Research Note

Inactivation of Lactobacillus plantarum in Apple Cider, Using Radio Frequency Electric Fields†

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ABSTRACT

Radio frequency electric fields (RFEF) nonthermal processing effectively inactivates gram-negative bacteria in juices, but has yet to be shown effective at reducing gram-positive bacteria. Apple cider containing Lactobacillus plantarum ATCC 49445, a gram-positive bacterium, was RFEF processed under the following conditions: field strength of 0.15 to 15 kV/cm, temperature of 45 to 55°C, frequency of 5 to 65 kHz, treatment time of 170 μs, and holding time of 5 to 50 s. The effect of refrigerating the inoculated cider prior to processing, the extent of sublethal injury, and the effect of storing the treated cider for 35 days were investigated. The population of L. plantarum was reduced by 1.0 log at 15 kV/cm, 20 kHz, and 50°C, with a 5-s hold time. There is a synergistic effect between RFEF and heat above 50°C. Inactivation significantly \( P < 0.05 \) increased as frequency was decreased from 65 to 5 kHz. Inactivation increased linearly with field above 8 kV/cm. Holding cider at 55°C after RFEF treatment for 5 and 50 s resulted in 2.5- and 3.1-log reductions, respectively. The surviving population was composed of 1.4-log sublethally injured cells. Storing processed cider at 4°C for 35 days steadily and significantly \( P < 0.05 \) reduced L. plantarum from 4.5 to 0.9 log CFU/ml. The electrical energy density was 51 J/ml. This provides the first evidence that nonthermal RFEF processing inactivates gram-positive bacteria, and that surviving cells may die off during refrigerated storage.

The radio frequency electric field (RFEF) process is one of many nonthermal processes recently developed to improve the safety and quality of liquid foods. It essentially consists of quickly applying electrical energy to food at field strengths in excess of approximately 5 kV/cm. Nonthermal inactivation of microorganisms is thought to occur due to electroporation of the cell membranes. RFEF processing has the potential to be commercially adopted on a large scale because it is a continuous operation that can treat high flow rates.

RFEF processing has been shown to inactivate Escherichia coli in saline water (22), in apple juice (8, 9), in orange juice (11), and in apple cider (10). Substantial progress has been made in understanding the effect of RFEF processing on this gram-negative bacterium. However, research is totally lacking on the effect of RFEF processing on gram-positive bacteria.

Many aspects of RFEF processing have been studied including temperature, electric field strength, frequency, treatment time, sublethal injury, kinetics, and energy requirements (8–11, 24). Yet, several areas of RFEF processing have not been researched with any type of microorganism, let alone a gram-positive bacterium. These include the effect of storing contaminated (inoculated) food for a couple of days prior to RFEF processing, the effect of storing RFEF-processed food for several weeks, and the effect of adding a thermal treatment after RFEF processing. An understanding of these subjects is necessary before the RFEF process can be commercialized.

In large cider-producing facilities, apples are pressed to make cider, which is then typically stored in tanks and blended before being thermally pasteurized. It has been reported that storing apple cider inoculated with E. coli for 24 h significantly increases the thermal \( D \)-value of the bacterium (15).

RFEF processing imparts some thermal energy to cider, which raises the temperature of the cider during processing. It may be preferable to hold the cider at this elevated temperature for a brief period to aid microbial inactivation before cooling. Inserting a holding tube into the RFEF process does not require any additional energy.

Commercially produced cider is refrigerated after pasteurization for up to a month or more before being consumed. Under suitable conditions, a few remaining cells could multiply and result in an infective dose.

The objective of this study was to determine the effect of RFEF nonthermal processing on Lactobacillus plantarum, a gram-positive bacterium. In addition, the effects of storing inoculated cider for various amounts of time before RFEF processing and of storing cider for various amounts of time after RFEF processing were studied. Finally, the

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potential benefit of combining RFEF processing with thermal processing was investigated.

