Korean J. Food Sci. An. Vol. 35, No. 6, pp. 800~806(2015)
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DOI http://dx.doi.org/10.5851/kosfa.2015.35.6.800
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

Inactivation of *Escherichia coli*, *Saccharomyces cerevisiae*, and *Lactobacillus brevis* in Low-fat Milk by Pulsed Electric Field Treatment: A Pilot-scale Study

Gun Joon Lee, Bok Kung Han[1], Hyuk Joon Choi[1], Shin Ho Kang[2], Seung Chun Baick[2], and Dong-Un Lee*

Department of Food Science and Technology, Chung-Ang University, Anseong 17546, Korea
[1]BK Bio Co. Ltd., Seongnam 13403, Korea
[2]R&D Center, Seoul Dairy Co-op, Ansan 15407, Korea

Abstract

We investigated the effects of a pulsed electric field (PEF) treatment on microbial inactivation and the physical properties of low-fat milk. Milk inoculated with *Escherichia coli*, *Saccharomyces cerevisiae*, or *Lactobacillus brevis* was supplied to a pilot-scale PEF treatment system at a flow rate of 30 L/h. Pulses with an electric field strength of 10 kV/cm and a pulse width of 30 μs were applied to the milk with total pulse energies of 50-250 kJ/L achieved by varying the pulse frequency. The inactivation curves of the test microorganisms were biphasic with an initial lag phase (or shoulder) followed by a phase of rapid inactivation. PEF treatments with a total pulse energy of 200 kJ/L resulted in a 4.5-log reduction in *E. coli*, a 4.4-log reduction in *L. brevis*, and a 6.0-log reduction in *S. cerevisiae*. Total pulse energies of 200 and 250 kJ/L resulted in greater than 5-log reductions in microbial counts in stored PEF-treated milk, and the growth of surviving microorganisms was slow during storage for 15 d at 4°C. PEF treatment did not change milk physical properties such as pH, color, or particle-size distribution ($p<0.05$). These results indicate that a relatively low electric-field strength of 10 kV/cm can be used to pasteurize low-fat milk.

Keywords: pulsed electric field, low-fat milk, microbial inactivation, pasteurization, physical property

Received February 16, 2015; Revised October 3, 2015; Accepted October 26, 2015

Introduction

Milk is susceptible to both spoilage and pathogenic microorganisms, and current regulations require milk to be heat pasteurized to ensure public safety and to increase shelf life. Heat processing methods used to pasteurize milk are classified as low-temperature long-time (LTLT) treatments, in which milk is pasteurized at 63°C for 30 min, and high-temperature short-time (HTST) treatments, in which milk is typically heated to 72-100°C for 0.01-15 s. An ultrahigh temperature (UHT, 2-4 s at 135°C) method confers long shelf life without the requirement for refrigerated storage (Food and Drug Administration, 2011). Although heat inactivation of enzymes and microorganisms increases milk shelf life, it has deleterious effects on the organoleptic and nutritional properties of milk. The most common organoleptic change is the generation of "cooked flavor" caused by the liberation of sulfhydryl groups from some sulfur-containing amino acids (In and Jung, 2001; Yoo *et al*., 2013).

Pulsed electric field (PEF) treatment is a promising alternative to conventional thermal preservation processes for liquid foods (Buckow *et al*., 2013; Knorr *et al*., 2001; Zhang *et al*., 1995). A PEF is a high-voltage electric field applied for a short period of time (a few to tens of microseconds), with PEF treatment processing parameters including the electric field strength (kV/cm), treatment temperature (°C), pulse width (μs), and pulse frequency (Hz). Electrical breakdown of the membrane surrounding the cell, which acts as a capacitor filled with a dielectric medium, is the mechanism by which microbes are inactivated by a PEF treatment (Zimmermann, 1986). Both the cell cytoplasm and the liquid medium surrounding the cell have greater dielectric constants than the cell membrane, and the difference between the dielectric constants on either side of the membrane results in a transmembrane potential that induces electrical breakdown of the

