A mild pulsed electric field condition that improves acid tolerance, growth, and protease activity of *Lactobacillus acidophilus* LA-K and *Lactobacillus delbrueckii* subspecies *bulgaricus* LB-12

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ABSTRACT

Pulsed electric field (PEF) processing involves the application of pulses of voltage for less than 1 s to fluid products placed between 2 electrodes. The effect of mild PEF on beneficial characteristics of probiotic bacteria *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* is not clearly understood. The objective of this study was to determine the influence of mild PEF conditions on acid tolerance, growth, and protease activity of *Lb. acidophilus* LA-K and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. A pilot plant PEF system (OSU-4M; The Ohio State University, Columbus) was used. The PEF treatments were positive square unipolar pulse width of 3 μ s, pulse period of 0.5 s, electric field strength of 1 kV/cm, delay time of 20 μ s, flow rate of 60 mL/min, and 40.5°C PEF treatment temperature. Both *Lb. acidophilus* LA-K and *Lb. bulgaricus* LB-12 subjected to mild PEF conditions were acid tolerant until the end of the 120 min of incubation, unlike the *Lb. bulgaricus* control, which was not acid tolerant after 30 min. The mild PEF-treated *Lb. acidophilus* LA-K and *Lb. bulgaricus* LB-12 reached the logarithmic phase of growth an hour earlier than the control. Mild PEF conditions studied significantly improved acid tolerance, exponential growth, and protease activity of both *Lb. acidophilus* LA-K and *Lb. bulgaricus* LB-12 compared with the control. The mild PEF conditions studied can be recommended for pre-treating cultures to enhance these desirable attributes.

Key words: yogurt, pulsed electric field, probiotic

INTRODUCTION

High-intensity pulsed electric fields (PEF), typically 20 to 80 kV/cm (Barbosa-Cánovas et al., 1999), have been shown to inactivate microorganisms and have the potential to replace thermal processing for liquid foods

(Qin et al., 1995) with minimal effect on nutritional and organoleptic qualities (Quass, 1997; Sampedro et al., 2005). Mild electric field intensity conditions form reversible pores in the cellular membrane, whereas drastic electric field intensity conditions lead to cellular death due to the irreversibility (Heinz and Knorr, 2000; Jayaram, 2000). Aronsson et al. (2001) reported that electrical treatments could affect cell physiology. Wang and Sastry (1998) have shown that the presence of a moderate electric field (MEF; 1 V/cm) improved juice yield from cellular tissue. Cho et al. (1996) concluded that MEF accelerates growth in the early stage, but inhibits growth at the late stage of the fermentation of *Lactobacillus acidophilus*. The application of MEF (1 V/cm) at a frequency of 60 Hz has been shown to alter the metabolic activity of the microbial cells by increasing their enzymatic activity (Loghavi et al., 2007). Mild PEF have been reported as high as 16.7 kV/cm (Pol et al., 2000).

Lactobacillus acidophilus is a probiotic bacterium widely used as an adjunct culture in approximately 80% of the yogurts in the United States (Hutkins, 2006). *Lactobacillus delbrueckii* ssp. *bulgaricus* is a representative lactic acid bacterium that is used as a starter culture extensively in the production of the most popular types of fermented milk (Hartley and Denariáz, 1993). Chandan (1999) explained that the consumption of yogurt has increased in recent years, mainly because of its nutritional value and the beneficial health effects of yogurt cultures. Several studies reported *Lb. acidophilus* and *Lactobacillus bulgaricus* as probiotics (Reid, 2000; Guarner et al., 2005; Scholz-Ahrens et al., 2007; Apostolidis et al., 2007; Kligler et al., 2007). Survival within the gastrointestinal tract is one of the preconditions for microorganisms to develop any beneficial effect after consumption (Gibson et al., 2000). To provide health benefits, probiotics must overcome chemical barriers such as acid in the stomach (Gibson et al., 2000; Dunne et al., 2001). It is necessary that probiotic bacteria should be able to survive the low pH values of the stomach for at least 90 min (Berrada et al., 1991; Gibson et al., 2000; Dunne et al., 2001).

