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Pulsed electric field influences on acid tolerance, bile tolerance, protease activity and growth characteristics of Lactobacillus acidophilus LA-K

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PULSED ELECTRIC FIELD INFLUENCES ON ACID TOLERANCE, BILE TOLERANCE, PROTEASE ACTIVITY AND GROWTH CHARACTERISTICS OF *LACTOBACILLUS ACIDOPHILUS* LA-K

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ABSTRACT

Pulsed electric field processing represents a promising non-thermal technology which involves the application of pulses of high voltage (20-80 kV/cm) for less than one second to fluid foods placed between two electrodes. During the application of certain PEF conditions microbial inactivation occurs by causing a pore formation and destruction of the cell membranes. Lactobacillus acidophilus is an important probiotic bacterium used for the production of fermented dairy products. The objective of this study was to study the influence of pulsed electric field (PEF) on the growth characteristics, bile tolerance, acid tolerance, and protease activity of Lactobacillus acidophilus LA-K. Freshly thawed Lactobacillus acidophilus LA-K was suspended in peptone and treated in a pilot plant PEF system (OSU-4M). The treatments were pulse width (3, 6 and 9 μs), pulse period (10,000; 20,000 and 30,000 μs), voltage (5, 15 and 25 kV/cm) and flow rate (10, 60 and 110 mL/min). Control was run through PEF system at 60 mL/min without receiving any pulsed electric field condition. Growth and bile tolerance on control and treatment samples were determined hourly throughout 16 hours of incubation. Acid tolerance was determined at 0, 5, 10 and 15 minutes of incubation. Protease activity was determined at 0, 12 and 24 hours of incubation. The experimental design was a repeated measure design. Data were analyzed using the PROC GLM of the Statistical Analysis Systems (SAS). Differences of least square means were used to determine significant differences at $P < 0.05$ for main effects (pulse width, pulse period, voltage, flow rate) and interaction effects (pulse width * time, pulse period * time, voltage * time, and flow rate * time). Bipolar pulse width and pulse period significantly lowered acid tolerance and bile tolerance as well as slowed log stage growth of Lactobacillus acidophilus LA-K. Voltage significantly influenced acid tolerance, bile tolerance and growth of Lactobacillus acidophilus LA-K. Bipolar pulse width, pulse period and voltage did not influenced protease activity of Lactobacillus acidophilus LA-K. Flow rate

significantly influenced bile tolerance and growth of Lactobacillus acidophilus LA-K. Flow rate did not significantly influenced acid tolerance and protease activity of Lactobacillus acidophilus LA-K.

CHAPTER 1: INTRODUCTION

1.1 Pulsed Electric Field

Thermal processing methods, such as pasteurization, are commonly used in the food industry to increase shelf life and maintain food safety by inactivating spoilage and pathogenic microorganisms. Although thermal processing methods provide safer foods, these can also unfavorably affect the taste, color, flavor, and nutritional quality of foods (Qin *et al.*, 1995a). Nowadays with the increasing consumer demand for fresh food or minimally processed food products, there is a growing interest in non-thermal processes for food preservation.

The food industry has investigated several promising non-thermal pasteurization techniques, for example the utilization of pulsed electric fields, oscillating magnetic field pulses, microwave induced electromagnetic fields, high hydrostatic pressure, electron ionizing radiation, intense light pulses, and others (Mertens and Knorr, 1992).

Since high-intensity pulsed electric fields (PEF) represents one of the promising alternatives to the process for pasteurizing certain liquid foods, including milk and milk products (yogurt and fortified yogurt drinks), this non-thermal technique is gaining popularity in the food industry (Qin *et al.*, 1995a). High intensity pulsed electric field (PEF) processing involves the application of pulses of high voltage (typically 20-80 kV/cm) for short time periods (less than 1 second) to fluid foods places between two electrodes (Barbosa-Cánovas *et al.*, 1999). Application of PEF is restricted to foods products that can withstand high electric fields, have low electrical conductivity, and do not contain or form bubbles (e.g., liquid foods as milk or fruit juices) (Calderon-Miranda *et al.*, 1999). PEF technology is considered better than heat treatment of foods because it

achieves high microbial inactivation, avoids or greatly reduces detrimental changes in the sensory and physical properties of food, and inactivates some enzymes (Quass, 1997). Pulsed electric field is more energy efficient than thermal pasteurization. This nonthermal technology would add only $0.03-$0.07 to final food costs (Ramaswamy *et al.*). The total additional cost for orange juice treated with PEF, is 0.01 Euro per liter when compared to heat pasteurization (Smit, 2004; Bartels and Mastwijk, 2003).

