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Behavior of Pulsed Electric Fields Injured *Escherichia coli* O157:H7 Cells in Apple Juice Amended with Pyruvate and Catalase

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Abstract

Pulse Electric Field (PEF) treatment has been used to inactivate bacteria in liquid foods. However, information on the behavior of PEF injured *Escherichia coli* bacteria in media during storage at 5 and 23°C are limited. In this study, we investigated the fate of *E. coli* O157:H7 cells at 6.8 log CFU/ml in apple juice treated with PEF at 7.2 kV/cm and 32.2 kV/cm, 18.4 A with pulse width of 2.6 μs at 25, 35, 45 and 55°C, at a flow rate of 120 ml/min. The juices collected were amended with pyruvate and catalase (0 to 0.1%) and then stored at 5 and 23°C for 24 h. Periodically (0, 3, 6, and 24 h), 0.1 ml of the treated sample was plated on Sorbitol MacConkey Agar (SMAC) and Tryptic Soy Agar (TSA) amended with catalase and pyruvate to determine percent injury, viability loss and behavior of injured cells. PEF voltage at 32.2 kV/cm and treatments at 35, 45 and 55°C led to significant decline in the surviving cell populations than treatment at 7.2 kV/cm. The injured populations in control media were higher than media amended with pyruvate and catalase suggesting possible recovery of PEF injured *E. coli* cells.

Keywords: PEF; Injury; Inactivation; *E. coli*; Apple juice; Catalase and pyruvate

Introduction

Food manufacturers and distributors are responding to consumers' demand for food products that receive no heat or minimal heat treatment, safe, fresh and convenient (Ukuku and Geveky, 2010). In some cases foods may be improperly processed and/or contaminated with spoilage bacteria or human bacterial pathogens during processing, storage or distribution (Alex et al., 2001; Cody et al., 1999). Thermal processing used by the juice industry to inactivate food borne pathogens impairs the characteristic flavor of juices (Linton et al., 1999; Mazzotta, 2001). Therefore there is a need for alternative processing treatments that can achieve a 5 log reduction of these pathogens (Sizer and Balasubramaniam, 1999; Mazzotta, 2001) without causing adverse effect on the flavor of the juice.

There are several reports on non-thermal pasteurization process using pulse electric fields (PEF) to inactivate bacteria in liquid foods (Hulsheger, et al., 1983; Jeyamkondan, et al., 1999; Zhao et al., 2008; Dutreux et al., 2000). In these studies, a set of PEF operating parameters that achieved 99.999% (5 logs) reduction of *E. coli* in apple cider was determined, and the kinetics of bacterial inactivation established (Min et al., 2003). However, the current knowledge of PEF processes and its mechanisms for inactivation of bacterial pathogen is limited. It was hypothesized that PEF inactivation is caused by rupture of bacteria membrane structure through application of high voltage electric fields to the bacteria (Zimmerman et al., 1976; Zimmerman, 1986). Most aerobic and facultative bacteria that utilize oxygen produce hydrogen peroxide (H_2O_2), which is toxic to their own enzyme systems. Therefore, this class of bacteria produces catalase in small amount during logarithmic growth (Martin et al., 1976) to counteract the toxic effect of H_2O_2. The catalase produced by bacteria is attached to the intracellular membrane (Kovacs et al., 1966; Lin, 1963) and if the membrane structure is ruptured as proposed, then the catalase attached at this site becomes dysfunctional. In this study, we hypothesized that the addition of membrane associated pyruvate and catalase to PEF injured *E. coli* cells in apple juice will aid cellular repair of PEF injured *E. coli* populations. The results obtained in this study will provide more information leading to the understanding of the mechanism of PEF inactivation of bacteria.