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Cellular stress begins in the stomach, which has a pH as low as 1.5 to 2 (Lankaputhra and Shah 1995). Once the cells have survived these hurdles, they can colonize and grow to enough numbers to produce the beneficial effect to the host.

Yogurt cultures have been regarded as lesser-value probiotics, due to their inadequate survival in in vitro acid resistance studies. However, recent studies have shown that yogurt cultures are able to survive gastrointestinal passage in vivo (Lick et al., 2001; Brigidi et al., 2003; Callegari et al., 2004); this, in addition to accumulating evidence of positive health effects of these cultures (Piaia et al., 2003) has further highlighted the importance of viability and the probiotic potential of traditional yogurt cultures.

Lactobacillus acidophilus and *Lb. delbrueckii* ssp. *bulgaricus* are probiotic bacteria with several health benefits, including enhancement of the immunocompromised immune system (Miettinen et al., 2000; Isolauri et al., 2002; Guarner et al., 2005); reduction of various types of diarrhea in humans (Guerrero et al., 1996; Sanders and Klaenhammer, 2001); replacement of various pathogenic organisms in the intestine, such as *Staphylococcus aureus* and *Escherichia coli*, leading to a healthy microbiological balance (Watkins and Miller, 1983); reduced serum cholesterol levels in humans (Gilliland et al., 1985; Roberfroid, 2000; Sanders and Klaenhammer, 2001); improving symptoms of lactose intolerance (Rizkalla et al., 2000; Labayen et al., 2001; Pelletier et al., 2001); balancing of intestinal microflora (Sanders and Klaenhammer, 2001); reduction of the incidence of colon cancer (Shahani et al., 1983; Biasco et al., 1991; Roberfroid 2000), antihyperglycemic and antihypertensive effects (Apostolidis et al., 2007); treatment of urogenital infections and vaginosis in women (Reid, 2000); and alleviation of Crohn's disease (Sanders and Klaenhammer, 2001).

Enhancing the probiotic/beneficial characteristics of the microorganisms is desirable, as it improves the performance, efficiency, and use of these bacteria. This also is a step toward a future generation of more efficient probiotics. Just as high temperatures in pasteurization are used to kill/reduce numbers of microorganisms and comparatively milder temperatures in incubations are used to grow/enhance numbers of microorganisms, mild PEF conditions might enhance desirable characteristics of probiotic bacteria. The effects of mild PEF on the probiotic characteristics of the beneficial bacteria *Lb. acidophilus* LA-K and *Lb. delbrueckii* ssp. *bulgaricus* LB-12 are not well understood. The objective of this study was to determine the effect of mild PEF conditions on acid tolerance, growth, and protease activity of *Lb. acidophilus* LA-K and *Lb. delbrueckii* ssp. *bulgaricus* LB-12.

MATERIALS AND METHODS

Sample Preparation for Control and Mild PEF Treatment

Frozen cultures of *Lb. delbrueckii* ssp. *bulgaricus* LB-12 and *Lb. acidophilus* LA-K (Chr. Hansen's Laboratory, Milwaukee, WI) were stored at -35°C . The log count per milliliter of the frozen culture was 10 for *Lb. acidophilus* and 12 for *Lb. bulgaricus*. Most of the studies on the effect of PEF on microorganisms have been performed in buffer or model solutions; therefore, the control and mild PEF treatment samples for the growth, acid tolerance, and protease activity analyses were prepared by inoculating 10 mL of freshly thawed pure frozen concentrated stock solution culture of *Lb. delbrueckii* ssp. *bulgaricus* LB-12 and *Lb. acidophilus* LA-K individually into 990 mL of sterile 0.1% peptone water to make a 1% (vol/vol) solution and treated individually in a pilot plant PEF system (OSU-4M; The Ohio State University, Columbus). For protease activity analysis, sterile skim milk (previously autoclaved at 121°C for 15 min) was inoculated at 10% (vol/vol) individually with *Lb. delbrueckii* ssp. *bulgaricus* LB-12 and *Lb. acidophilus* LA-K for the control and mild PEF treatment samples.