MATERIALS AND METHODS

Microorganisms, inoculum, and media. L. plantarum ATCC 49445 was maintained on deMan Rogosa Sharpe (MRS) agar (Remel, Lenexa, KS) at 4°C. Prior to inoculation of product, the organism was cultured in MRS broth (Remel), with shaking at 37°C for 16 to 18 h. Pasteurized apple cider containing no preservatives was obtained from a local producer, Zeigler Beverage (Lansdale, PA). The cider was sampled to determine background microbiota, and the levels ranged from less than 0.3 to 2.1 log CFU/ml. The cider was inoculated from the stationary-phase culture of L. plantarum to give a population of approximately 7 log CFU/ml. RFEF processing of the inoculated cider began either immediately, or after a 2- to 48-h storage time at 4°C to simulate actual conditions found in the cider industry. The pH (Orion 420A, Thermo Electron Corp., Beverly, MA) and conductivity (Oakton Acorn CON 5, Cole-Parmer, Vernon Hills, IL) of the cider were 3.8 and 2.16 mS/cm, respectively.

RFEF equipment. Geveke and Brunkhorst (7) have previously described the RFEF power supply system used in this investigation. The RFEF power supply produced a peak voltage of 3 kV over a frequency range of 5 to 65 kHz. It consisted of four 1-kW radio frequency amplifiers (model 1000A, Industrial Test Products, Port Washington, NY) and four step-up transformers (Industrial Test Products) connected in series. A function generator (model AFG 310, Tektronix, Beaverton, OR) drove the amplifiers. The RFEF power supply was connected to a treatment chamber.

The treatment chamber was made of Rexolite, a transparent, cross-linked polystyrene copolymer (C-Lec Plastics, Philadelphia, PA). The chamber was designed to converge the flowing apple cider into a narrow area in order to reduce the power requirement (8, 18, 19). Cider entered and exited the Rexolite chamber through the annuli of cylindrical stainless steel electrodes (part SS-400-1-OR, Swagelok, Solon, OH). Between the electrodes in the treatment chamber, there was a thin partition with a channel of circular cross section through the center. The diameter and length of the channel were 0.10 and 0.20 cm, respectively. The channel design included a 0.20-cm space between the end of each of the electrodes and the central channel to reduce the potential for arcing. The volume formed by the space was 0.19 cm³.

One electrode on the treatment chamber was grounded. The other electrode on the treatment chambers was connected to the RFEF power supply. On exiting the treatment chamber, the cider flowed through a 1.8-m section of plastic tubing having an internal diameter of 3.2 mm. The purpose of this plastic tubing was to electrically isolate the treatment chamber from the surrounding equipment and to ensure that the maximum field is achieved within the chamber.

The supplied voltage and current to the RFEF treatment chamber were measured by using an oscilloscope (model TDS224, Tektronix), current probe (model 411, Pearson Electronics, Palo Alto, CA), and a voltage divider (model VD15–8.3-A-KB-A, Ross Engineering, Campbell, CA). The nominal maximum electric field strength used in the study was 15 kV/cm, obtained by dividing the peak voltage, 3.0 kV peak, by the length of the central gap, 0.20 cm. The minimum field strength used in the study was 7.5 kV/cm.

The temperature and flow rate of the apple cider entering the treatment chamber was regulated by using a miniature-scale high-temperature, short-time processing system (model FT74-30-Mk-III-33-34, Armfield, Jackson, NJ). The system included a feed tank, processing cavity feed pump, plate and frame heat exchanger, thermocouples, and an electrically powered hot water boiler and pump. Cider was pumped from the feed tank at a flow rate of 33 liters/h through the heat exchanger and to the RFEF treatment chamber. The cider was exposed to RFEF in the chamber for 170 μs. A back pressure of 1.7 atm gauge (172.25 kPa) helped to eliminate arcing by minimizing bubble formation within the cider. The Reynolds number within the treatment chamber was calculated based on the flow rate, a density of 1 g/ml, and a viscosity of 0.01 g/(cm·s). The Reynolds number was 12,000, which is in the turbulent flow region.