*Corresponding author: Dong-Un Lee, Department of Food Science & Technology, Chung-Ang University, Anseong, 17546, Korea. Tel: +82-31-670-3034, Fax: +82-31-675-4835, E-mail: dong-un.lee@cau.ac.kr

membrane. If the transmembrane potential exceeds 1 V (i.e., the critical membrane voltage), irreversible permeabilization is induced, which leads to the release of substances from the cell's interior and ultimately causes cellular malfunction or death. Many studies on the application of PEF treatments to liquid foods have been carried out. The effect of a PEF on the plasma membrane of microbes enables the preservation of liquid foods by inactivating microorganisms without detrimental effects on the quality of foods such as wine (Guerrero-Beltrán *et al*., 2010) or fruit and vegetable juices (Agcam *et al*., 2014; Rivas *et al*., 2006).

Multiple studies on PEF treatments have been conducted to verify the efficacy of inactivating microorganisms inoculated into various milk products (Sampedro *et al*., 2005), but most of these studies were conducted using lab-scale PEF systems and very high electric-field strengths. Qin *et al*. (1998) studied inactivation of *Escherichia coli* in simulated milk and obtained 6-log reductions in cell numbers after treatment in five steps with each step consisting of 10 pulses at 60 kV/cm. Rowan *et al*. (2001) studied the effects of batch-type PEF treatments using an electric field strength of 30 kV/cm on the inactivation of *Mycobacterium paratuberculosis* in sterilized milk and obtained a 5.9-log reduction in cell number using a PEF treatment with 2500 pulses at 50°C. A *Listeria innocua* population suspended in skim milk exhibited a 1.9-log reduction with an electric field strength of 30 kV/cm and a 2.1-log reduction with an electric field strength of 50 kV/cm (Calderon-Miranda *et al*., 1999). In skim milk inoculated with *Staphylococcus aureus*, the microbial co- unt decreased significantly when the PEF treatment time was increased from 0 to 450 μs. A 3.7-log reduction was obtained using an electric field strength of 35 kV/cm and a treatment time of 450 μs, although the system was a lab-scale test with a flow rate of 60 mL/min (Evrendilek *et al*., 2005). Treatment at 30 kV/cm and 116 kJ/kg resulted in a 5.5-log reduction in *Lactobacillus rhamnosus* numbers in buffer solution, whereas inactivation in milk showed only a 2-log reduction (Jaeger *et al*., 2009).

To transfer the inactivation results obtained using model systems to actual food production systems, a pilot study was required to determine the appropriate PEF treatment parameters. Electric field strengths of 20 kV/cm or higher could result in greater reductions microbial populations, but high field strengths are difficult to achieve on an industrial scale. Although PEF treatment has been the subject of many studies, studies addressing electric field strength in pilot-scale PEF treatments have been limited. Therefore, we supposed that sufficient microbial reductions could be obtained at relatively low electric-field strength if total pulse energy is increased. The objective of this study was to determine the applicability of PEF treatments using a relatively low electric field-strength for inactivating microorganisms in low-fat milk.

Materials and Methods

Low-fat milk

Commercial low-fat milk was obtained from the Seoul Dairy Cooperative (Korea) and sterilized thermally at 130°C for 3 s. The milk was stored at room temperature before use.

Microorganisms

E. coli (ATCC 8739), *Saccharomyces cerevisiae* (ATCC 26603), and *L. brevis* (ATCC 13648) strains were obtained from the Korean Culture Center of Microorganisms (KCCM, Korea). *E. coli* was grown on Nutrient Agar (Difco, USA) at 36°C for 3 d. A single *E. coli* colony was then transferred to Nutrient Broth (Difco) and grown in a shaking incubator at 36°C for 24 h. *L. brevis* was grown on MRS agar (Difco) at 36°C for 3 d. A single *L. brevis* colony was then transferred to MRS broth and grown in a shaking incubator at 36°C for 24 h. *S. cerevisiae* was grown on potato dextrose agar (Difco) at 32°C for 3 d. A single *S. cerevisiae* colony was then transferred to potato dextrose broth (Difco) and grown in a shaking incubator at 32°C for 24 h. Each of the microorganisms was inoculated into sterilized low-fat milk just before PEF treatment at initial microbial counts of 1.0-5.0×10^8 CFU/mL.