Experimental Design

Sterile peptone water (0.1% wt/vol) was individually inoculated with *Lb. delbrueckii* ssp. *bulgaricus* LB-12 and *Lb. acidophilus* LA-K. The mild pulsed electric field treatment consisted of electric field strength of 1 kV/cm, pulse width of 3 μs , pulse period of 0.5 s., flow rate of 60 mL/min, and treatment temperature of 40.5°C . The control treatment consisted of samples passed through the PEF equipment at 60 mL/min. without exposure to PEF (Figure 1). The control and the mild PEF-treated samples were tested for acid tolerance, growth, and protease activity. Acid tolerance was determined by inoculating the control and the mild PEF-treated samples in the acidified de Man, Rogosa, and Sharpe (MRS) broth (Difco; Becton, Dickinson and Co., Sparks, MD) and plating every 30 min up to 120 min. Growth was determined by plating the control and the mild PEF-treated samples hourly for 25 h of incubation of *Lb. acidophilus* LA-K and for 25 h of incubation of *Lb. delbrueckii* ssp. *bulgaricus* LB-12. Protease activity of the control and the mild PEF-treated samples were determined by measuring the optical density (OD) value at 0, 12, 24, 36, and 48 h of incubation. The experimental design was a repeated-measures design. Determinations were in duplicate and 3 replications were conducted for each experimental condition.

Figure 1. Flow diagram of the experimental design. PEF = pulsed electric field.

PEF Equipment and Treatment Conditions

The equipment used to apply the different PEF conditions in this study was an integrated continuous fluid handling pilot plant processor (OSU-4M) holding unipolar square wave pulses. The PEF processor consists of 4 (2 pairs) co-field tubular treatment chambers; each chamber contains 2 stainless steel electrodes separated by a gap of 0.29 cm. After passing the sample through a pair of treatment chambers, the sample was cooled in coils that were submerged in a water bath maintained at 40.5°C. The flow rate was controlled by a micro pump (R) gear pump (model 75211-30; Cole-Palmer Instrument Co., Vernon Hills, IL); the temperature (40.5°C) in the treatments was controlled by an Iso-temp refrigerated circulator (Fisher Scientific, Hanover Park, IL). Prior to testing, the PEF equipment was

cleaned by flushing with soap water, distilled water, and 5% sodium hypochlorite solution as a sanitizer [50 mL of active chlorine (4,000 ppm) in 1,000 mL of distilled water] with exposure time of 45 min, and then rinsed thoroughly with sterile distilled water using a micro pump. The autoclaved distilled water (at room temperature) in the final run was collected and tested for the presence of any contamination by serial dilution and plating using plate count agar (Petrifilm plates; 3M, St. Paul, MN) and the Petrifilm plates were incubated aerobically at 32°C for 48 h and colonies were counted (Champagne et al., 2009). Zero counts were found, which indicated that the preliminary sanitization method successfully sterilized the PEF equipment.

Care was taken to ensure that no air bubbles were in the PEF system (Beveridge et al., 2005). The mild PEF treatment conditions were positive square unipo-

Figure 2. Influence of mild pulsed electric field (PEF) conditions on the acid tolerance of (A) *Lactobacillus acidophilus* LA-K and (B) *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. Results are means of 3 replications and error bars represent SE.

