Membrane Damage and Viability Loss of *Escherichia coli* K-12 in Apple Juice Treated with Radio Frequency Electric Field

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**ABSTRACT**

The need for a nonthermal intervention technology that can achieve microbial safety without altering nutritional quality of liquid foods led to the development of a radio frequency electric fields (RFEF) process. In order to understand the mechanism of inactivation of bacteria by RFEF, apple juice purchased from a wholesale distributor was inoculated with *Escherichia coli* K-12 at 7.8 log CFU/ml and then treated with RFEF. The inoculated apple juice was passed through an RFEF chamber operated at 20 kHz, 15 kV/cm for 170 μs at a flow rate of 540 ml/min. Treatment condition was periodically adjusted to achieve outlet temperatures of 40, 45, 50, 55, and 60°C. Samples at each outlet temperature were plated (0.1 ml) and the number of CFU per milliliter determined on nonselective and selective agar media was used to calculate the viability loss. Bacterial inactivation and viability loss occurred at all temperatures tested with 55°C treatment, leading to 4-log reductions. No significant effect was observed on bacterial population in control samples treated at 55°C with a low-RFEF (0.15 kV/cm) field strength. These observations suggest that the 4-log reduction in samples treated at 15 kV/cm was entirely due to nonthermal effect. RFEF treatment resulted in membrane damage of the bacteria, leading to the efflux of intracellular ATP and UV-absorbing materials. Populations of injured bacteria recovered immediately (<30 min) from the treated apple juice averaged 0.43 log and were below detection after 1 h of RFEF treatment and determination using selective plates (tryptic soy agar containing 5% sodium chloride). The results of this study suggest that mechanism of inactivation of RFEF is by disruption of the bacterial surface structure leading to the damage and leakage of intracellular biological active compounds.

Enterohemorrhagic *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* are recognized foodborne pathogens (6, 15, 25, 26, 31) and are capable of surviving in low-acid foods like fruit juices (6, 8, 9). Outbreaks involving *E. coli* O157:H7 in apple cider (12, 21) and *Salmonella* in orange juices (10, 27) have raised concerns about the safety of consuming unpasteurized fruit juices. Physical and chemical treatments have been used in food processing to eliminate or at least reduce the presence of pathogenic and spoilage microorganisms in foods (5, 34, 36–38). Thermal processing is used by the juice industry to inactivate foodborne pathogens; however, it impedes the characteristic flavor of juices (6). Therefore, there is a need for alternative processing treatments that can achieve a 5-log reduction of these pathogens (27, 35), without causing adverse effect on the flavor of the juice.

Several nonthermal technologies have been commercialized, including UV processing of apple cider (41). A pasteurization process using radio frequency electric fields (RFEF) has been developed recently (17–20). In these studies, a set of RFEF operating parameters that achieved 99.999% (5-log) reduction of *E. coli* in apple cider was determined, and the kinetics of bacterial inactivation established. However, data on the exact mechanism of bacterial inactivation using RFEF are limited. We hypothesized that RFEF inactivation is caused by rupture of bacteria membrane structure by exerting irreversible high-voltage electric fields on the bacteria. If the membrane structure of the bacteria is damaged beyond self-repair, then the bacteria may lose its biological activities due to leakage of intracellular metabolites. In this study, we investigated the effect of RFEF on sublethal injury of *E. coli* and leakage of intracellular ATP and UV-absorbing materials from the bacteria. Bioluminescence ATP assay has been used to measure direct membrane damage of *Candida albicans* treated with ketoconazole and tioconazole (3), and the authors reported accumulation of extracellular ATP in media containing cells treated with ketoconazole and tioconazole. Similarly, Uku-ku and Shelef (40) reported a dose-dependent effect on cell numbers and leakage of intracellular ATP of *L. monocytogenes* treated with nisin, ampicillin, and streptomycin.

Results presented in this study are intended to serve as a guide to understanding the mechanism of inactivation of bacteria by RFEF and to provide a scientific basis for an effective comparison of this technology against conventional thermal processing technology.

Similarly, the results may help juice processors, research institutions and the regulatory agency to understand the mechanism of bacteria inactivation by RFEF method.

**MATERIALS AND METHOD**

Test strains and preparation of inocula. *E. coli* K-12 (ATCC 23716) from the U.S. Department of Agriculture, Agri-
cultural Research Service, Eastern Regional Research Center culture collection were used in this study. Individual cell cultures were 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, Kans.) with incubation at 37°C for 16 to 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 (pH 7.2) solution. Finally, the washed cells were resuspended in 100 ml of phosphate-buffered saline (10⁹ CFU/ml) and used as the inoculum.