Materials and Method

Test strains and preparation of inocula

Apple juice -related *E. coli* O157:H7 outbreaks (SEA13B88) and (Oklahoma) from the U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center culture collection was used in this study. 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 10 min at 5°C. The cell pellets were washed with equal volume (100 ml) of sterile phosphate-buffered saline (PBS, pH 7.2) solution. Finally, individual washed cells were combined and resuspended in 100 ml PBS at 10^9 CFU/ml and used as the inoculum.

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Sample preparation

Apple juice concentrate (1.30 L) purchased from a local store was mixed with deionize sterile water (8.30 L) at room temperature (~23°C). *E. coli* prepared as above was added to the apple juice at approximately10^7 CFU/ml, mixed and a serial dilution was prepared. A 0.1 ml of the diluted samples was plated in duplicate on TSA and SMAC with incubation at 36°C for 24 h to determine the initial number of colony forming unit (CFU) in the apple juice before PEF treatment. Similarly, the apple juice without the inoculated populations of *E. coli* bacteria was plated on TSA and SMAC with incubation at 36°C for 24 h to determine if *E. coli* is present on the sample.

Pulsed electric fields treatment and processing variables

Inoculated apple juice was allowed to stand at room temperature for up to 2 h before being pumped through the pulsed electric field (PEF) chamber (0.29 electrode-gap distance, with a treatment volume of 0.012 cm^3) at a rate of 120 ml/min. A peak voltage of 7.4 kV/cm, peak current of 18.4 ampere and a pulse width of 2.6 µs at 55°C were applied. In another study, we increased the peak voltage to 32.2 kV/cm, while the treatment parameters listed above were maintained as is. The maximum treatment temperature was kept below 57°C, using a cooling coil, which was connected between each pair of chambers and submerged in an iced water bath. Thermocouples were attached to the stainless-steel coils, 2.5 cm away from the PEF zones along the flow direction. The temperatures of the inlet and outlet of each pair of chambers were recorded every 0.1 s. The characteristics of the electric pulses delivered such as shape, polarity, width, difference of potential as well as the electric current generated across the electrodes and the pulse frequency were monitored using a digital oscilloscope (model THS720, Tektronix Inc., Beaverton, OR, USA). The water bath temperature was adjusted to give similar outlet temperatures as stated above. Samples (~ 20 ml) at each outlet temperatures were individually collected and analyzed for bacterial cell survivors, injured cells and viability loss as stated below.

Preparation of pyruvate and catalase

Pyruvate and catalase (Sigma-Aldrich, St. Louis MO, USA) were individually dissolved in ddH_2O, filter sterilized using 0.45µ 150 ml analytical filter unit (Nalgnene, Rochester, NY) and added to the PEF treated apple juice containing injured *E. coli* cells at a final concentration of 0, 0.01, 0.05 and 0.1% each. Also, tryptic soy agar (TSA) and Sorbitol MacConkey Agar (SMAC) plates were amended with pyruvate and catalase at a final concentration of 0, 0.01, 0.05 and 0.1% each.

Microbial injury and viability loss

To determine the initial number of surviving *E. coli* population including injured cells after PEF treatment of apple juice, an aliquot (0.1 ml) of the apple juice described above was plated on TSA, TSA+0.1% Catalase (TSA-cat), TSA+ 0.1% Pyruvate (TSA-pyr), Sorbitol MacConkey Agar (SMAC) and SMAC+ 0.1% Pyruvate (SMAC-pyr) and SMAC+ 0.1% Catalase plates. All plated samples were incubated at 36°C for 48 h to determine the colony forming unit (CFU/ml). The difference in populations of *E. coli* enumerated on selective versus non-selective media was considered as injured cells, and the percent injury was calculated using this formula:

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

The number CFU/ml on selective agar media was used to calculate the viability loss which is defined as the differences in log CFU/ml of bacteria between control and PEF-treated samples (Linton et al., 1999).