Table 1. Mean square (MS) and *P*-values for treatment, time, and their interaction for acid tolerance (log cfu/mL), growth (log cfu/mL), and protease activity (absorbance units) of *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*

Source	<i>Lactobacillus acidophilus</i>						<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>					
	Acid tolerance		Growth		Protease activity		Acid tolerance		Growth		Protease activity	
	MS	<i>P</i> -value	MS	<i>P</i> -value	MS	<i>P</i> -value	MS	<i>P</i> -value	MS	<i>P</i> -value	MS	<i>P</i> -value
Mild PEF ¹	6.819	0.0001	0.1558	0.0001	0.0405	0.0001	42.2933	0.0001	0.1515	0.0001	0.0459	0.0001
Time	16.845	0.0001	13.6507	0.0001	13.6507	0.0001	26.6712	0.0001	6.8889	0.0001	0.4213	0.0001
Mild PEF × time	0.713	0.0002	0.0035	0.017	0.0073	0.0001	5.3245	0.0001	0.0206	0.0001	0.0027	0.0001
Error	0.0778		0.0002		0.0003		0.0329		0.0049		0.0002	

¹PEF = pulsed electric field.

lar pulse widths of 3 μs, pulse periods of 0.5 s., electric field strength of 1 kV/cm, delay time of 20 μs, and flow rate of 60 mL/min with 40.5°C mild PEF treatment temperature. These conditions were chosen based on extensive preliminary trials.

Analytical Procedures

Acid Tolerance. The acid tolerance of the 2 cultures was determined by the method proposed by Pereira and Gibson (2002) with slight modifications. The control and mild PEF-treated samples were inoculated in acidified MRS broth previously adjusted to pH 2 using 1 *N* HCl. The inoculated acidified MRS broth was incubated at 37°C for *Lb. acidophilus* LA-K and 43°C for *Lb. delbrueckii* ssp. *bulgaricus* LB-12 for 120 min. Every 30 min up to 120 min, 1 mL of the inoculated broth was serially diluted in peptone water (0.1% wt/vol) and pour plated. The cultures *Lb. acidophilus* LA-K and *Lb. delbrueckii* ssp. *bulgaricus* LB-12 were enumerated using lactobacilli MRS agar and pH-modified lactobacilli MRS agar, respectively (Dave and Shah, 1996). The MRS agar was prepared according to the manufacturer instructions. The pH of the MRS agar was adjusted to 5.2 using 1 *N* HCl (Dave and Shah, 1996). The Petri plates were incubated anaerobically at 37°C for 48 h for *Lb. acidophilus* LA-K and anaerobically at 43°C for 72 h for *Lb. delbrueckii* ssp. *bulgaricus* LB-12. After the incubation period, the colonies were counted. Plating was in duplicate and 3 replications were conducted for each experimental condition.

Growth. Growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 and *Lactobacillus acidophilus* LA-K was determined by the method proposed by Lin and Young (2000), with slight modifications. Control and mild PEF-treated samples were inoculated separately into MRS broth, which was previously autoclaved at 121°C for 15 min at pH 6.5 ± 0.2. Growth of the cultures was determined hourly for 25 h of incubation of *Lb. acidophilus* LA-K at 37°C and *Lb. delbrueckii* ssp. *bulgaricus* LB-12 at 43°C. One milliliter of the inoculated broth was serially diluted in peptone water (0.1% wt/vol) and pour plated. The cultures *Lb. acidophilus* LA-K and *Lb. delbrueckii* ssp. *bulgaricus* LB-12 were enumerated using lactobacilli MRS agar and pH-modified lactobacilli MRS agar, respectively (Dave and Shah, 1996). The MRS agar was prepared according to the manufacturer instructions. The pH of the MRS agar was adjusted to 5.2 using 1 *N* HCl (Dave and Shah, 1996). The Petri plates were incubated anaerobically at 37°C for 48 h for *Lb. acidophilus* LA-K and anaerobically at 43°C for 72 h for *Lb. delbrueckii* ssp. *bulgaricus* LB-12. After the incubation period, the colonies were counted. Duplicate

Table 2. Least squares means for acid tolerance (log cfu/mL), growth (log cfu/mL), and protease activity (absorbance units) of *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*

Treatment	<i>Lactobacillus acidophilus</i>			<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>		
	Acid tolerance	Growth	Protease activity	Acid tolerance	Growth	Protease activity
Mild PEF ¹	6.0039 ^a	10.4891 ^a	0.7048 ^a	4.49971 ^a	10.7816 ^a	0.4357 ^a
Control	5.0503 ^b	10.4204 ^b	0.6313 ^b	2.12503 ^b	10.7046 ^b	0.3574 ^b

^{a,b}Least squares means with different letters within the same column are significantly different at $\alpha = 0.05$.

