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Research Note

Inactivation of *Pseudomonas fluorescens* in Skim Milk by Combinations of Pulsed Electric Fields and Organic Acids

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ABSTRACT

Pseudomonas fluorescens suspended in skim milk was inactivated by application of pulsed electric fields (PEF) either alone or in combination with acetic or propionic acid. The initial concentration of microorganisms ranged from 10^5 to 10^6 CFU/ml. Addition of acetic acid and propionic acid to skim milk inactivated 0.24 and 0.48 log CFU/ml *P. fluorescens*, respectively. Sets of 10, 20, and 30 pulses were applied to the skim milk using exponentially decaying pulses with pulse lengths of 2 μs and pulse frequencies of 3 Hz. Treatment temperature was maintained between 16 and 20°C. In the absence of organic acids, PEF treatment of skim milk at field intensities of 31 and 38 kV/cm reduced *P. fluorescens* populations by 1.0 to 1.8 and by 1.2 to 1.9 log CFU/ml, respectively. Additions of acetic and propionic acid to the skim milk in a pH range of 5.0 to 5.3 and PEF treatment at 31, 33, and 34 kV/cm, and 36, 37, and 38 kV/cm reduced the population of *P. fluorescens* by 1.4 and 1.8 log CFU/ml, respectively. No synergistic effect resulted from the combination of PEF with acetic or propionic acid.

Pseudomonas fluorescens is a gram-negative organism and the most important psychrotrophic bacterium responsible for deterioration of refrigerated milk *(20). Pseudomonas* is considered the major cause of microorganism spoilage in pasteurized milk, which reaches the end of its shelf life (8 to 11 days) after manufacturer storage at the recommended temperature of 4°C *(20). Pseudomonas* is well adapted to survival in the milk-processing environment, regardless of the location of production, and adheres strongly to surfaces of milk-processing equipment *(20).* The most common treatment used to inactivate *Pseudomonas* in milk is commercial pasteurization (72 to 80°C for 10 to 20 s) *(21).* However, the increasing consumer demand for minimally processed foods is directing the attention of researchers at public institutions and food industries around the world to the development of nonthermal technologies capable of reducing the use of heat while maintaining the safety of processed food for human consumption *(12).* Among the new nonthermal technologies dealing with the preservation of foods, pulsed electric field (PEF) treatment relies on intense electric fields for the inactivation of microorganisms and is an effective method for inactivating microorganisms in liquid foods such as fruit juices, milk, liquid whole eggs, and certain model foods *(1, 2, 5, 7, 10, 13–15, 17, 19, 22).* During the PEF process, lysis of microorganisms occurs through irreversible structural changes in the membranes that lead to pore formation and disruption of the semipermeable membranes *(3, 18).* According to Sale and Hamilton *(16),* membrane destruction in many cellular systems occurs when the induced membrane potential exceeds the critical value of 1 V, which corresponds approximately to 10 kV/cm for *Escherichia coli.* Ho et al. *(7)* used PEF to inactivate *P. fluorescens* suspended in selected aqueous solutions. Six-log reductions of this microorganism were achieved with an applied electric field intensity of 10 kV/cm and 10 2-μs pulses at 2-s intervals.

The inactivation of microorganisms by the combination of organic acids and PEF was studied by Liu et al. *(11),* who found that a combination of organic acids and PEF may facilitate the entry of undissociated molecules into the bacterial cell and thus increase bactericidal action. The shorter chain organic acids such as acetic, benzoic, citric, lactic, propionic, and sorbic acids and their salts are most commonly used in food preservation. Acetic acid is widely used as a preservative, and its inhibitory action results from neutralizing the electrochemical gradient of the cell membrane and denaturing the microbial proteins *(4).* Considering the intracellular acidification property of this organic acid, which will result in the loss of cell viability or in cell destruction, the aim of this study was to evaluate the effect of combining PEF with acetic and propionic acids on the inactivation of *P. fluorescens* suspended in skim milk.

MATERIALS AND METHODS

Microbe preparation. *Pseudomonas fluorescens* (ATCC 17926, American Type Culture Collection, Rockville, Md.) was grown according to American Type Culture Collection procedures.