The temperature of the apple cider rises in the RFEF treatment chamber due to ohmic (resistance) heating in 170 μs. The temperatures of the cider entering and exiting the RFEF treatment chamber were measured with 3.2-mm-diameter chromel-alumel thermocouples (Omega Engineering, Inc., Stamford, CT). The treatment chamber outlet temperatures selected were 45, 50, and 55°C. Product samples in test tubes were immediately cooled to less than 30°C by using ice water. To determine the effect of temperature alone, the treatment chamber (described above) was replaced with a low-electric-field chamber that heated the cider to the desired temperatures, 45 to 55°C. The low-field chamber consisted of two stainless steel electrodes (part SS-400-1-OR, Swagelok) inserted into a 20.3-cm length of 0.64-cm-inner-diameter plastic tubing. The field strength used for the thermal treatments was 0.15 kV/cm. The temperature rise occurs in 700 ms.

Energy calculations. The processing energy was calculated by using the applied voltage and current as measured by the oscilloscope. The energy density of the process was calculated by dividing the energy by the flow rate.

Enumeration of survivors. Appropriate dilutions of the product samples were made in 0.1% buffered peptone water (Difco, Becton Dickinson, Sparks, MD) with a minimum 1-ml transfer. Duplicate samples were then poured plated with MRS agar, and the plates were incubated at 37°C for 48 h. Plates with 30 to 300 colonies were enumerated by using a manual colony counter ( Colony Counter 920, Bantex, Burlingame, CA). Bacterial injury by RFEF treatment was determined by pour plating treated samples with MRS plus 5% NaCl, incubating at 37°C for 72 h, and enumerating. Preliminary studies determined that healthy stationary-phase L. plantarum cells recovered in equivalent numbers on MRS, and MRS plus 5% NaCl. Shin and Pyun (20) used MRS agar plus 4% NaCl to determine the level of injured L. plantarum treated by pulsed microwave radiation. The authors spread plated cells directly on the surface of the medium. In the present study, it was determined that, by pour plating, the concentration of NaCl could be increased to 5% and still recover a maximal population of healthy cells, while inhibiting growth of injured cells. Bacterial inactivation was defined as the difference in log CFU per milliliter of microorganisms between untreated and treated samples on nonselective media (MRS). Injury was defined as the difference in log CFU per milliliter of treated microorganisms between untreated and treated samples on nonselective (MRS) and selective media (MRS plus 5% NaCl).

Storage after RFEF processing. To evaluate the long-term effect of RFEF processing on L. plantarum in apple cider, treated and untreated (control) samples were packaged in sterile 10-ml test tubes inside a sanitary laminar flow hood equipped with a HEPA air filter (Forma Scientific, Inc., Marietta, OH). The closed tubes were stored at 4 and 10°C for 35 days. Viable cells were counted at selected days throughout this period on MRS agar, as described above.
FIGURE 1. Effect of storage time at 4°C prior to RFEF processing (under conditions of a 15-kV/cm electric field, 20-kHz frequency, a 170-μs treatment time, a 55°C outlet temperature, and a 5-s hold time). The initial concentration of cells was 7.03 log CFU/ml.

FIGURE 2. Effect of frequency (under conditions of a 15-kV/cm electric field, a 170-μs treatment time, a 55°C outlet temperature, and a 5-s hold time). The initial concentration of cells was 7.03 log CFU/ml.

Replication and statistics. Each RFEF experiment was performed in triplicate. Results were expressed as the means of these values ± standard deviations. The significance of differences in results obtained was calculated by using the Student's t test statistical function in Excel (Microsoft Corp., Redmond, WA).

Kinetics modeling. The relationship between microbial inactivation and the electric field strength was modeled by using the following equation (13): \( \ln(\text{inactivation}) = a(E - E_c) \), where \( a \) is a constant, \( E \) is the electric field strength, and \( E_c \) is the critical electric field strength below which inactivation does not occur.