¹PEF = pulsed electric field.

plating was conducted. Experiments were replicated 3 times.

Protease Activity. The protease activity of 2 cultures was determined by *o*-phthaldialdehyde (OPA) spectrophotometric method proposed by Oberg et al. (1991), with slight modification. Control and mild PEF-treated samples were inoculated separately into sterile skim milk, which was previously autoclaved at 121°C for 15 min. Samples were incubated at 40°C for 0, 12, 24, 36, and 48 h. After incubation, 2.5 mL from each sample was mixed with 1 mL of distilled water individually and was transferred into test tubes containing 5 mL of 0.75 *N* TCA (Fisher Scientific, Fair Lawn, NJ) and were vortexed. After 10 min at room temperature, the acidified samples were filtered through a Whatman number 2 filter paper (Whatman Inc., Clifton, NJ). Duplicate aliquots from each TCA filtrate were analyzed by OPA testing using a UV-visible dual-beam spectrophotometer (Nicolet Evolution 100; Thermo Scientific, Madison, WI). The OPA final solution was prepared by combining the following reagents and diluting to a final volume of 50 mL with distilled water: 25 mL of 100 mM sodium borate (Fisher Scientific), 2.5 mL of 20% (wt/wt) SDS (Fisher Scientific), 40 mg of OPA reagent (Alfa Aesar, Ward Hill, MA) dissolved in 1 mL of methanol (Sigma, St Louis, MO), and 100 μ L of β -mercaptoethanol (Sigma). One hundred and fifty microliters of each TCA filtrate was mixed with 3 mL of OPA reagent in a 3-mL cuvette, and the absorbance at 340 nm was read. Absorbance of the OPA final solution with the noninoculated sterile skim milk was subtracted from each sample reading. The OPA reagent was used as a blank to calibrate the UV-visible spectrophotometer.

Statistical Analysis

Data were analyzed using the PROC MIXED model of SAS (SAS Institute Inc., Cary, NC). Significant differences ($P < 0.05$) among mild PEF and control treatments were analyzed using the Tukey adjustment and differences of least squares means were used to de-

termine significant differences for the main effect (mild PEF) and interaction effect (mild PEF $\times$ time).

RESULTS AND DISCUSSION

Acid Tolerance

The acid tolerance of *Lb. acidophilus* LA-K is shown in Figure 2A. Cultures subjected to mild PEF conditions, as well as the control, were acid tolerant until the end of the 120 min of incubation, which, on average, is the time the food spends in the acidic environment of the human stomach (Lankaputhra and Shah, 1995). The time effect was significant (Table 1). A significant ($P < 0.0001$) decrease was observed in the viable counts after each incubation time of 30 min. The mild PEF treatment effect was also significant ($P < 0.0001$; Table 1). From min 30 to 120 the acid tolerance of *Lb. acidophilus* LA-K subjected to mild PEF conditions was significantly ($P < 0.001$) higher than the control (Table 2). Dunne et al. (2001) reported that *Lb. acidophilus* was more acid resistant at pH 1.2 and 2.5 for 30 min compared with *Bifidobacterium bifidum*, which did not tolerate the acidic conditions after 5 min. Liong and Shah (2005) observed that *Lb. acidophilus* exhibited more acid tolerance than *Lactobacillus casei* at pH 2. Mild electrical stimulation treatment that alters the synthesis of molecular chaperones may affect bacterial resistance to acid stress (Lado et al., 2004). Exposure to acid stress induced de novo synthesis of several heat shock-like proteins by *Lactobacillus paracasei* F19 and 50:1 and *Lactobacillus plantarum* 2592, F5, and F26 strains, which cross-reacted with stress proteins; this may protect other surface proteins and adhesions during transport through the gastrointestinal tract (Ljungh-Wadström et al., 2007).