Pulsed electric field (PEF) treatments

PEF treatments were conducted using a 5 kW pulse generator (HVP-5, DIL, Germany) equipped with a continuous treatment chamber. The pulse generator produced bipolar, rectangular pulses with a wide range of variation in parameter values: peak voltage, 120 kV; pulse width, 530 μs; and pulse frequency, 11000 Hz. The continuous treatment chamber was stainless steel with a colinear structure. The inner diameter of the treatment chamber was 1.0 cm with a gap distance of 1.0 cm between the electrodes. The total specific energy input (W) was used as a parameter to describe the intensity of the PEF treatment and was calculated according to Eq. (1) based on field strength (U, kV/cm) and current (I, A) signals as well as the mass flow rate (m, L/h) measured during the treatment.

$$W_{specific} = \frac{1}{\dot{m}} \int U(t) \cdot I(t) dt \quad (1)$$

Based on preliminary trials, we applied pulses with an electric field strength of 10 kV/cm and a pulse width of 30 μs. Milk was supplied to the treatment chamber using a peristaltic pump (323 Du, Watson Marlow, USA) at a flow rate of 30 L/h. The inlet temperature was adjusted by flow-through stainless steel coils immersed in a water bath, and the inlet temperature of the milk samples was maintained at either 30 or 40°C.

Determination of microorganism survival after PEF treatment

After PEF treatment, 1.0 mL volumes of untreated control and PEF-treated samples were diluted serially into 9.0 mL of sterilized Ringer's solution. The number of surviving *E. coli* cells was determined by spreading 0.1 mL of each dilution onto Nutrient Agar and counting the cells after incubation at 36°C for 24 h. *L. brevis* cells were plated on MRS agar and incubated at 36°C for 24 h, and *S. cerevisiae* cells were plated on potato dextrose agar and incubated at 32°C for 24 h before counting. All measurements were made in triplicate.

Color measurements

The colors of the PEF-treated milk samples were measured as Commission Internationale de l'Éclairage (CIE) L^* (lightness), a^* (±, redness/greenness), and b^* (±, yellowness/ blueness) values using a colorimeter (UltraScan Pro, HunterLab, USA), which was calibrated using a standard white surface calibration plate (L^* = 97.49, a^* = -0.13, b^* = 0.04). Each sample was measured three times. The color difference (ΔE) between the control and PEF-treated samples was calculated as follows.

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

Particle-size distribution

The particle-size distributions of the milk samples were determined using a laser diffraction particle-size analyzer (Mastersizer 3000, Malvern Instruments Ltd., UK). Milk samples were poured into the inlet chamber, and the particle size and distribution were measured automatically by laser diffraction.

Statistical analysis

The data are presented as the mean of three measurements ± the standard deviation. Data were analyzed by ANOVA and Duncan's multiple range comparison test using SPSS ver. 20.0 software (SPSS Inc., USA). $p<0.05$ was regarded as indicating statistical significance.