PEF injured *E. Coli* bacteria during storage

The population of untreated *E. coli* cells and those surviving the PEF treatments of apple juice amended with or without 0, 0.01, 0.05 and 0.1% catalase and pyruvate were investigated during storage at 5 and 23°C. Periodically (0 , 3, 6, and 24 h), an aliquot (0.1 ml) of each sample was plated on TSA, TSA+0.1% catalase (TSA-cat), TSA+ 0.1% pyruvate (TSA-pyr), Sorbitol MacConkey Agar (SMAC) and SMAC+ 0.1% pyruvate (SMAC-pyr) and SMAC+ 0.1% catalase plates to determine the number of colony forming unit (CFU/ml). Untreated PEF inoculated apple juice was used as controls for each experiment.

Data analysis

All experiments were performed in triplicate with duplicate samples being analyzed at each sampling time. Data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System Program (SAS Institute, Cary, NC, USA). Significant differences ($p<0.05$) between mean values of number of cells in media amended with or without catalase and pyruvate were determined by the Bonferroni least significant difference (LSD) method (Miller, 1981).

Results and Discusion

Untreated *E. coli* cells

There was no *E. coli* population determined in freshly prepared apple juice before the inoculation and PEF treatments. The populations of *E. coli* cells inoculated in apple juice before PEF treatment averaged 6.8 and 6.5 logs on TSA and SMAC, respectively. After PEF treatment at 7.2 kV/cm, the populations of *E. coli* cells in apple juice were reduced by 1.2 log CFU/ml and the surviving populations in apple juice amended with 0, 0.1, 0.05 and 0.1% pyruvate or catalase increased slightly during storage at 23°C for 24h (Figure 1). The populations of surviving *E. coli* cells in apple juice amended with catalase were slightly higher than in juices amended with pyruvate but again these numbers were not significantly ($p<0.05$) different. Populations in juices amended with 0.1% catalase or pyruvate were slightly higher than the rest of the concentration therefore a concentration of 0.1% for catalase and pyruvate was used for the rest of the study. The results of this study indicate that a trace amount of catalase in apple juice enhanced the recovery and survival of PEF injured *E. coli* cells during storage at 23°C for 24 h.

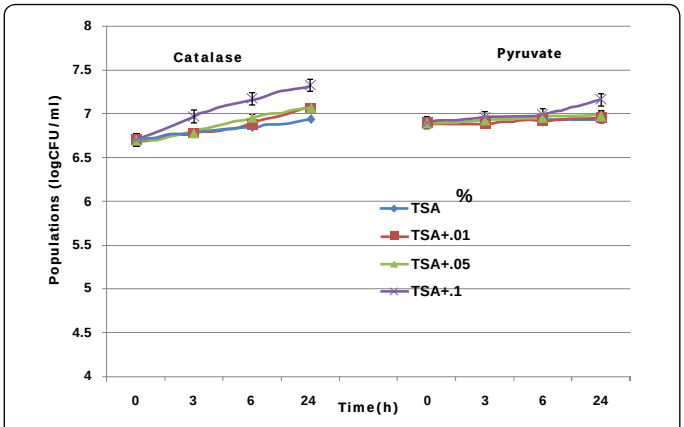

Figure 1: Recovery of control (untreated) *E. coli* O157:H7 cells on TSA plates containing different concentrations of pyruvate and Catalase during incubation at 23°C for 24 h. Values are means plus/minus standard deviation of mean on three experiments with duplicate determinations.