Sample preparation. A 1.3-liter container of apple juice concentrate purchased from a local store was mixed with 8.3 liters of deionized sterile water at room temperature (~23°C). E. coli K-12 prepared as above (10⁹ CFU/ml) was added to the apple juice, mixed, and a serial dilution was prepared. Then, 0.1 ml of these diluted samples was plated in duplicate on TSA, with incubation at 35°C for 24 h to determine the initial bacterial count.

RFEF treatment and processing variables. Inoculated apple juice was allowed to stand at room temperature for up to 2 h before being pumped through the RFEF chamber (0.1-cm diameter, 0.2-cm gap (19)) at a rate of 540 ml/min. The residence time of inoculated treated and untreated apple juice in the holding tube after the treatment chamber was 1.6 s. The radio frequency used throughout the entire test was set at 20 kHz, with a supplied peak-to-peak voltage of 6 kV. The inlet temperatures to the treatment chamber were adjusted to achieve outlet temperatures of 23, 36, 40, 45, 50, and 55°C. In another study designed to investigate the influence of temperature alone, the 0.2-cm gap treatment chamber was replaced with a 20-cm gap treatment chamber, and the RFEF chamber was turned off. The water bath temperature was adjusted to give a similar outlet temperatures stated above up to 60°C. Samples (~20 ml) at each outlet temperatures were individually collected and analyzed for viability, injury, and leakage of intracellular substances such as UV-absorbing materials and ATP.

Microbial injury and viability loss. An aliquot (1 ml) of apple juice treated as described above was plated on TSA, TSA plus 3% NaCl (TSA3), TSA plus 5% NaCl (TSA5), and sorbitol MacConkey agar (SMAC) plates and incubated at 36°C for 48 h. When necessary, depending on the RFEF treatment, samples were diluted in 0.1% peptone water (PW) before plating onto the agar plates. The difference in populations of E. coli enumerated on selective versus nonselective media was considered as injured cells, and the percent injury was calculated using this formula: [(1 − colonies on selective agar)/(counts on nonselective)] × 100. The concentrations of NaCl used to detect sublethal injury caused by RFEF correspond to the highest amount that did not affect the growth of untreated cells. The number of colony-forming units (CFU per milliliter) on nonselective and selective agar media was used to calculate the viability loss which is defined as the differences in log CFU per milliliter of bacteria between control and RFEF disks treated samples (25). Untreated RFEF-inoculated apple juice was used as the control for each experiment.

Leakage of bacteria intracellular UV-absorbing materials. To quantify the intracellular UV materials released from E. coli K-12, treated and untreated apple juice containing the UV materials were measured at 260 and 280 nm with a Spectrophotometer (DUR 530, Beckman Coulter, Fullerton, Calif.) (7, 42, 45).

Bioluminescence ATP assay. To determine any increase in extracellular (somatic) ATP of apple juice after RFEF treatment, portions (0.1 ml) of sample prepared for microbiological analysis were mixed with 0.1 ml of luciferin-luciferase (Sigma) reagent using an ATP bioluminescent assay kit (Turner Design, Sunnyvale, Calif.). Bioluminescence ATP assays were performed using a TD-20/20 (DL Ready) Luminometer (Turner Design). The generated light signal was measured after a 3-s delay time and a 14-s integration time. Assays of standard amounts of purified ATP (Turner Design) were used to calculate ATP levels, and ATP concentrations in samples were expressed as log femtogram (fg) per milliliter. Controls for background luminescence consisting of 50 μl of apple juice were run, and the readings were subtracted from readings for ATP determination. Possible inhibition of the luciferase reaction by residues from the apple juice and/or RFEF treatment was corrected by the addition of known amounts of ATP standard to the reaction vial followed by addition of the luciferase enzyme (40).

SEM. Aliquots (50 μl) of cellular suspensions in treated apple juice were deposited on 12-mm-diameter glass coverslips. After about 30 s, the coverslips were gently immersed into 2-ml volumes of 2.5% glutaraldehyde–0.1 M sodium imidazole buffer solution (pH 7.0) in a multi-well plate, sealed, and stored for further processing. For scanning electron microscopy (SEM) preparation, the coverslips were washed with buffer solution, dehydrated by exchange with graded ethanol solutions (50%, 80%, and absolute), and critical point dried from liquid CO₂. The dry coverslips 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, Oreg.) operated in the secondary electron imaging mode at an instrumental magnification of 25,000× to visualize topographical details of cell surfaces.

Data analysis. All experiments were done 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, N.C.). The SAS program was used to determine significant differences in survival, viability loss, inactivation, UV-absorbing substances, and ATP concentrations. Significant differences (P < 0.05) between mean values of number of cells and ATP concentrations were determined by the Bonferroni least significant difference (LSD) method (28).