Several theories have been proposed to explain microbial inactivation by PEF. The most commonly accepted theory is electroporation, which is the phenomenon in which the lipid bilayer and proteins of cell membrane exposed to high intensity electric field pulses are temporarily destabilized (Chang *et al.,* 1992). One major consequence of electroporation is an event called electropermeabilization, which is a dramatic increase in permeability (or conductivity) and, in some cases, mechanical rupture of the membrane. The electropermeabilization can be reversible or irreversible, depending on the degree of membrane organizational changes (Wouters *et al.,* 2001). Irreversible structural changes in the cell membrane include pore formation and destruction of the semipermeable barrier of the cell membrane, which protects the microorganism from the surrounding environmental conditions (Lin *et al*., 2002). Leakage of intracellular contents results from disrupted cell membranes, which leads to the loss of cell metabolic activities. Microbial cells that have lost the ability to grow and divide in a nutrient medium are considered to be inactivated (Jeyamkondan *et* al., 1999). Relationship between membrane permeabilization and inactivation kinetics of different *Lactobacillus* species of food origin was studied by Wouters *et al.,* (2001). In this study the permeabilization of cells showed to increase when increasing the energy input, which was achieved by applying an

increasing number of pulses with electric field strength of 2.5 V/μm. This increased membrane permeability corresponded to a higher inactivation.

Genetic engineering techniques have been used to improve desired characteristics of lactic acid bacteria. Techniques such as transduction, conjugation, fusion and transformation have been used to transfer plasmid to produce genetically modified strains of *Streptococcus* and *Lactococcus* (De Vos and Simons, 1994). However, gene transfer systems for *Lactobacillus* using conjugation and protoplast transformation have shown low efficiency and lack of reproducibility (De Vos and Simons, 1994). Electroporation is being extensively used as one of the easiest methods for cell hybridization and electrofusion in the areas of genetic engineering and biotechnology (Chang *et al.*, 1992). *Lactobacillus casei* was the first lactobacilli to be transformed reproducibly by electroporation at high frequency and efficiency (Chassy and Flickinger, 1987). PEF has been applied to microbial cells in order to cause electroporation of cell membranes. Foreign materials, such as DNA, are then added to infuse into the cell. When the PEF is removed, microbial cells repair their membranes, sealing the electropores. In this situation the pore formation in the cell membrane by PEF is reversible, which occurs at low field strength and energy inputs. The same principle is used for inactivation of microorganisms by PEF, which is accomplished by increasing the intensity of treatment, thereby resulting in the irreversible breakdown of the cell membrane (Jeyamkondan *et* al., 1999).

Microbial inactivation increases with an increase in the electric field intensity, number of pulses, pulse duration, temperature of the medium and ionic strength of the medium (Qin *et al.*, 1998). Jayaram et al. (1992) applied PEF to inactivate *Lactobacillus*

brevis cells and concluded that the cell destruction was primarily due to the field induced rupture of the cell wall and not because of ohmic heating. In a study conducted by Sakurauchi and Kondo (1980) it was observed that *Bacillus subtilis* spores and vegetative cells of *Escherichia coli* could be inactivated under long treatment times (several seconds). In contrasts to irreversible effects of PEF on cells, relatively little information concerning reversible PEF effects on cell membranes are available.