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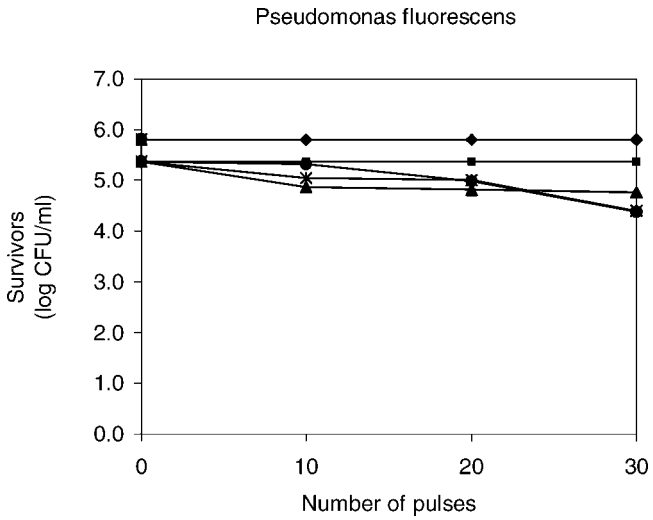

FIGURE 1. *Survivior plots for the inactivation of* P. fluorescens *in skim milk by PEF in combination with acetic acid. Control (♦), acetic acid (■), PEF at 31 kV/cm (▲), PEF at 33 kV/cm + 0.2% acetic acid (●), PEF at 34 kV/cm + 0.3% acetic acid (*).*

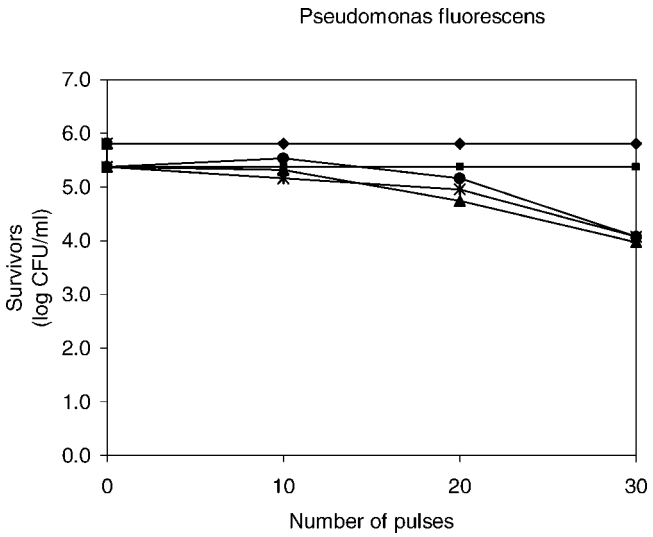

FIGURE 2. *Survivior plots for the inactivation of* P. fluorescens *in skim milk by PEF in combination with acetic acid. Control (♦), acetic acid (■), PEF at 38 kV/cm (▲), PEF at 36 kV/cm + 0.2% acetic acid (●), PEF at 37 kV/cm + 0.3% acetic acid (*).*

Tryptic soy broth enriched with 0.6% yeast extract (TSBYE; Difco Laboratories, Becton Dickinson, Sparks, Md.) was used as the growth medium. One milliliter of frozen *P. fluorescens* culture was thawed in 50 ml of TSBYE and then agitated continuously at 190 rpm in a temperature-controlled shaker (model BSB-332A-1, GS Blue Electric, Blue Island, Ill.) at 30°C for 24 h to reach the stationary phase. Harvested cultures were stored at −70°C with 1 ml of 20% glycerol until uses in PEF experiments.

Preparation and inoculation of milk samples. Whole raw milk was obtained from the Washington State University Creamery (Pullman, Wash.). Milk was separated into cream and skim milk (0.2% fat) at 4°C using a separator (model 612, DeLaval, Lund, Sweden). The skim milk was sterilized at 121°C for 15 min and then refrigerated until use. Sterilized samples of skim milk (pH 6.7) were collected into a 2-liter Erlenmeyer flask. Acetic acid was added to the milk at 0.2% (pH 5.2) and 0.3 % (pH 5.0). Milk samples were also acidified with 0.2% (pH 5.3) and 0.3% (pH 5.0) propionic acid. Acidified milk samples were gently agitated with a magnetic stirrer for about 15 min, then 2 ml of inoculum was pipetted into an Erlenmeyer flask to attain an initial *P. fluorescens* concentration of 10^5 to 10^6 CFU/ml.

PEF treatment. Inoculated milk samples were treated with PEF using a continuous coaxial treatment chamber consisting of two stainless steel electrodes with an electrode gap of 0.66 cm. Samples were pumped through the treatment chamber at a rate of 8.33 ml/s. A pilot plant PEF system (Physics International, San Leandro, Calif.) was used to generated high-voltage PEFs. Input voltages of 35 and 40 kV were used to provide electric field intensities at 31, 33, and 34 kV/cm and 36, 37, 38 kV/cm; these electric field intensities correspond to pulse voltages of 21.8, 22.4, 23.8, 24.4, 25.1, and 26.4 kV, respectively. Sets of 10, 20, and 30 pulses were applied to the skim milk using exponentially decaying pulses with pulse lengths of 2 μs and pulse frequencies of 3 Hz. Treatment temperature was maintained between 16 and 20°C. PEF-treated skim milk at given electric field intensities in the absence of organic acids were used as control.