The acid tolerance of the *Lb. delbrueckii* ssp. *bulgaricus* LB-12 is shown in Figure 2B. The viability of *Lb. bulgaricus* LB-12 in the control samples was lost after 30 min of incubation in acidified MRS broth at pH 2. The mild PEF treatment had a significant ($P < 0.0001$) effect on acid tolerance (Table 1). The viable counts

Figure 3. The influence of mild pulsed electric field (PEF) conditions on the growth characteristics of (A) *Lactobacillus acidophilus* LA-K and (B) *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. Results are means of 3 replications and error bars represent SE.

of the bacterium subjected to mild PEF conditions were significantly ($P < 0.0001$) higher at every 30 min of incubation from min 30 to 120 compared with the control. The mild PEF treatment significantly ($P <$

0.0001) increased the acid tolerance of the bacterium compared with the control (Table 2). The time effect was significant (Table 1). A significant ($P < 0.0001$) decrease was observed in the viable counts after each

Figure 4. The influence of mild pulsed electric field (PEF) conditions on the protease activity of (A) *Lactobacillus acidophilus* LA-K and (B) *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. OD = optical density. Results are means of 3 replications and error bars represent SE.

incubation time of 30 min. Bacterial resistance to acid stress may have been influenced by the mild electrical stimulation treatment that alters synthesis of molecular chaperones (Lado et al., 2004).

Growth

The growth of *Lb. acidophilus* LA-K is shown in Figure 3A. The mild PEF treatment had a significant ($P < 0.0001$) influence on the growth of *Lb. acidophilus* LA-K (Table 1). The mild PEF-treated culture reached the logarithmic phase of growth an hour earlier than the control. Growth of *Lb. acidophilus* LA-K when subjected to the mild PEF treatment was significantly higher than the control (Table 2). The exponential phase of the growth of the control was between h 4 and 12, whereas that of the bacterium subjected to mild PEF treatment was between h 3 and 14 (Figure 3A). Liong and Shah (2005) studied cholesterol-removal ability of lactobacilli strains and also observed growth of the bacterium *Lb. acidophilus* to be predominant in the first 9 to 15 h, after which it reached a stationary phase. Electrical treatment could affect cell physiology (Aronsson et al., 2001). The application of MEF (1 V/cm) produced a shorter lag phase than conventional (control) fermentation of *Lactobacillus acidophilus* OSU at the same temperature (Loghavi et al., 2008). However, recent studies under controlled temperature conditions have shown that MEF accelerates growth in the early stage, but inhibits growth at the late stage of fermentation of *Lb. acidophilus* (Cho et al., 1996).

The growth of *Lb. delbrueckii* ssp. *bulgaricus* LB-12 is shown in Figure 3B. The mild PEF treatment had a significant ($P < 0.0001$) influence on the growth of the bacterium (Table 1). The mild PEF-treated cultures reached the logarithmic phase an hour earlier than the control. The control and the mild PEF treatment studied were significantly different from each other (Table 2). In the exponential phase of growth, from h 3 to 10 for mild PEF-treated culture and from 4 to 10 h for the control, the viable bacterial counts of the bacterium subjected to mild PEF conditions were significantly ($P < 0.001$) higher than those of the control at h 4 and 5. During the stationary phase of growth (from h 10 to 18) the mild PEF-treated culture had no significant ($P > 0.05$) difference in the viable bacterial counts compared with the control. At h 25, the mild PEF-treated culture had significantly ($P < 0.0001$) higher viable bacterial counts than the control. Mild PEF treatment significantly increased the growth of *Lb. delbrueckii* ssp. *bulgaricus* LB-12 compared with the control. Simova et al. (2006) analyzed the growth profile of *Lactobacillus bulgaricus* HP1 inoculated in autoclaved reconstituted skim milk and reported that the growth reached the

exponential phase in the first 5 h and reached the stationary phase in 8 to 12 h.