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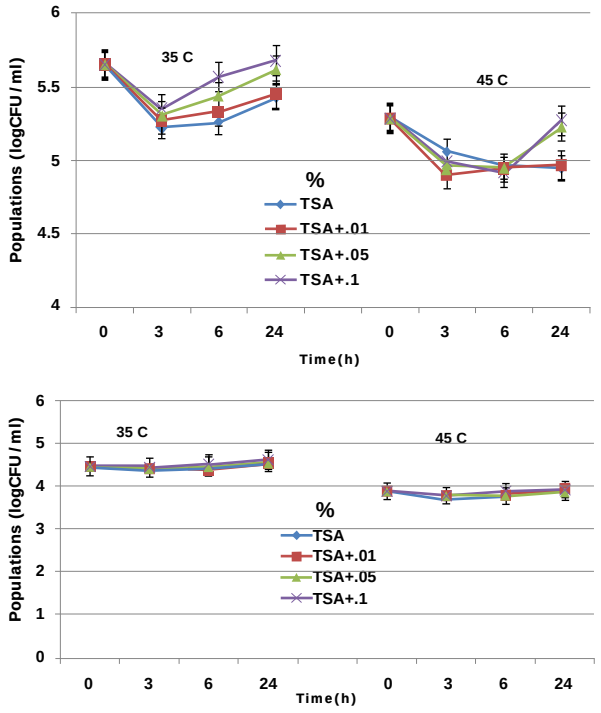

Figure 2: Recovery of PEF treated *E. coli* O157:H7 cells on TSA plates containing different concentrations of pyruvate (2B) and Catalase (2A) during incubation at 23°C for 24 h. Values are means plus/minus standard deviation of mean on three experiments with duplicate determinations.

	TSA + Pyruvate	TSA	SMAC + Pyruvate	SMAC
IC	6.75 ± 0.15^{A}	6.76 ± 0.15^{A}	6.85 ± 0.14^{A}	6.69 ± 0.16^{A}
T1 (35°C)	4.96 ± 0.12^{BA}	4.95 ± 0.14^{BA}	4.72 ± 0.14^{BC}	4.85 ± 0.13^{BB}
T2 (45°C)	3.93 ± 0.14^{CA}	3.89 ± 0.12^{CA}	2.87 ± 0.12^{CB}	2.69 ± 0.12^{CC}
T3 (55°C)	3.95 ± 0.12^{CA}	3.90 ± 0.10^{CA}	2.82 ± 0.10^{CA}	2.42 ± 0.12^{CB}

TSA = tryptic soy agar plates
TSA + Pyruvate = TSA plus Pyruvate (0.1%)
SMAC = Sorbitol McConkey agar plates
SMAC + Pyruvate = Sorbitol McConkey agar plates plus Pyruvate (0.1%)
Values are means ± SD of three experiments with duplicate determinations per experiment.
Means in each row and column not followed by the same letter are significantly ($p<0.05$) different

Table 1: Effect of PEF treatment temperature on populations of *Escherichia coli* in different media plates containing 0.1% pyruvate.

	TSA + Catalase	TSA	SMAC + Catalase	SMAC
IC	6.76 ± 0.15^{AA}	6.78 ± 0.15^{AA}	6.82 ± 0.14^{AA}	6.66 ± 0.15^{AA}
T1 (35°C)	5.16 ± 0.12^{BA}	5.14 ± 0.12^{BA}	5.01 ± 0.13^{BA}	5.08 ± 0.12^{BA}
T2 (45°C)	3.86 ± 0.10^{CA}	3.54 ± 0.10^{CA}	2.59 ± 0.10^{CB}	2.48 ± 0.12^{CB}
T3 (55°C)	3.65 ± 0.12^{CA}	3.32 ± 0.12^{CA}	2.54 ± 0.10^{CB}	2.36 ± 0.10^{CB}

TSA = tryptic soy agar plates
TSA + Catalase = TSA plus Catalase (0.1%)
SMAC = Sorbitol McConkey agar plates
SMAC + Catalase = Sorbitol McConkey agar plates plus Catalase (0.1%)
Values are means ± SD of three experiments with duplicate determinations per experiment.
Means in each row and column not followed by the same letter are significantly ($p<0.05$) different

Table 2: Effect of PEF treatment temperature on populations of *Escherichia coli* in different media plates containing 0.1% Catalase.