Microbial assays. Before and after PEF treatment, each milk sample was analyzed by transferring 1 ml of sample into 9 ml (0.1%, wt/vol) of sterile peptone solution. Serial dilutions at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were made for each test, and two samples of each dilution were prepared in tryptic soy agar enriched with 0.6% yeast extract. The plates were incubated at 30 ± 1°C for 48 ± 2 h before being counted. All plates were observed under UV light at 365 nm *(6)* to detect fluorescence and to determine whether the colonies were *P. fluorescens.*

Statistical analysis. Each PEF treatment was repeated three times, with duplicate analyses for each replication. An analysis of variance was performed using the SAS *(16)* general linear model procedure. Tukey's Studentized range test was used for comparisons between sample means. Differences were considered significant at $P < 0.05$.

RESULTS

***P. fluorescens* survivors.** Survival of *P. fluorescens* as a function of different electric fields (31, 33, and 34 kV/cm; 36, 37, 38 kV/cm) and acetic or propionic acid concentrations (0.2 and 0.3%) are presented in Figures 1 through 4. The initial numbers of microorganisms were 6.3×10^5 and 1.1×10^6 cells per ml.

Electric field intensity. PEF treatments at 31 and 38 kV/cm with 30 pulses of 3 Hz for 2 μs in the absence of organic acids reduced the microbial counts in skim milk by 1.0 to 1.8 log CFU/ml (Figs. 1 and 2) and 1.2 to 1.9 log CFU/ml (Figs. 3 and 4), respectively. Combinations of acetic acid (0.2 and 0.3%) and PEF treatments at 33 and 34 kV/cm and 36 and 37 kV/cm with 30 pulses of 3 Hz for 2 μs reduced the population of *P. fluorescens* by 1.4 and 1.7 log CFU/ml, respectively (Figs. 1 and 2). No synergistic effect between electric field intensity and presence of acetic acid was observed ($P = 0.1001$ and 0.8668, respectively) among the interaction factors, indicating that the reduction of viable *P. fluorescens* cells was due to PEF treatment, not acetic acid. Combinations of propionic acid (0.2 and 0.3%) with PEF at 33 and 34 kV/cm and 36 and 37 kV/cm with 30 pulses of 3 Hz for 2 μs reduced the population of *P. fluorescens* by 1.7 and 1.8 log CFU/ml, respectively (Figs.

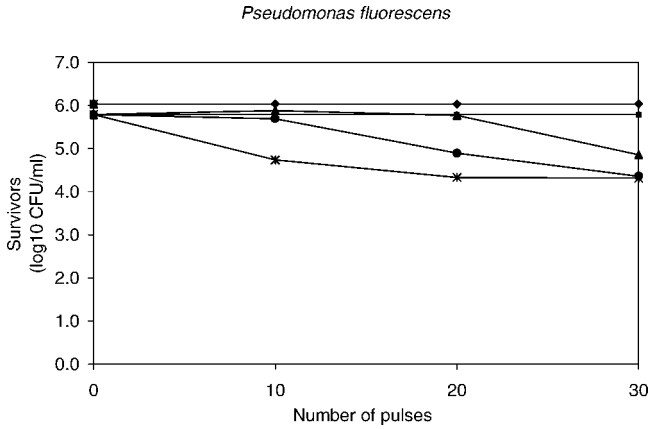

FIGURE 3. *Survivior plots for the inactivation of* P. fluorescens *in skim milk by PEF in combination with propionic acid. Control (♦), propionic acid (■), PEF at 31 kV/cm (▲), PEF at 33 kV/cm + 0.2% propionic acid (●), PEF at 34 kV/cm + 0.3% propionic acid (*).*