Several studies exist, where, if treatment could have a kill effect on a bacterium, then high initial inoculum levels were used [for example, *Lb. paracasei* initial inoculation was 2.2×10^9 cfu/mL (Bayrock and Ingledew, 2001), *Listeria monocytogenes* initial inoculation was 10^9 cfu/mL (Mytilinaios et al., 2012), *Salmonella enteritidis* initial inoculation was 10^8 cfu/mL (Hoffman et al., 2001), and *Rhodotorula minuta* initial inoculation was 10^{10} cfu/mL (Patiño-Vera et al., 2005)]. It is well reported in the literature that PEF is used as a hurdle technology having a kill effect on microorganisms (Qin et al., 1995; Zhang et al., 1995; Pothakamury et al., 1996; Reina et al., 1998; Barbosa-Cánovas et al., 1999; Rowan et al., 2001; Sobrino-López et al., 2006). Hence, initial counts in this study were high because if a rapid kill effect by PEF occurred, the reduction in logarithms over a few hours at least could be recorded. Moreover, in this study, despite the high initial inoculum level, growth still occurred in both bacterial controls as evidenced by their lag, logarithmic, stationary, and decline phases.

Protease Activity

The OD values of the protease activity of *Lb. acidophilus* LA-K is shown in Figure 3A. The mild PEF effect was significant (Table 1). The protease activity of the bacterium subjected to mild PEF treatment was significantly ($P < 0.05$) higher than the control (Table 2). A significant ($P < 0.0001$) increase in the protease activities between each time point were found when comparing among time points 12, 24, 36, and 48 h for the control samples and the mild PEF samples as well (Table 1). Mild PEF treatment significantly ($P < 0.0001$) enhanced the protease activity of *Lb. acidophilus* LA-K (Table 2). *Lactobacillus acidophilus* LA-K subjected to mild PEF conditions exhibited significantly ($P < 0.0001$) the highest protease activity at 36 h of incubation at 40°C compared with the control (Figure 4A; Table 2). Bendicho et al. (2005) studied PEF treatment on *Bacillus subtilis* protease and reported that an enhancement in proteolytic activity was found when the PEF treatment was carried out in milk. In general, enzymes require more severe high-intensity PEF treatment than microorganisms to obtain significant inactivation (Bendicho et al., 2002, 2003).

The OD values of the protease activity of *Lb. delbrueckii* ssp. *bulgaricus* LB-12 is shown in Figure 4B. At h 0, 12, 24, 36, and 48, the protease activities of the bacterium subjected to mild PEF treatment were significantly ($P < 0.0001$) higher compared with the control. Significant ($P < 0.0001$) differences were ob-

served in the protease activity between 0, 12, and 24 h for the control samples and mild PEF samples as well (Table 1). The mild PEF treatment effect was significant (Table 1). Mild PEF treatment significantly ($P < 0.0001$) improved the protease activity of *Lb. bulgaricus* LB-12 (Table 2). *Lactobacillus bulgaricus* LB-12 in both the control and the mild PEF treated cultures exhibited significantly ($P < 0.0001$) the highest proteolytic activity at 24 h of incubation compared with 36 and 48 h of incubation at 40°C. In general, mild PEF processing affects protease activity and an enhancement in the initial activity was detected (Giner et al., 2000, 2002; Bendicho et al., 2002, 2003; Min et al., 2003). Pilar and Antonio (2006) suggested that the PEF could originate small conformational changes leading to enhanced proteolytic activity.

CONCLUSIONS

Mild PEF treatment under study significantly improved the acid tolerance, exponential growth, and protease activity of both *Lb. acidophilus* LA-K and *Lb. delbrueckii* ssp. *bulgaricus* LB-12 compared with the control. These identified mild PEF conditions can be used for pretreating cultures to enhance these desirable attributes.

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