Results and Discussion

Inactivation of inoculated microorganisms by continuous PEF treatment

Inactivation curves for *E. coli* (A), *L. brevis* (B), and *S. cerevisiae* (C) inoculated into low-fat milk are shown in Fig. 1. The initial counts of each microorganism were adjusted to 1.0-5.0×10^8 CFU/mL. The *E. coli* inactivation curves were biphasic beginning with a lag phase (or shoulder) followed by a phase of rapid decline. When the inlet temperature of the continuous treatment chamber of the pulse generator was 30°C, the *E. coli* microbial count was reduced rapidly by a PEF energy of 150 kJ/L and a PEF energy of 200 kJ/L resulted in a 4.5-log reduction in the *E. coli* microbial count. With an inlet temperature of 40°C, the *E. coli* population was reduced rapidly by a PEF energy of 100 kJ/L, and a PEF energy of 200 kJ/L caused a 5.2-log reduction in the number of *E. coli*. The *L. brevis* inactivation curves exhibited characteristics similar to those of *E. coli*. Inactivation was limited below a PEF energy of 100 kJ/L, but was rapid at higher PEF values. *S. cerevisiae* was more susceptible to PEF treatment than *E. coli* or *L. brevis*, exhibiting a short lag phase and 6.0-log reductions in *S. cerevisiae* numbers were observed at a PEF energy of 200 kJ/L. In addition, the *S. cerevisiae* inactivation curves were the same for inlet temperatures of 30 and 40°C. Because the *E. coli*, *L. brevis*, and *S. cerevisiae* inactivation curves exhibited nonlinear, biphasic characteristics, a modified Gompertz equation was used to fit the microbial inactivation points as a function of applied electric energies, and indicated as fitted lines in Fig. 1. All fitted lines showed good agreement with inactivation data (r^2 >0.95).

Many efforts have been made to verify the effect of PEF treatments on the inactivation of microorganisms inoculated into milk and milk products. However, most previous studies used high electric field levels, typically in the range of 30-60 kV/cm, which are impractical for industrial applications. An electric field strength of 36.7 kV/cm applied to yoghurt yielded a 1-2-log reduction in *Lactobacillus* and *S. cerevisiae* (Dunn and Pearlman, 1987), an electric field strength of 35 kV/cm applied to skim milk gave a 3.7-log reduction in *S. aureus* (Evrendilek *et al*., 2005), an electric field strength of 50 kV/cm applied to skim milk gave a 2.1-log reduction in *L. inno-*

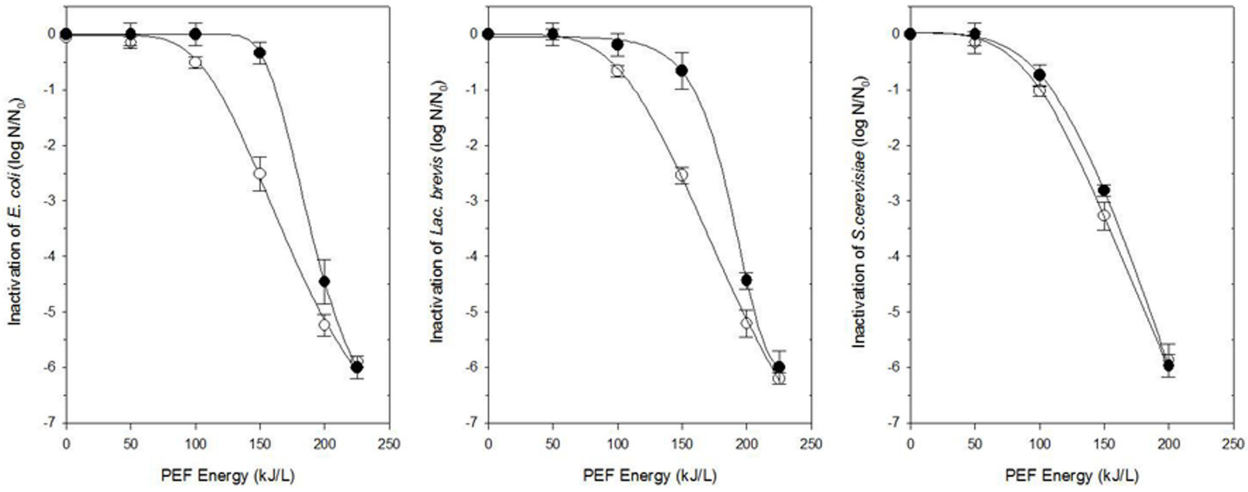

Fig. 1. Inactivation of (A) *Escherichia coli*, (B) *Lactobacillus brevis*, and (C) *Saccharomyces cerevisiae* by pulsed electric field (PEF) treatment after inoculation of low-fat milk. PEF treatments were conducted at a field strength of 10 kV/cm with various pulse frequencies resulting in PEF energies of 50-225 kJ/L.

cua (Calderon-Miranda *et al*., 1999), and an electric field strength of 60 kV/cm applied to simulated milk yielded a 6-log reduction in *E. coli* (Qin *et al*., 1998).