RESULTS AND DISCUSSION

Storage prior to RFEF processing. In the real world, apple cider is rarely contaminated with bacteria immediately before being pasteurized. More probably, the cider, which is acidic, will become contaminated by the bacteria several hours before it is pasteurized. The effect of storing inoculated cider for various amounts of time prior to RFEF processing has never been studied. Therefore, a series of experiments were performed to determine the effect of varying this time on inactivation. Inoculated cider was processed immediately and after 2, 4, and 6 h of storage at 4°C. The RFEF operating conditions were selected based on a previous study with E. coli (8) to give a 2- to 3-log reduction. The conditions used were an electric field strength of 15 kV/cm, a frequency of 20 kHz, a treatment chamber outlet temperature of 55°C, a treatment time of 170 μs, and a hold time of 5 s. When apple cider was inoculated with L. plantarum and immediately processed with RFEF, the population of L. plantarum was reduced by 2.54 ± 0.40 log. Storing the inoculated cider for 2 h before treatment did not significantly (\( P > 0.05 \)) alter the reduction of L. plantarum, as shown in Figure 1. As the time after inoculation was increased from 2 to 6 h, bacterial inactivation increased significantly (\( P < 0.05 \)). However, RFEF processing 48 h after inoculation resulted in the same (\( P > 0.05 \)) level of reduction as that obtained immediately after inoculation. The reason for this phenomenon is unclear, but will be investigated in future studies. During thermal processing, a slightly different behavior has been observed. When E. coli was inoculated into apple cider and the inoculated cider stored at refrigerated temperature for 0 to 24 h prior to thermal treatment, the D-value significantly increased at 6 and 24 h (15). For all of the remaining experiments in the present study, the inoculated cider was stored at 4°C for 2 h prior to RFEF processing.

Frequency. The effect of frequency on microbial inactivation was determined. The RFEF system is capable of operating at frequencies from 5 to 65 kHz. In this study, frequencies of 5, 20, 35, 50, and 65 kHz were investigated. The relationship between inactivation and frequency appeared to be sigmoidal (Fig. 2). Inactivation increased significantly (\( P < 0.05 \)) as the frequency decreased from 50 to 20 kHz. This phenomenon has been observed before (8, 9, 11). These results are interesting because they suggest that the RFEF process could be even more effective at frequencies lower than 5 kHz. However, there is a lower limit to the frequency that can be used with the present treatment chamber (10). In physics, the period is defined as the duration of one cycle in a repeating event. The residence time in the treatment chamber should be equal to or greater than the period of the RFEF power supply to minimize temperature fluctuations within the chamber. The period is also defined as the reciprocal of the frequency. Hence, with the 170-μs treatment time used in the present study, the recommended minimum frequency is 5.9 kHz. At frequencies below approximately 4 kHz, the RFEF system began to operate erratically. The cause for the decrease in effectiveness at higher frequencies may be due to the time lag between the applied voltage and the induced voltage across the bacterial cell membrane (10). The membrane, due to its dielectric properties, stores electrical energy and permits the flow of voltage to a degree dependant on the frequency. Although it is very difficult to calculate exactly the induced transmembrane voltage, it is clear that the voltage is significantly reduced as the frequency is increased in the range of frequencies from 5 to 65 kHz.
of 100 kHz (16). As the frequency is raised much above 20 kHz, the induced transmembrane voltage may be reduced to a point where fewer pores in the membrane are formed, and fewer cells rupture (Fig. 2). Nearly all of the previous studies on RFEF have used a frequency close to 20 kHz (7–11, 22, 23). This frequency was chosen to use for all of the remaining experiments in the present study.

**Temperature.** A series of experiments were performed to determine the effect of temperature on inactivation. The population of *L. plantarum* in apple cider was reduced by 0.74 ± 0.42 log after being RFEF processed at a peak electric field of 15 kV/cm, a treatment time of 170 μs, a treatment chamber outlet temperature of 45°C, and a hold time of 5 s (Fig. 3). When the cider was thermally heated to 45°C and held for 5 s, the population of *L. plantarum* was unaffected (Fig. 3). RFEF inactivation significantly (*P < 0.05) improved as the temperature increased from 45 to 55°C. The RFEF inactivation at 50°C was 1.04 ± 0.23, while the 50°C thermally heated sample again determined that there was no thermal inactivation. The inactivation at 55°C was 2.51 ± 0.36 log. Although at 55°C there was some thermal inactivation—0.23 ± 0.06 log—the vast majority of the RFEF inactivation was due to nonthermal effects. The combined effect of RFEF and temperature is synergistic. This phenomenon has been reported with RFEF processing (9). In addition, a strong synergism between PEF and temperature was observed during processing of *L. innocua* NCTC 11289 in a phosphate buffer at an electric field of 47 kV/cm (25).