Behavior of PEF injured *E. coli* cells in media amended with pyruvate or catalase

The effect of 7.2 kV/cm PEF treatment at 35°C and 45°C on the population of *E. coli* cells in apple juice is shown in (Figure 2A). The populations of the surviving *E. coli* cells averaged 5.7 log and 5.3 log CFU/ml, respectively. The *E. coli* populations decreased during storage at 23°C for 3 h in all apple juice amended with catalase including the control. At 6 h of storage, *E. coli* cells begin to revive and by 24 h the populations were approximately close to the initial numbers started with. The initial populations of surviving *E. coli* cells in apple juice treated at 45°C averaged 5.4 log CFU/ml. Again, all populations declined to <5.0 log CFU/ml at 3 h during storage and the numbers remained the same up to 24 h with the exception of juices amended with 0.05 and 0.1% catalase. In apple juice similarly treated and amended with pyruvate, the *E. coli* cell populations did not decline but remained relatively the same up to 24 h of storage at 23°C (Figure 2B).

The results of PEF treatment at 32.2 kV/cm and treatment temperatures at 35, 45 and 55°C on the surviving *E. coli* cell populations determined on TSA and SMAC plates is shown (Table 1). The surviving treated PEF treatment at 35°C reduced the surviving *E. coli* populations in apple juice to an average of 4.95 log and 4.96 log CFU/ml s on TSA and TSA-pyr, respectively. However, the populations on SMAC and SMAC-pyr plates were 4.85 and 4.92 log CFU/ml, respectively. PEF treatment at higher temperatures (45 and 55°C) resulted to a significant reduction of the surviving populations. The *E. coli* populations determined on TSA plates after PEF treatment at 45 and 55°C averaged 3.89 and 3.90 log CFU/ml and 2.69 and 2.42 log CFU/ml on SMAC plates, respectively. Recovery of PEF injured *E. coli* cells on TSA- pyr averaged 3.93 and 3.95 log CFU/ml at 45 and 55°C, respectively. Populations determined on SMAC- pyr plates were slightly lower and averaged 2.87 and 2.82 log CFU/ml at 45 and 55°C, respectively. *E. coli* cell populations recovered on TSA and SMAC amended with or without catalase showed similar trend observed on plates amended with pyruvate (Table 2). The addition of 0.1% pyruvate (Table 1) or catalase (Table 2) to the agar plates slightly enhanced the recovery of *E. coli* cell populations, however, the number of cells determined on TSA and SMAC plates containing pyruvate and catalase were not significantly ($p<0.05$) different. The surviving population of *E. coli* cells determined immediately after PEF treatments of apple juice decreased as treatment temperature decreased from 55°C to 35°C., respectively.

Approximately 20, 93 and 97 % of the surviving *E. coli* populations determined in apple juice immediately after PEF treatment at 35, 45 and 55°C were injured (Figure 3). In this study, both catalase and pyruvate and storage at 23°C for 24 h appeared to help recovery of PEF injured *E. coli* cells (Figure 3). Storage of PEF treated *E. coli* cells in apple juice amended with 0.1% catalase and pyruvate at 5°C for 24 h led to further decline of injured cells irrespective of the presence or absence of catalase or pyruvate (Figure 4). However, the decline of injured *E. coli* cells in apple juice amended with catalase and pyruvate was slower than the populations in the control. Addition of 0.1% pyruvate to the PEF treated apple juice decreased the populations of injured *E. coli* cells from 20 to 2.3 %, 94 to 9.6% and 97 to 12.2 % in juices treated at 35, 45 and 55°C, respectively. Again, catalase appeared to be more effective in repair and recovery of PEF injured *E. coli* cells than the pyruvate. For example, the injured *E. coli* populations recovered and the percent injured cells determined averaged 4.7, 4.7 and 7.1% in 35, 45 and 55°C, respectively in PEF treated apple juices. Though this effect appeared to be minimal in