Our results indicate that PEF treatments with an electric field strength of 10 kV/cm inactivated *E. coli*, *L. brevis*, and *S. cerevisiae* in milk. The inactivation efficacy of our PEF treatment was comparable to those shown in previous studies in which the total electric energy exceeded 200 kJ/L. The lower electric field strength used in this study would reduce the system costs of industrial applications or increase the capacity of PEF treatments, which is an important factor in the scale-up to industrial levels when a high level of production is required.

Effects of PEF treatment on the physical properties of low-fat milk

We treated low-fat milk with PEF energies of 150, 200, and 250 kJ/L to determine if changes in pH and color occurred (Table 1). The PEF treatments did not alter the pH value, which was 6.61 in the control milk, and the color properties were unaffected. The L^* or a^* values remained essentially constant after the PEF treatment, the b* values exhibited little change, and the small ΔE values indicated that the treatments did not change the color of low-fat milk. Previous reports also indicated that PEF treatment does not change the color of skim milk (Michalac *et al*., 1999), or milk protein concentrate fortified soybean milk (Li *et al*., 2002).

The particle-size distribution of milk is complex. Fat globules are distributed as an emulsion, proteins are distributed as a colloidal suspension, and lactose is dissolved in the milk whey. We measured the effects of the PEF treatments on the particle-size distribution of milk using a laser diffraction size analyzer (Table 2). The average particle size (Dv_{50}) of the low-fat milk was 0.70 mm with a span value of 1.22. PEF treatments as high as 250 kJ/L resulted in average particle size (Dv_{50}) of 0.72 mm, which is regarded as marginal change.

Qin *et al*. (1995) examined the physicochemical properties and sensory attributes of milk after treatment with

Table 1. Effect of PEF treatments on the pH and color property of low fat milk

Samples	pH	Color property			
		L^*	a^*	b^*	ΔE
Control	6.61 ± 0.03 [a]	85.45 ± 0.02 [a]	–3.29 ± 0.00 [a]	6.44 ± 0.01 [a]	-
PEF-150[1)]	6.61 ± 0.02 [a]	85.38 ± 0.00 [a]	–3.39 ± 0.01 [a]	6.20 ± 0.03 [a]	0.27
PEF-200[2)]	6.61 ± 0.01 [a]	85.65 ± 0.01 [a]	–3.30 ± 0.01 [a]	6.39 ± 0.01 [a]	0.21
PEF-250[3)]	6.63 ± 0.04 [a]	85.28 ± 0.03 [a]	–3.38 ± 0.00 [a]	6.44 ± 0.02 [a]	0.19

[1)]PEF treatment with a total specific energy input of 150 kJ/L. [2)]PEF treatment with a total specific energy input of 200 kJ/L. [3)]PEF treatment with a total specific energy input of 250 kJ/L. Values are expressed as the mean±standard deviation. No significant difference was observed between means (within the same property) designated by the same letter (Duncan's, $p<0.05$).

Table 2. Effects of PEF treatments on the particle-size distributions and specific surface areas of low fat milk

Samples	$D_{[4,3]}$ (mm)	Dv_{10} (mm)	Dv_{50} (mm)	Dv_{90} (mm)	Span	Specific surface area (m^2/kg)
Control	0.785 ± 0.005^c	0.424 ± 0.004^b	0.695 ± 0.006^c	1.274 ± 0.005^a	1.22 ± 0.01^a	30229 ± 241^a
PEF-150	0.803 ± 0.002^{ab}	0.431 ± 0.002^a	0.718 ± 0.003^b	1.209 ± 0.007^b	1.21 ± 0.01^b	29524 ± 105^b
PEF-200	0.797 ± 0.023^b	0.429 ± 0.002^{ab}	0.717 ± 0.003^b	1.219 ± 0.006^b	1.22 ± 0.01^a	29419 ± 697^b
PEF-250	0.807 ± 0.002^a	0.431 ± 0.002^a	0.723 ± 0.002^a	1.212 ± 0.004^b	1.21 ± 0.00^b	29401 ± 92^b