The microbiological inactivation results obtained in this study on *L. plantarum*-inoculated apple cider can be compared with those obtained using similar RFEF equipment to process apple juice containing *E. coli* (8). The nonthermal inactivation of *E. coli* was 1.3 log at an electric field of 15 kV/cm, a treatment time of 170 μs, and a treatment chamber outlet temperature of 50°C. In the present study under the same conditions, the population of *L. plantarum* in apple cider was reduced by 1.0 log. Thus, it may be inferred that gram-positive bacteria are slightly more resistant to RFEF processing than are gram-negative bacteria.

This has also commonly been observed in pulsed electric field (PEF) processing (1, 14). However, additional RFEF studies utilizing other genera of bacteria are required before a more general conclusion can be made.

**Electric field strength.** The effect of electric field strength on microbial inactivation was determined. The field strengths investigated were 7.5, 10.0, 12.5, and 15.0 kV/cm (Fig. 4) at constant RFEF conditions of a 20-kHz frequency, a 170-μs treatment time, a 55°C outlet temperature, and a 5-s hold time. The microbial inactivation significantly (*P < 0.05) declined as the field strength decreased. The inactivation decreased steadily from a high of 2.51 ± 0.36 log reduction at 15 kV/cm to a low of 0.28 ± 0.12 log at 7.5 kV/cm. The data were described by using a first-order kinetics model (Fig. 4). The correlation coefficient was 0.93. The critical electric field strength, τ, is the minimum field required to irreversibly rupture the cell membrane, according to the electric field strength model (13). The calculated τ for *E. coli* in apple cider are 13.5 and 4.0 kV/cm at 55 and 60°C, respectively (10). These latter values were acquired by using a pilot plant RFEF system that had minimum field strength limited to 20 kV/cm; hence, significant extrapolation was required. The RFEF system used in the present study does not suffer from this drawback. Therefore, it is recommended that future studies of the critical electric field strengths of other microorganisms be performed by using the present system. Also, additional field strength data may lead to a model with a higher correlation coefficient.

**Injury and storage after RFEF processing.** Because RFEF processing is a relatively new nonthermal technology, very little research has been done on evaluating sublethal injury, and none has been done on storage after processing. The injury of *L. plantarum* in apple cider was 1.44 ± 0.25 log (or 94%) of surviving cells subsequent to being RFEF processed at an electric field of 15 kV/cm, a fre-
FIGURE 5. Effect of storage temperature on shelf life of RFEF-processed apple cider (under conditions of a 15-kV/cm electric field, a 20-kHz frequency, a 170-μs treatment time, and 55°C outlet temperature, and a 5-s hold time). Viable cells were counted on MRS agar.

FIGURE 6. Effect of hold time (under conditions of a 15-kV/cm electric field, a 20-kHz frequency, a 170-μs treatment time, and a 55°C outlet temperature). The initial concentration of cells was 7.03 log CFU/ml.

quency of 20 kHz, a treatment time of 170 μs, a treatment chamber outlet temperature of 55°C, and a hold time of 5 s. The RFEF injury of E. coli in apple juice at very similar operating conditions (15 kV/cm, 20-kHz frequency, 170-μs treatment time, and 55°C) was 0.4 log (24). The present study corroborates that RFEF processing causes substantial sublethal injury. The occurrence of injury in PEF processing, which is similar to RFEF processing, has been questioned. Many PEF studies determined either that there was little injury, <0.25 log (2), or no injury (3, 21). However, recent studies have shown that substantial injury occurs. More than 3 log survivors were injured when E. coli was PEF processed in McIlvaine buffer at pH 4.0 for 400 μs at 19 kV cm (4). It seems plausible that PEF and RFEF processing should cause injury in addition to inactivation, but more research with other microorganisms, media, and operating conditions, is needed.