1.2 Factors Affecting the Microbial Inactivation with Pulsed Electric Field (PEF)

The factors that affect the microbial inactivation with PEF depend on (1) the process (electric field intensity, pulse width, treatment time and temperature, and pulse waveshapes), (2) microbial entity (type and growth stage of microorganism), and (3) treatment media (pH, antimicrobials, ionic compounds, conductivity) (Anonymous, 2000).

1.2.1 Process Factors

a) Electric Field Intensity or Strength. Electric field intensity, which is one of the main factors influencing microbial inactivation, is determined by the voltage (kV) across the electrodes and the distance between the electrodes (cm). Increasing the gap will require higher voltage to obtain the desired electric field strength (Zhang et al., 1994). The microbial inactivation increases with an increase in the electric field intensity, above the critical transmembrane potential (Qin et al., 1998). Pore formation will occur when a certain threshold value of the transmembrane potential formed is exceeded, which was found to be in the range of 1 V. (Zimmerman 1996). To achieve this transmembrane potential an electric field strength above 30 kV/cm is required for most bacteria in liquid systems (Ulmer et al., 2002).

b)Pulse Waveshape. Electric field pulses may be applied in the form of exponential decaying, square wave, oscillatory pulses or bipolar. In terms of pulse shape, square wave pulses are more energy and lethally efficient than exponential decaying pulses (Qin et al., 1994). Oscillatory pulses are the least efficient for microbial inactivation because they prevent the cell from being continuously exposed to a high intensity electric field for an extended period of time (Jeyamkondan et al., 1999). In terms of pulse polarization, bipolar pulses are more lethal than monopolar pulses. Bipolar pulses produce alternating changes in the movement of charged molecules, which cause a stress in the cell membrane and enhance its electric breakdown. Moreover, bipolar pulse reduces deposition of solids on the electrode surface, decreases food electrolysis, and is energy efficient (Barbosa-Cánovas et al., 1999).

c) Treatment Time. Treatment time has been earlier defined as the product of the number of pulses and the pulse width (Zhang *et al.,* 1994). The pulse width is defined as the time where the peak field is maintained for square wave pulses or the time until decay to 37% for exponential decay pulses. Pulse width influences microbial reduction by affecting the electric field intensity. Longer pulse widths decrease electric field intensity, which result in higher inactivation; however, an increase in pulse width may also result in an unwanted food temperature increase. Hülsheger *et al*. (1981) developed a mathematical model that relates microbial survival fraction with PEF treatment time. The inactivation of microorganisms increases with an increase in treatment time (Hülsheger *et al*., 1983). In a recent study conducted by Min *et al.,* (2003) the treatment time for the inactivation kinetics of tomato juice lipoxygenase by pulsed electric field was calculated with the following formula:

Treatment time = volume of 1 chamber (mL)/flow rate (mL/sec) * pulse per second * number of chamber * pulse width.

d) Treatment Temperature. Constant electric field strength increases microbial inactivation as well as increases the temperature in foods. For this reason, proper cooling is necessary to maintain food temperatures far below those generated by thermal pasteurization (Anonymous, 2000). In a study made by Vega- Mercado *et al.* (1996), *E. coli* reduction was observed to increase from 1 to 6.5 log reduction cycles with a temperature change from 32 to 55 °C.

1.2.2 Microbial Factors

a) Type of Microorganisms. Microorganisms differ in their sensitivity to PEF. Hülsheger *et al*. (1983) reported that gram positive bacteria and yeast are less sensitive to PEF treatment than gram negative bacteria when few pulse numbers are applied. However, this statement disagrees with the results found by Qin *et al.* (1995b), where *Saccharomyces cerevisae* showed to be more sensitive to PEF treatment than gram negative bacteria. Sale and Hamilton (1967) also found that yeasts are more sensitive to electric fields than bacteria due to their large size.

b) Growth Stage of Microorganism. Logarithmic phase cells are more sensitive to stress than lag and stationary phase cells. Killing effect of PEF in the logarithmic phase is 30% greater than those in the stationary phase of growth (Gaskova *et al*., 1996). In the logarithmic phase the microbial growth is characterized by a high proportion of cells undergoing division, during which the cell membrane is more susceptible to the applied electric field. Hülsheger *et al*. (1983) reported that cell harvested from the logarithmic growth phase were more sensitive to PEF treatment than were those from the stationary

growth phase. Pothakamury *et al.* (1996) also reported that *E. coli* cells in the logarithmic phase were more sensitive than cells in the lag and stationary phase, when treated with PEF.