RESULTS AND DISCUSSION

Effect of RFEF and treatment temperature on cell population. There was no background microflora recovered in the uninoculated apple juice plated on TSA and SMAC. Survival of E. coli populations inoculated in apple juice treated with RFEF at different temperatures is shown in Figure 1. The population of E. coli cells recovered in apple juice after inoculation and before RFEF treatment averaged 7.8 log CFU/ml on nonselective agar plates. This population was slightly lower on selective agar plates (7.4 log CFU/ml). RFEF treatment at 23°C did not cause significant change in the population of the E. coli cells in apple juice. Significant changes in the population of E. coli were observed when RFEF treatment temperature was increased to 40°C and above (Fig. 1). At 45°C, the viability loss for E. coli in RFEF-treated apple juice was 3 log, and this population remained unchanged even at 55°C. The population
FIGURE 1. Survival of Escherichia coli K-12 (ATCC 23716) in apple juice treated with RFEF (15 kV/cm) at 23, 27, 40, 45, 50 and 55°C. Values are means of three determinations ± standard deviations.

of healthy E. coli cells that survived the RFEF treatment recovered using the selective agar plates at 45°C and above were significantly \( (P < 0.05) \) different than the numbers on nonselective plates. At 55°C, the healthy population of E. coli cells recovered in RFEF-treated apple juice averaged <1.5 log CFU/ml on selective agar plates.

**SEM observation of E. coli surface structure.** The effect of RFEF treatments on the membrane structure of the E. coli K-12 in apple juice observed using the SEM is shown in Figure 2. At room temperature (control), the surfaces of the cell membrane were smooth and cylindrical, showing profiles of the short, narrow rods with a uniform contour, smooth sides, and regular semicircular tips (Fig. 2A). As the temperature of the RFEF treatment was increased, the bacterial cell surface structure and shape changed. At 40°C, most cells had “pimples” surfaces with a few to many circular and irregularly shaped spots (20 to 30 nm) in diameter dotted along the cell body and at the tips (Fig. 2B). At 45°C, the cell membrane on many of the bacterial cells had a higher concentration of smaller spots than at 40°C, and the contours along the cylindrical surfaces and cell tips were uneven (Fig. 2C). At 50°C, the membrane surfaces were differentiated into irregular folds, dents, and pits, and the cell profiles were usually deformed (Fig. 2D). The electric field strength of the RFEF at all temperatures tested deformed the bacterial surface structure leading to the leakage of intracellular substances and in some cases collapse of the bacteria surface structure (data not shown). Bacterial cell surfaces possess a net negative electrostatic charge by virtue of ionized phosphoryl and carboxylate units on outer cell envelope macromolecules (44). Escherichia coli K-12 used in this study is a gram-negative bacteria that has lipopolysaccharide (LPS) and protein units on its outer layer. The LPS and protein unit forms a highly charged surface that is stabilized by cation binding (35). Similarly, the gross effects of electrical interaction with biological cells are well known and a macroscopic intravascular electrode maintained at a constant current intensity of 1 mA was found to induce thrombosis and injury on the vascular wall, ranging from minimal lesion of endothelium to almost total necrosis of the vascular wall (43). These particular phenomena could explain the variance observed on the E. coli bacterial cell surface structure after the RFEF treatment (Fig. 2).
Leakage of bacterial intracellular materials. The initial extracellular ATP in untreated apple juice determined immediately before RFEF treatment averaged 1.4 log fg/ml. The bulk of this ATP comes from plant and to some extent from the environment. The effect of RFEF treatment on the extracellular ATP concentration of treated apple juice at different temperatures is shown in Figure 3. ATP plays a key role in the energy status of the cell and in regulating enzyme activity; therefore, any leakage of intracellular ATP into the extracellular media would be an indication of damage to the cytoplasmic membrane. This observation precludes non-RFEF samples treated at 40°C and above. In this study, we are able to show changes on E. coli surface structure due to the effect of RFEF-temperature treatment.

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Leakage of bacterial UV-absorbing substances measured at 260 and 280 nm is shown in Figure 4. Similar to ATP determination, an increased intracellular UV-absorbing substance that leaked out of the bacteria into the apple juice extract was measured at $A_{260\,nm}$ and $A_{280\,nm}$. The average values of protein materials determined at $A_{280\,nm}$ in RFEF samples treated at 27°C was 0.18, and this value did change at 40°C and slightly decreased at 45°C and above. There was an average reading of 0.19 ± 0.02 of nucleic acid–containing materials determined at $A_{260\,nm}$ in RFEF treated apple at 27°C. This value was slightly higher in inoculated untreated apple juice but was not significantly different at this temperature. However, this initial value increased to 2 at 40°C and 2.4 at 45°C after which there was a rapid decline of these substances at higher treatment temperatures. This rapid decline may be attributed to the electrical charge produced by the RFEF that may have interacted with the substances as they leaked out of the bacterial membrane. This observation precludes non-RFEF samples treated at 40°C and above (data not shown). This is the first study to document cellular leakage of biological molecules from bacteria treated with RFEF. An earlier study used a model system of lipid vesicle consisting of phosphatidyl choline only. In that study, the authors observed an increased leakage of phosphatidyl choline throughout the course of the study (34).