Values are expressed as the mean±standard deviation. No significant difference was observed between means (within the same property) designated by the same letter (Duncan's, $p<0.05$).

pulses in three steps with an electric field strength of 40 kV/cm, a pulse width of 2 μs, and 6-7 pulses, and observed no physicochemical or sensory changes relative to a sample treated with thermal pasteurization. A separate study also showed that PEF treatment had little effect on the physical properties of milk, although slight changes in physical properties may have resulted from electrical breakdown within the treatment chamber caused by the higher field strength used (Evrendilek *et al*., 2005). The results of Bendicho *et al*. (1999) also indicated a potential for destruction of riboflavin, thiamine, cholecalcipherol, and tocopherol in milk subjected to higher electric field strengths. These possible changes induced by higher electric field strengths and our results of little changes in color and particle size distribution might explain the advantage of PEF treatment at reduced electric field strength.

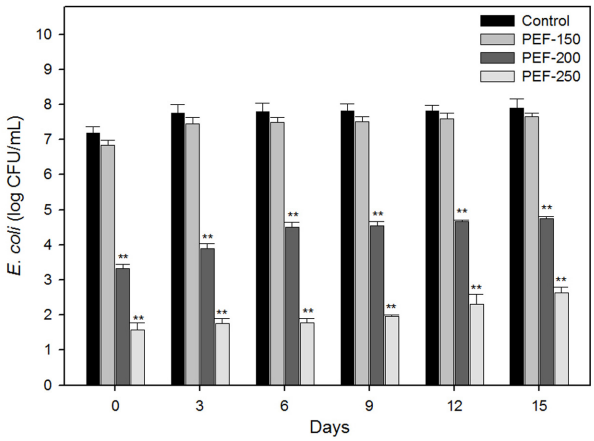

Fig. 2. Effects of pulsed electric field (PEF) treatments on the survival and growth of *E. coli* inoculated into low-fat milk. Untreated control and PEF-treated samples were stored at 4°C for 15 d. *$p<0.05$, **$p<0.01$ using Student's *t*-test with n=3.

Inactivation and growth of inoculated microorganisms during storage for 15 d

The survival and growth rates of *E. coli* inoculated into low-fat milk after PEF treatments are shown in Fig. 2. Low-fat milk was inoculated with *E. coli* at a concentration of 1.58×10^7 CFU/mL. The microbial counts of *E. coli* were reduced immediately by the PEF treatments and log reductions of 0.34, 3.87, and 5.62 were observed after treatment with PEF energies of 150 (PEF-150), 200 (PEF-200), and 250 (PEF-250) kJ/L, respectively. The *E. coli* microbial counts remained essentially constant in the control and the PEF-150-treated samples during the of 15-d storage period, resulting in high microbial counts at the low storage temperature. The PEF-200 treatment immediately reduced the *E. coli* microbial counts to 2.09×10^3 CFU/mL followed by a slow increase to 5.62×10^4 CFU/mL after 15 d. The PEF-250 treatment reduced the number of *E. coli* to 3.72×10^1 CFU/mL followed by a slow increase to 4.37×10^2 CFU/mL after 15 d. The *L. brevis* (Fig. 3) and *S. cerevisiae* (Fig. 4) survival and growth curves exhibited similar characteristics to those of *E. coli*. The PEF-200 and PEF-250 treatments resulted in 5-log reductions in the microbial counts, and the populations of surviving microorganisms grew slowly during the 15-d storage period.

