular tips. The bacterial cell surface structure of the TDT disk treated cells (B) showed uniform cylindrical shaped surfaces with a few to many circular and irregularly shaped spots (20-30 nm) in diameter dotted along the cell body. The membrane surfaces of bacteria pressurized at 350 MPa were differentiated into irregular folds, dents and pits, and in most cases the cell surface profiles were usually deformed (C). The 350MPa pressure treatment at 35°C resulted to injury which deformed the bacterial surface structure and in some cases, collapse of the bacteria surface structure.

Leakage of intracellular materials

In this study, we measured the initial extracellular ATP level of apple juice before treatment to establish the base line. Any increase of the initial extracellular ATP after treatments would be an indication of leakage of bacterial intracellular ATP due to membrane damage. The initial extracellular ATP values of apple juice before and after pressure and TDT disk treatments at 25, 35, 45, 50, 55 and 60°C for 20 min are shown in figure 4. The initial ATP level in apple juice before treatment averaged 1 log10 fg/ml. Pressure treatments at 25°C and above led to increased level of extracellular ATP in apple juice. Increase in treatment temperature led to higher level of extracellular ATP and at 55°C, the ATP level stabilized. For the TDT disks treatment, only at 45°C or above did we observe a higher value of extracellular ATP and unlike the HHP, ATP level did not stabilize at 55°C or above. The increase in ATP in both treated samples indicates surface injury or membrane damage to the bacterial cells which led to leakage of intracellular ATP of the injured bacteria. However, the increase of ATP determined in apple juice pressurized at 23-45°C was significantly ($P<0.05$) different than the values determined in TDT disk treated samples.

Leakage of intracellular UV-absorbing materials determined at Ab280 nm is shown in figure 5. The average UV-absorbing materials determined at Ab280 nm in untreated apple juice averaged 0.03 and the value were not significantly ($P>0.05$) different when juice was inoculated with *E. coli* O157:H7 and *Salmonella* spp. The results obtained for samples pressurized at 350 MPa were significantly ($P<0.05$) different from those values obtained using TDT disks similarly treated at 25, 35, 45, 50, 55 and 60°C. The UV materials for *Salmonella* spp. were slightly lower than the values determined for *E. coli* O157H:H7 bacteria. Relationship between injury and leakage of ATP from *Salmonella* spp. and *E. coli* O157:H7 similarly treated with HPP is shown in figure 6. Leakage of intracellular ATP of *Salmonella* spp. and *E. coli* O157:H7 in apple juice treated with HHP showed similar trend and were not significantly ($p>0.05$) different. The percent injured *Salmonella* spp. and *E. coli* O157:H7 in HPP treated apple juice were low at 55 and 60°C while total ATP determined in the juice was higher. The increased ATP values in apple juice observed at 55 and 60°C may have come from dead cells as previously reported by Ukuku and Shelef [29]. HHP injured *E. coli* O157:H7 and *Salmonella* spp. in apple juice did not recover during storage at 5°C, especially in apple juice pressurized at 45°C and above (Data not shown). *E. coli* O157:H7 and *Salmonella* spp. population in control apple juice stored at room temperature (22°C) for a week

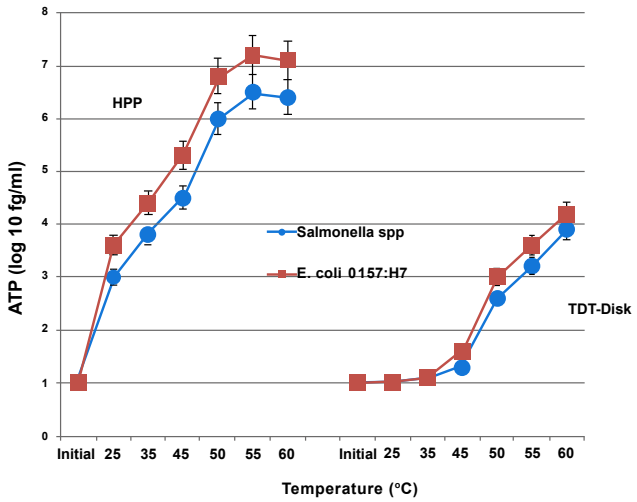

Figure 4: Intracellular ATP leakage of Escherichia coli O157H:7 in apple juice pressurized at 350 MPa and thermal death time disk treated apple juice at 25, 35, 45, 50, 55 and 60°C for 20min. Values are means of three determinations ± standard deviation.