1.3 Microbial Inactivation Studies by PEF

Inactivation of microorganisms in liquid media using PEF has been extensively studied. Various authors have focused on studying *Escherichia coli.* Among these studies we can mention the inactivation of *Escherichia coli* in simulated milk ultrafiltrated (SMFU) studied by Pothakamury *et al.,* (1996). In this study, 4 to 5 log reductions were obtained when applying 60 pulses, 16 kV/cm and 300 µs. These results were compared with those obtained by using heat (82.2 ⁰C, 171 s), which produced 5 log reductions, but caused degradation of organoleptic and nutritive characteristics. Qin *et al.,* (1998) achieved more than a 6 log cycle reduction in *E. coli* suspended in SMFU using electric field intensity of 36 kV/cm and 50 pulses PEF treatment. The temperature of the chamber in this study was maintained below 40 ⁰C during the PEF treatment, which is lower than the temperature of commercial pasteurization (70-90 ⁰C) for milk. Grahl *et al.,* (1992) reported the influence of pulse number in microbial inactivation of *E. coli*. They were able to reduce the populations of *E. coli* in UHT milk by 1, 2 and 3 log cycles when 5, 10, and 15 pulses at 22 kV/cm were applied. Barbosa-Cánovas *et al.,* (1999) reported that the inactivation of *E. coli* in SMFU was not affected when the concentration of microorganisms was varied from 10^3 to 10^8 cfu/mL after being subjected to 70 kV/cm, 16 pulses, and a pulse width of 2 µs. The killing effect of PEF on *Escherichia coli* ATCC 8739 suspended in an orange juice and milk beverage was studied by Rivas *et al.,* (2006). Bipolar square pulse widths with a pulse width of 2.5 µs, electric field strengths from 15

to 40 kV/cm, and treatment times from 0 to 700 µs were applied in this study. The found a maximum of 3.83 log reductions with 15 kV/cm and 700 µs, and no significant differences at the electric field strength range from 25-40 kV/cm.

Effect of PEF in many other pathogens has also been studied. Fernádez- Molina *et al.,* (1999) reported 2.6 and 2.7 log reductions for different microorganisms such as *Listeria innocua* in raw skim milk (0.2% milk fat) and *Pseudomonas fluorescens* with 2 µs, 100 pulses and 50 kV/cm at room temperature. The influence of the food composition was shown by Calderon-Miranda (1998) in studies where *L. innocua* was reduced by 2.4 and 3.4 log cycles reductions in raw skim milk and liquid whole egg, respectively, under the same experimental conditions. Michalac *et al.,* (1999) studied inactivation of *Pseudomonas fluorescens* in UHT skim milk subjected to PEF treatments. They achieved 1 log reduction with 35 kV/cm, 3 µs, and a total treatment time of 90 µs.

Pulsed electric field influence on *Listeria monocytogenes* Scott A (10^7) inoculated in whole milk (3.5% milk fat), semi skimmed milk (2% milk fat), and skim milk (0.5% milk fat) was studied by Reina *et al.,* (1998). In the first experiment they studied three different treatment times (100, 300, and 600 µs) and the different fat content of the samples. They observed that inactivation increased when treatment time increased, reaching 3 log reductions. However, the fat content did not showed a difference in the inactivation. In a second experiment they studied the effect of 25 and 35 kV/cm and treatment time on inactivation of *L. monocytogenes* in whole milk. They observed no differences in the degree of inactivation between the two field strengths at short times (100 µs), but there were differences at longer treatment times; more inactivation was produced with the higher field strength.