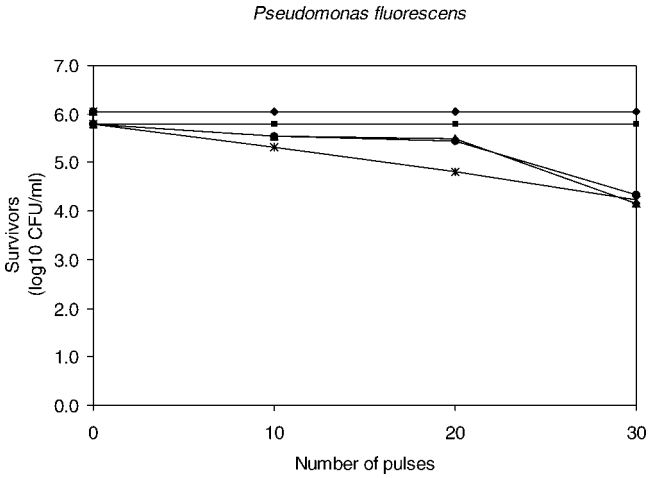

FIGURE 4. *Survivior plots for the inactivation of* P. fluorescens *in skim milk by PEF in combination with propionic acid. Control (♦), propionic acid (■), PEF at 38 kV/cm (▲), PEF at 36 kV/cm + 0.2% propionic acid (●), PEF at 37 kV/cm + 0.3% propionic acid (*).*

3 and 4). No significant relationship was observed ($P = 0.2549$ and 0.7432, respectively) between propionic acid and the number of pulses for PEF treatment at 31 to 34 kV/cm and 36 to 38 kV/cm. Mean number of bacteria surviving were significantly different ($P < 0.05$) among treatments with 0.0, 0.2, and 0.3% propionic acid at 31 to 34 kV/cm, but no differences were observed ($P > 0.05$) between 0.0 and 0.2% propionic acid.

DISCUSSION

The inactivation of microorganisms by PEF results from the electrochemical instability of the cell membrane *(10).* In the present study, the effectiveness of PEF depended on the applied electric field intensity and number of pulses. As the field intensity increased from 31 to 38 kV/cm and the number of pulses increased from 10 to 30, the survival of *P. fluorescens* cells was significantly reduced ($P = 0.0001$) from 1.0 to 1.9 log CFU/ml (Figs. 1 through 4).

During PEF inactivation of *P. fluorescens,* the electric field intensity increased in the presence of organic acids (Figs. 1 through 4). The treatment temperature was maintained between 16 and 20°C by means of a cooling system with two cooling coils connected on opposite ends of the PEF treatment chamber. This system caused the pulse voltage to increase with a consequent rise in the electric field intensity.

Liu et al. *(11)* reported a synergistic killing effect when *E. coli* was treated with PEFs and organic acids. This effect was observed in the presence of both benzoic and sorbic acids at pH 3.4 but not at pH 6.4. Log reductions of 5.6 and 4.2 were achieved in that study, suggesting that lowering the pH of the medium induces microbial inactivation. In our experiments, the effect of combining acetic or propionic acid with PEF was not significant ($P > 0.05$) following PEF treatment at 31 to 34 kV/cm and 36 to 38 kV/cm with 30 pulses of 3 Hz for 2 μs (Figs. 1 and 2); the number of surviving *P. fluorescens* cells was reduced by only 1.4 and 1.7 log, respectively. Mean numbers of *P. fluorescens* survivors in skim milk treated with PEF at the three acetic acid concentrations were not significantly different ($P > 0.05$). Thus, no synergistic effect was observed between PEF and acetic acid on the inactivation of *P. fluorescens* in skim milk. Similarly, no synergistic effect ($P > 0.05$) was observed between propionic acid and PEF treatment at 31 to 34 kV/cm and 36 to 38 kV/cm. Thus, inactivation of *P. fluorescens* in skim milk was due to the high-intensity electric fields and the number of pulses applied and not to the combined effect of propionic acid and acetic acid with PEF treatment. *P. fluorescens* was reduced by 1.7 and 1.8 log CFU/ml, which indicates that increases in the propionic acid concentration and in electric field intensity do not improve the inactivation of *P. fluorescens* in skim milk. The initial concentrations of *P. fluorescens* ranged from 10^5 to 10^6 CFU/ml. When cell membranes are electroporated and then ruptured, cell fluid escapes, resulting in increased bulk fluid conductivity and decreased local fluid conductivity because the membrane constituents have low conductivity. Cluster cells form a protective layer also of low conductivity. The live cells surrounded by this protective layer will not be exposed to the same electric field strength as the unprotected cells *(21).* Therefore, the probability that protected cells will survive is much greater than that for unprotected cells, resulting in a lowered inactivation rate. The reduction in lethality with increased bacterial concentration is attributed to the increased ability of cells to form clusters *(8, 9).*

PEF treatment alone was more effective than the combination of PEF with acetic or propionic acid for inactivation of *P. fluorescens* in skim milk. PEF treatment inactivated 1.9 log CFU/ml compared with 1.7 and 1.8 log CFU/ml following combined PEF and acid treatments. The inactivation of *P. fluorescens* in skim milk by combinations of organic acids and PEF depends on the electric field intensity, the number of pulses, and the pH of the medium.

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