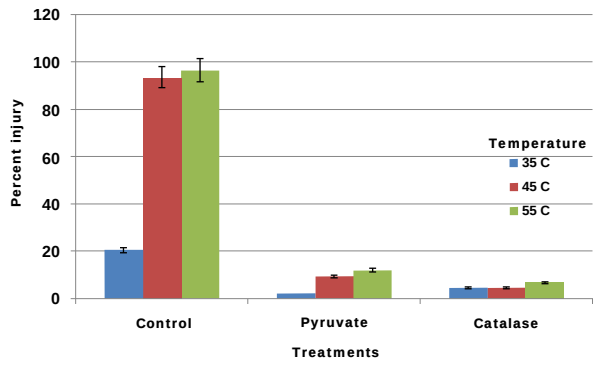

Figure 3: Effect of pyruvate and Catalase on the populations of PEF injured *E. coli* O157:H7 cells treated at different temperatures Values are means plus/minus standard deviation of mean on three experiments with duplicate determinations.

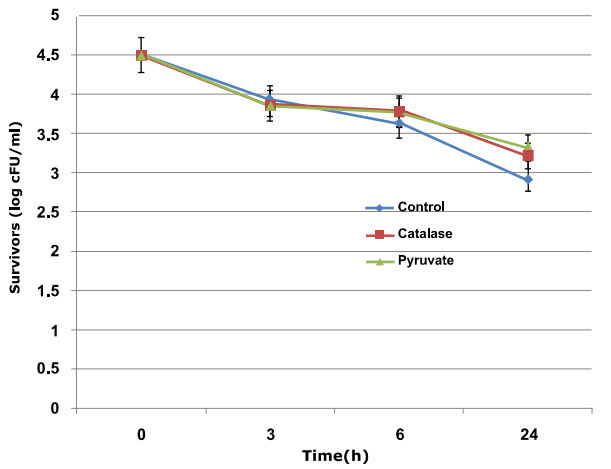

Figure 4: Effect of refrigeration (5°C) storage for 24 h on PEF injured *E. coli* O157:H7 cells in apple juice amended with 0.1% pyruvate and Catalase Values are means plus/minus standard deviation of mean on three experiments with duplicate determinations.

plate count data, the results of this study is in agreement with the Mackey and Seymour (1987) study that reported a protective effect of catalase and peroxidase in heat injured *E. coli* cells.

It has been reported that PEF inactivation is caused by rupture of bacteria membrane structure through application of high voltage electric fields to the bacteria (Zimmerman et al., 1976; Zimmerman, 1986). This effect may have caused the bacteria membrane bound catalase, to be disrupted and non functional. It has been reported that accumulation of H_2O_2 is a universal phenomenon associated with cellular injury following sublethal stress (Martin et al., 1976) and this contributes to the cell death. As a result of the PEF treatment, it is possible that the dysfunctional membrane catalase and or pyruvate was not able to clear/destroy the accumulated H_2O_2. Therefore, the injured *E. coli* cells were not able to convert cellular substrates needed for cell metabolism which ultimately led to the death of PEF injured *E. coli* cells. The slight recovery of injured *E. coli* cells observed in apple juice was a result of the catalase or the pyruvate added to the PEF treated apple juice. Other researchers have proposed a mode of action of sodium pyruvate to be through the degradation of the metabolic by-product hydrogen peroxide (H_2O_2), rather than through supplementation of a required nutrient (Baired-Parker and Davenport, 1965). This is the first study to look into the behavior of PEF injured *E. coli* cells in the presence of catalase or pyruvate towards understanding the mechanism of inactivation of bacteria by PEF. Further study in this area is needed to clearly understand and elucidate the mechanism of PEF inactivation of bacteria in liquid foods. In conclusion, the results of the present study suggest that catalase and pyruvate did provide minimal aid to cellular repair of injured PEF treated *E. coli* cells in apple juice stored at room temperature (~23°C) for 24 h. Also, the results showed that immediate storage of PEF treated apple juice at 5°C would enhance the microbial safety of the treated apple juice

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