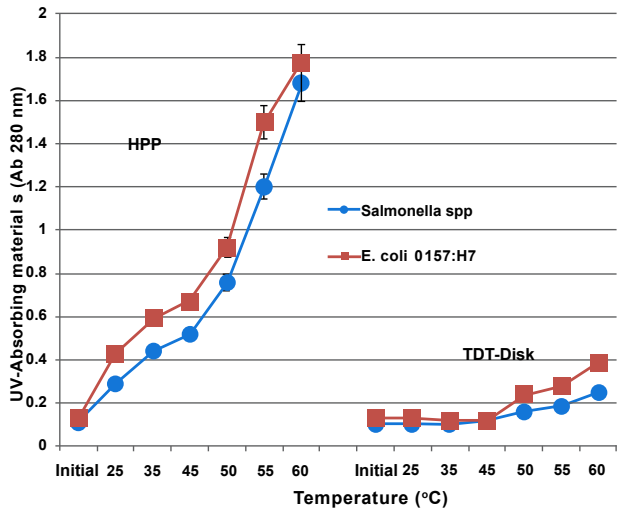

Figure 5: Leakage of intracellular UV-substances of Salmonella spp. and E. coli O157H:7 in 350 MPa pressurized apple juice at 25, 35, 45, 50, 55 and 60°C for 25 min. Measurement was performed at Ab_{280} nm. Values are means of three determinations ± standard deviation.

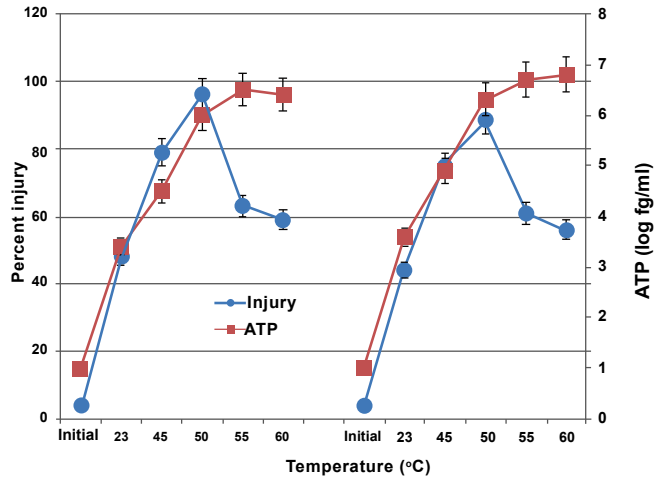

Figure 6: Relationship between injury and leakage of ATP from *Salmonella* spp. and *E. coli* O157:H7 similarly treated with HPP.

survived but slightly increased. Also, the surviving populations of HPP treated *E. coli* O157:H7 and *Salmonella* spp. in apple juice stored at 5°C declined further during storage and the numbers in apple juice treated above 45°C for 20 min were below detection limits (<3 CFU/ml) by day 5.

The use of high-hydrostatic pressure (HHP) at low pH to inactivate *E. coli* O157:H7 in fruit juices and other fruit products has been reported [31,24,18,27]. In this study, the authors achieved a 5-log reduction in the populations of all bacteria tested. Similarly, Garcia-Graells et al. [4] reported a 5-log decrease in the population of the surviving high-pressure-resistant mutants of *E. coli* in apple juice (pH 3.3) after 300 MPa pressure treatments at 20°C for 15min. In our study, 300 to 400 MPa pressure treatments at all temperatures tested for 20 min were equally effective in inactivation of *E. coli* O157:H7 and *Salmonella* spp inoculated in apple juice. In this study, we did achieve a 5 log reduction of both *E. coli* O157:H7 and *Salmonella* spp. in all treated apple juice (pH 3.1). Further log reductions of *E. coli* O157:H7 and *Salmonella* spp. was achieved during storage and this phenomenon was attributed to dying of the injured/damaged bacteria cells due to the acidity of juice. These results are within the 6-log inactivation range reported for *E. coli* O157: H7 in orange juice (pH 3.9) pressurized at 550 MPa for 5 min at 20°C [24]. The log reduction for *E. coli* O157: H7and *Salmonella* in all pressurized apple juice stored at 5°C was below detection limits (<3 CFU/ml) (Data not shown). Based on this observation, we can conclude that cold storage (5°C) and the acidity of the apple juice contributed to the inactivation of the HHP injured bacterial populations. Other researchers have reported similar findings in fruits juices [4] and phosphate buffered saline [22].

The pressure treatments used in this study caused injury and leakage of intracellular UV-substances including ATP of inoculated bacteria leading to its accumulation in the apple juice (Figure 5 and 6). The results obtained in this study suggest disruption of intact bacterial membranes that allowed leakage of internal biologically active compounds. Leakage of bacterial intracellular substances as a result of membrane damage by antimicrobial agents has been reported [32-34,]. These authors concluded that the accumulation of extracellular ATP in media containing nisin, ampicillin and streptomycin resulted from injured *L. monocytogenes* cells in the samples. Similarly, other researchers have measured leakage of nucleic acids and proteins from microwave-injured bacteria and reported that the intracellular UV absorbing substances that leaked out from the bacteria into the cellular extracts were mostly nucleic acids with some proteins [26,27,6]. The increase in UV-absorbing materials in pressurized samples suggests leakage of intracellular proteins and nucleic acids. In conclusion, the data from this study indicate the treatment temperature and pressure played a role in deformation of bacterial cell structures that led to inactivation. Also, the sensitization of injured cells to subsequent acid during incubation/storage provided valuable information needed for future development of appropriate combined HHP processes parameters required to enhance the microbiological safety of apple juice.

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