Other authors have focused on the study of *Staphylococcus aureus*. Pothakamury *et al.,* (1995) inoculated *Staphylococcus aureus* in SMFU achieving between 3 and 4 log reductions by the application of 60 pulses, 16 kV/cm, and 300 µs. Evrendilek *et al.,* (1999) studied inactivation of this bacterium (10^8 cfu/mL) in reconstituted, pasteurized nonfat dry milk (10% solids) by treatment with PEF. They achieved 3.5 log reductions using 29 kV/cm, 4 µs, and 71.3 µs of total treatment time. Sobrino-López *et al.,* (2006) reached a 4.5 log reduction in *Staphylococcus aureus* by applying 150 bipolar pulses of 8 µs each at 35 kV/cm. They found bipolar pulses to be more effective than monopolar pulses.

There are also works that focus on studying the inactivation of enzymes especially those produced by *Bacillus* and *Pseudomonas*, which spoil milk during processing. Barbosa-Cánovas *et al.,* (1998) reported that 80% of the protease from *Pseudomonas fluorescens* M3/6 in skim milk was inactivated after PEF treatments of 18 kV/cm electric field strength for 98 pulses with 10 µs pulse duration. The inactivation of protease by *Bacillus subtilis* in whole milk, skim milk, and SMFU by PEF (19.7-35.5 kV/cm, 866 µs of treatment time, 4 and 7 µs of pulse width) was studied by Bendicho *et al.,* (2002). The authors observed that protease activity decreased when electric field strength, treatment time, and frequency increased. It was also found no differences in protease activity with a pulse width between 4 and 7 µs.

The *Lactobacillus* has also been studied by some authors. Pothakamury *et al.,* (1995) achieved between 4 and 5 log reductions in SMFU inoculated with *Lactobacillus delbrueckii,* after 40 pulses with 16 kV/cm and 10^4 µs of treatment time. *Lactobacillus brevis* in UHT milk achieved 4 log reductions with 12.6 kV/cm (Grahl and Markl, 1996).

Although pulsed electric field has successfully demonstrated the inactivation of pathogens in foods by causing irreversible electroporation on them, few or none studies can be found regarding PEF application to good bacteria. Pulsed electric field could be of great interest to the industry and medicine concerned in the genetic improvement of friendly bacteria.

1.4 *Lactobacillus acidophilus*

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO 2002). *Lactobacillus acidophilus* is a probiotic bacterium with several health benefits, including enhancement of immune system, reduction of various types of diarrhea in humans, alleviation of Crohn's disease, lower cholesterol, improve symptoms of lactose intolerance, and balancing of intestinal microflora through the growth modulation of bacteria present in the gastrointestinal tract (Sanders 2000). *Lactobacillus acidophilus* is used extensively for the production of fermented dairy products and is increasingly applied in the area of health improvement, as probiotics, in the form of yogurts and dietary supplements. To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu/g of a product (Robinson, 1987). However, studied have shown low viability of probiotics in market preparations (Shah and Lankaputhra, 1997).

Probiotics used as probiotic adjuncts are commonly delivered in a food system and, therefore, begin their journey to the lower intestinal tract via the mouth. To provide health benefits, probiotics must overcome physical and chemical barriers such as acid and bile in the gastrointestinal tract (Gibson *et al.,* 2002). Once the cells have survived these hurdles, they can colonize and grow to enough numbers to produce the beneficial effect

to the host. The time from entrance to release from the stomach was reported to be 90 min (Berrada *et al.*, 1991). However, further digestive processes have longer residence time. For this reason, it is important for the bacteria to be resistant to stressful conditions of the stomach and upper intestine.

Lactobacillus acidophilus possesses several characteristics that allow it to survive and grow in the intestinal tract. Among these is the ability to grow in the presence of bile. *Lactobacillus acidophilus* 27SC with relatively high bile tolerance was able to increase the number of facultative lactobacilli in the upper part of the small intestines of calves significantly better than *Lactobacillus acidophilus* C28 with low bile tolerance (Gilliand *et al.,* 1984). Bile tolerance of the bacterium is the survival of the bacterium during passage through the upper small intestine (Nousiainen *et al.,* 2004). This important bile tolerance characteristic in *Lactobacillus acidophilus* has been identified as important to maintain in preparation and storage of concentrated cultures for use as dietary adjuncts (Gilliand, 1979).