Commercial HTST-pasteurized milk typically lasts 2-3 wk before spoiling and produces 3-4-log reductions in normal microflora in raw milk. PEF treatment of low-fat milk with high electric field pulses of 36 kV/cm and treatment times of 84 μs yielded inactivation comparable to that obtained from heat treatment and a shelf life of 14 d at 4°C (Fernandez-Molina *et al*., 2005). Performing the PEF treatment immediately after HTST pasteurization gave a shelf life of 60 d, which is valuable when the locations of milk production and consumption are far apart (Sepulveda *et al*., 2005). Our results showed that a relatively low electric field of 10 kV/cm can be used to pasteurize low-fat milk if a proper total electric energy is supplied by increasing the pulse frequency.

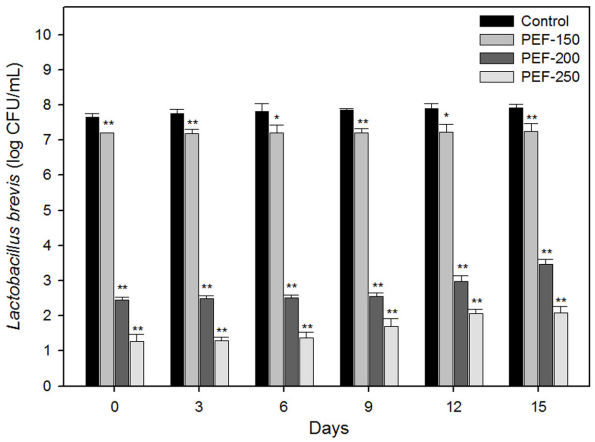

Fig. 3. Effects of pulsed electric field (PEF) treatments on the survival and growth of *L. brevis* inoculated into low-fat milk. Untreated control and PEF-treated samples were stored at 4°C for 15 d. *p<0.05, **p<0.01 using Student's t-test with n=3.

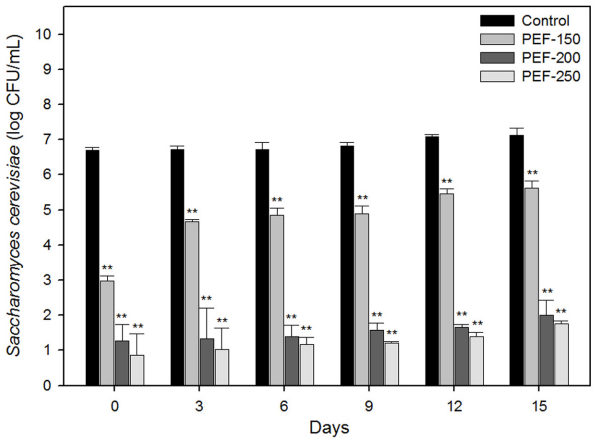

Fig. 4. Effects of pulsed electric field (PEF) treatments on the survival and growth of *S. cerevisiae* inoculated into low-fat milk. Untreated control and PEF-treated samples were stored at 4°C for 15 d. *p<0.05, **p<0.01 using Student's t-test with n=3.

Conclusion

The dependence of microbial inactivation by PEF treatment on electric field strength as well as pulse number and inlet temperature is well known. Most studies on PEF treatments have focused on increasing the electric field strength to increase the efficacy of microbial inactivation. As the use of high electric fields would result in high manufacturing costs for PEF equipment and reduced production capacity, we investigated the efficacy of PEF treatment of milk using a low electric-field strength of 10 kV/cm. Our results showed that PEF treatments with an electric field strength of 10 kV/cm and total electric energy exceeding 200 kJ/L inactivated *E. coli*, *L. brevis*, and *S. cerevisiae* in milk, resulting in greater than 5-log reductions in microbial counts, and revealed that the numbers of microorganisms remained constant during storage for 14 d at 4°C. The PEF treatment did not change the milk physical properties such as pH and color, and there was little change in particle-size distribution. These results indicate that a relatively low electric field of 10 kV/cm can be used to effectively pasteurize low-fat milk.

Acknowledgements

This research was supported by IPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

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