The effect of RFEF processing on microbial populations during storage has not been investigated. Apple cider containing L. plantarum was RFEF processed (electric field of 15 kV/cm, frequency of 20 kHz, treatment time of 170 μs, treatment chamber outlet temperature of 55°C, and hold time of 5 s) and was stored at 4°C for 35 days (Fig. 5). Similarly treated samples were also stored at 10°C to determine the effect of mild temperature abuse. In addition, inoculated, unprocessed (control) samples were also kept at the identical temperatures. The populations of L. plantarum cells in the controls stored at 4 and 10°C for 35 days were not significantly (P > 0.05) different from each other, nor did they significantly (P > 0.05) vary from their initial levels. The opposite effects were observed for the RFEF-processed samples. As previously described, the RFEF process resulted in an approximate 2.5-log inactivation, as indicated in Figure 5 (day 0). The population of processed L. plantarum cells stored at 4°C significantly (P < 0.05) declined over the 5-week period. The population steadily dropped from an initial level of 4.52 ± 0.36 log CFU/ml to a final level of 0.90 ± 0.50 log CFU/ml. This result is most likely related to the 1.44-log sublethal injury that occurred during the RFEF processing. The survivors of the RFEF processing are more sensitive to stress conditions after treatment such as a cold, acid incubation. A similar phenomenon has also been observed with PEF processing. A PEF treatment, which was not sufficient to inactivate 1 log E. coli O157:H7 in apple juice, injured a large proportion of cells that became sensitive to a subsequent storage under refrigeration (5). In the case of RFEF-processed apple cider stored at 10°C, the population of L. plantarum also significantly (P < 0.05) declined over the 35-day period (Fig. 5). The drop to 2.76 ± 0.48 log CFU/ml was significantly (P < 0.05) less than that for the samples stored at 4°C.

Hold time. The combined effect of RFEF processing at 55°C and then holding the product at that temperature for up to 50 s before cooling was investigated. Holding tubes having residence times of 5, 33, and 50 s were used, and the RFEF conditions were an electric field of 15 kV/cm, a frequency of 20 kHz, and a treatment time of 170 μs. The inactivation increased at the longest holding time by 0.56 log (Fig. 6). When the high-field treatment chamber was replaced with the low-field treatment chamber, and the cider was heated to 55°C and held for the same times, the amount of thermal inactivation increased by 0.53 log. Therefore, the cumulative effect of RFEF processing and thermal processing is additive rather than synergistic. However, the extra inactivation provided by the holding tubes does not require any additional energy.

Energy density. The RFEF processing energy was calculated for the condition that resulted in the maximum initial microbial inactivation with the shortest hold time, 2.51 ± 0.36 log. The operating parameters were a 15-kV/cm electric field, a 20-kHz frequency, a 170-μs treatment time, and a 55°C outlet temperature. The voltage and current measured by an oscilloscope were 6.0 kVpeak–peak and 0.62 Apeak–peak, respectively. The calculated energy for this particular condition was 0.47 kW. In continuous processes, a common method of comparing efficiencies is to calculate the energy per flow rate, or energy density. In this case, the energy density was 51 J/ml, which was obtained by dividing the energy by the flow rate, 33 liters/h. In a previous
study, the energy density of a RFEF process to inactivate 1.8 log \( E. coli \) in apple juice at an outlet temperature of 50°C was 100 J/ml (8). The cause for the lower energy density obtained in the present study probably is due to the higher operating temperature used. Several RFEF and PEF studies have concluded that processing temperature has a great, if not the greatest, influence on energy efficiency. The average RFEF energy density at an outlet temperature of 60°C was less than one-third that for 50°C (10). In PEF, raising the treatment temperature from 35 to 65°C reduced the energy density from approximately 100 to 40 J/ml (12). By comparison, the energy density for conventional thermal pasteurization with heat regeneration is 34 J/ml (6, 17).

The nonthermal RFEF process significantly reduced \( L. plantarum \) in apple cider at 50°C by 1.04 log. This provides the first evidence that RFEF processing is capable of inactivating a gram-positive bacterium. The electric field strength, frequency and temperature, hold time, as well as the storage time prior to processing and the storage time after processing all had significant effects on the level of inactivation. Of the surviving cells, 94% were injured. The population of RFEF-processed \( L. plantarum \) cells stored at 4°C significantly declined over a 5-week period to 0.9 log CFU/ml. The RFEF process operates at a lower temperature than does a typical thermal process, but requires more energy. The results of this study and previous studies point to the possibility of using nonthermal RFEF processing to pasteurize apple cider containing gram-negative and gram-positive bacteria.

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