Cellular stress begins in the stomach, which has a pH as low as 1.5 (Lankaputhra and Shah, 1995). Relatively few bacteria can tolerate this condition. During milk fermentation, acid stress is imposed to starter cultures since the hydrolysis of lactose results lactic acid accumulation and consequent acidification of the environment to pH as low as 4.0 (Piard and Desmazeud, 1991). Hood and Zottola (1998) observed that *Lactobacillus acidophilus* populations decreased rapidly at pH 2.0; however, there was no decrease in the number of viable cells at pH 4.0. Lorca *et al.,* (1998) found that he ability of *Lactobacillus acidophilus* CRL 639 to survive low pH conditions depends on the growth phase; stationary phase cells are naturally tolerant to acid, whereas

exponential phase cells need and adaptation step to induce acid tolerance. The survival during passage through the stomach and duodenum is known as acid tolerance (Nousiainen *et al.,* 2004).

After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids which are susceptible to destruction of bile salts (Jin *et al.,* 1998). After traveling through this harsh environment, the bacteria colonize the epithelium of the lower intestinal tract (Conway *et al.,* 1987). Therefore, strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile, attach to epithelium, and grow in the lower intestinal tract before they can start providing health benefits.

Cultured dairy products may have higher nutritional values than the corresponding raw materials. Possible explanations are the pre-digestion of lactose and proteins (Kurmann, 1988). Lactic acid bacteria possess a specific proteolytic activity which degrades the proteins in milk in free amino acids. For this reason, it is suggested that the proteolytic activity may be a good indicator to show the ability of *Lactobacillus acidophilus* to improve the nutritional value of milk products (Gurr, 1987; Kurmann, 1988).

The objective of this study was to study the influence of pulsed electric field on the growth characteristics, bile tolerance, acid tolerance, and protease activity of *Lactobacillus acidophilus* LA-K.

The hypothesis was whether certain pulse electric field conditions can improve the beneficial characteristics of probiotic bacterium *Lacobacillus acidophilus* LA-K.

CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental Design

Control and Pulsed Electric Field (PEF) treatment samples were inoculated with *Lactobacillus acidophilus* (F-DVS LA-K, Chr. Hansen's Laboratory, Milwaukee, WI, USA). The treatments were pulse width (3, 6, and 9 µs), pulse period (10,000 µs, 20,000 µs, and 30,000 µs), voltage (5, 10, and 15 kV/cm) and flow rate (10, 60, and 110 mL/min). Control was run through the PEF equipment at 60 mL/min without receiving any pulsed electric field treatment. Growth characteristics, bile tolerance, acid tolerance, and protease activity were determined in the control and PEF treatment samples. Growth characteristics and bile tolerance analyses were determined hourly throughout 16 hours of incubation. Acid tolerance was evaluated at 0, 5, 10 and 15 minutes of incubation. Protease activity was determined at 0, 12 and 24 hours of incubation. The experimental design was a repeated measure design. Three replications were conducted.

2.2 Control and PEF Treatment Samples Preparation

Control and PEF treatment samples for the growth, bile tolerance and acid tolerance analyses were prepared by inoculating 1% (v/v) of *Lactobacillus acidophilus* (F-DVS LA-K, Chr. Hansen's Laboratory, Milwaukee, WI, USA) in peptone water (0.1% wt/v) at room temperature (21 ⁰C). *Lactobacillus acidophilus* LA-K in control and PEF treatment samples for protease analysis was inoculated at 10% (v/v).