Effect of heat treatment alone. It is widely understood that application of heat pasteurization would inactivate bacteria in liquid food. In a separate study, we investigated the effect of heat treatment alone at extremely low RFEF by allowing inoculated apple juice samples to pass through the 20-cm RFEF chamber. The RFEF chamber temperature maintained at 55°C caused a viability loss of 1.5 log and a 27.8% injury on E. coli populations in the apple juice (Fig. 5). When the temperature inside the RFEF treatment chamber was reduced, viability loss and percent injury for E. coli decreased significantly, and at 27°C the population of bacteria recovered and the percent injury were not significantly ($P > 0.05$) different than the control (Fig. 5). The percent injury observed in RFEF-treated samples increased as the treatment temperatures increased and were significantly different than when temperature was used alone. And at 55°C RFEF treatment, the percent injury....
on E. coli population drastically reduced and was significantly lower than the numbers observed in samples treated without the RFEF turned on. The increased RFEF chamber temperature at 60°C showed a slight increase in the accumulation of extracellular ATP and a decreased population of E. coli in the RFEF-treated apple juice (Fig. 6). Again, the values of extracellular ATP determined when the chamber temperatures were set at 25°C to 50°C was not significantly different than the untreated values. However, at 55°C and above, an inverse relationship between decline in bacterial population and increase in extracellular ATP of treated apple juice was observed. Similarly, percent injury observed at these temperatures was minimal and did not cause leakage of cellular metabolites as seen when the RFEF was turned on. When the RFEF treatment chamber was changed from 0.2-cm gap (15 kV/cm) to 20-cm gap (0.15 kV/cm) and the chamber temperature set at 40°C, the population reduction determined with selective versus nonselective agar plates were not significantly (P > 0.05) different than when the chamber was set at 0.2 cm. The results of this study indicate that inactivation, injury, and viability loss of RFEF-treated E. coli populations in the apple juice at 15 kV/cm and outlet temperatures of <55°C is primarily due to nonthermal effect. When the intensity of the electric field was decreased to <1 kV/cm and the outlet temperature maintained at 50°C or less, the viability loss for E. coli averaged 0.3 log CFU/ml. Above this temperature, thermal effects contributed to minimal inactivation and less sublethal injury. When the RFEF treatment chamber temperature was set at 55°C or above, the efficacy of the RFEF treatment leading to bacterial injury, viability loss, leakage of intracellular materials, and inactivation was enhanced.

Similar inactivation of yeast in water and E. coli in apple juice at near ambient temperatures by RFEF has been reported (17-19). The electric energy for the RFEF process used was calculated at 300 J/ml. The results of the present study provide the first evidence that RFEF processing led to damage of the bacterial surface membrane structure, creating pores and causing leakage of intracellular bacterial substances that led to the viability loss and inactivation of the bacteria. Leakages of bacterial intracellular substances as a result of membrane damage by antimicrobial agents have been reported (3, 11, 23, 40)). In this study the authors concluded that the accumulation of extracellular ATP in media containing L. monocytogenes cells treated with nisin, ampicillin, and streptomycin resulted from the membrane damage caused by these compounds.

Other researchers have measured leakage of nucleic acid and protein of microwave-injured bacteria at 280 and 260 nm and reported that the intracellular UV absorbing substances that leaked out from the bacteria into the cellular extracts contained protein with nucleic acids being the most common contaminant in the extract (42, 45). Pyrimidines and purines, both nucleic acid compounds, are known to absorb UV light at a wavelength of 260 nm. Therefore, the presence of these materials determined in the RFEF-treated apple juice extract indicates damage to the cell at the membrane level (Figs. 3 and 4). However, it can be presumed that the charged energy produced by the RFEF was interacting preferentially with the UV-absorbing materials that leaked from the bacteria, as opposed to the leaked ATP. At this time, the detailed mechanisms of this interaction are not fully understood and are the subject of ongoing research.

The RFEF technique used in this study is nonthermal due to the fact that bacterial inactivation does not rely on heat application alone. However, an application of moderate heat to the RFEF as seen in this study provides a much greater effect than when used alone. Increasing the temperature to 55°C enhanced the inactivation of the bacteria, leading to a 99.99% reduction. The results of this study suggest that inactivation of bacteria by RFEF treatment was a result of induced structural surface changes on the bacterial membrane. These changes led to injury/damage, causing efflux/leakage of intracellular ATP, protein, and/or nucleic acid of the bacteria, which affected the energy status and the enzymatic activity of the bacterial cell, leading to cell death.
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