2.3 PEF Treatment Conditions

The pulsed electric field conditions analyzed in this study were bipolar pulse width, pulse period, voltage, and flow rate. These different pulsed electric field conditions were sequentially applied and evaluated. The first condition applied was

bipolar pulse widths of 3, 6 and 9 µs. The bipolar pulse width that showed the best result for growth, bile tolerance, acid tolerance, and protease activity was left constant for the second step. The best results for the various characteristics were determined as follows: a) for growth; a short lag phase followed by an early and prolonged log phase, b) for acid tolerance; the maximum viable counts over time, c) for bile tolerance; the maximum OD value (at 650 nm) hourly over 16 hours of incubated storage, and d) for protease activity; the maximum protease activity at 24 hours of incubation. The second step was three different pulse periods of 10,000; 20,000 and 30,000 µs. The pulse period that showed the best result for growth, bile tolerance, acid tolerance, and protease activity as described above was selected and left constant for the third step along with the bipolar pulse width selected in first step. The third step was three different voltages namely 5, 15 and 25kV/cm. The voltage that demonstrated best results for growth, bile tolerance, acid tolerance, and protease activity was chosen and left constant for the last step along with the bipolar pulse width and pulse period selected before. The fourth and last step was three different flow rates namely 10, 60, and 110 mL/min. The flow rate that showed best results for growth, bile tolerance, acid tolerance, and protease activity was selected along with the pulsed electric field conditions selected before. The sequential order of the pulsed electric field conditions applied was determined by considering the treatment time. For treatment time both bipolar pulse width and pulse period are important. For this reason, both bipolar pulse width and pulse period were studied first.

2.4 PEF Equipment

The equipment used to apply the different pulsed electric field conditions in this study was an integrated continuous fluid handling pilot plant PEF processor (OSU-4M,

Ohio State University, Columbus, OH) (Figure 1) holding bipolar square wave pulses

(Figure 2 and Figure 3). The PEF processor (Figure 4) consists of 4 treatment chambers;

each chamber contains 2 stainless steel electrodes separated by a gap of 0.29 cm. The

flow rate was controlled by a Micropump® gear pump (model 75211-30, Cole Palmer

Instrument Company, Vermon Hills, IL). The room temperature in the treatments was

controlled by an Isotemp Refrigerated Circulator (Fisher Scientific).
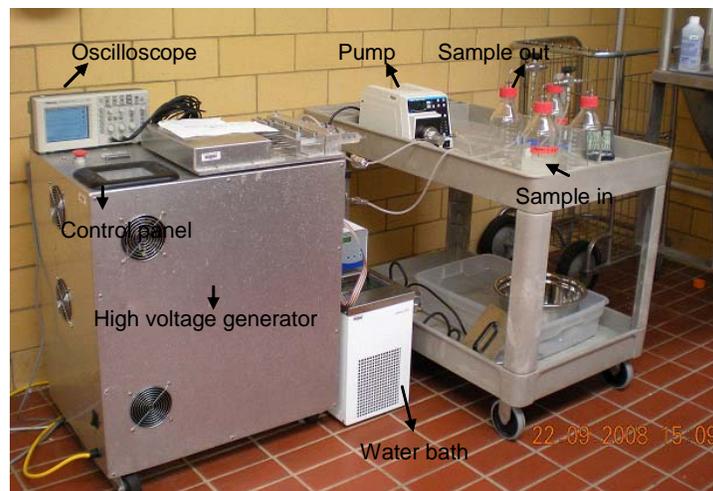
Figure 1. Pulsed electric field processor
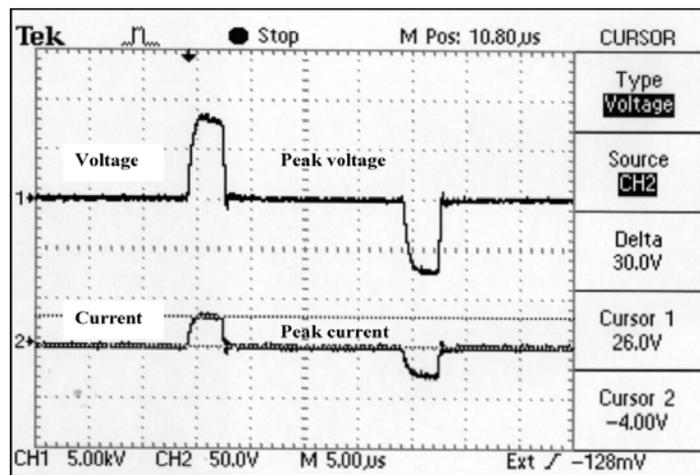
Figure 2. Bipolar square wave pulses seen on the PEF system (Min *et al.,* 2003)
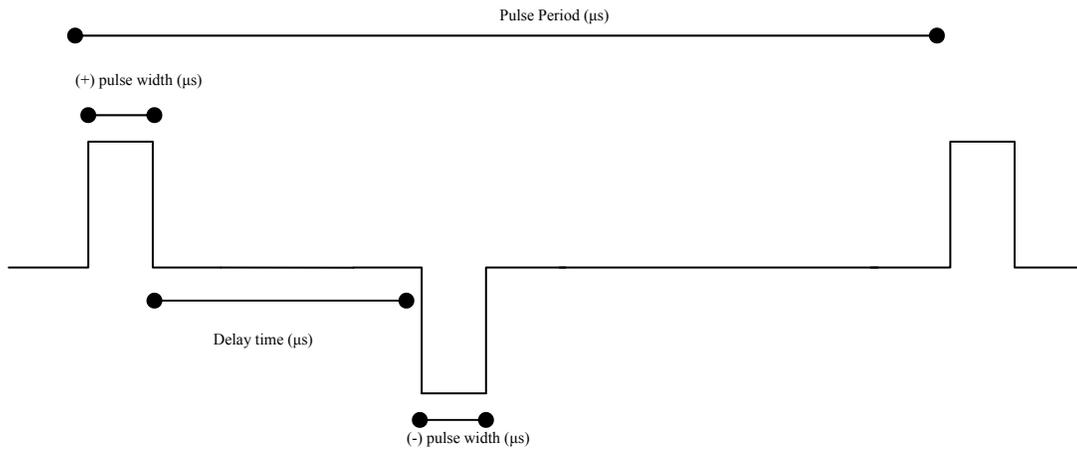
Figure 3. A bipolar square wave pulse
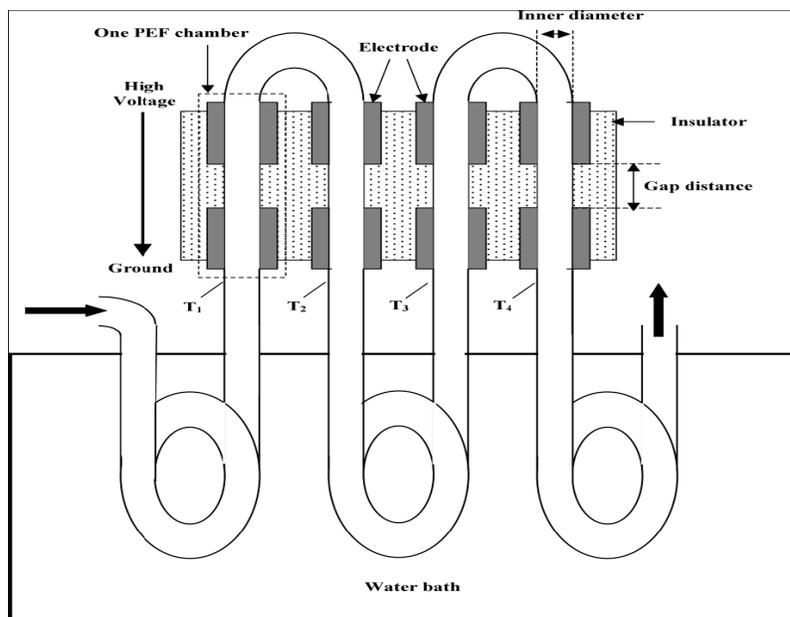
Figure 4. Flow arrangement of 4 pulsed electric field treatment chambers (Min *et al.,* 2003)

2.5 Analytical Procedures

2.5.1 Growth Test

Growth of *Lactobacillus acidophilus* LA-K was analyzed according to Loghavi *et al.,* (2007). The growth was monitored by measuring the optical density at 600 nm (OD_{600}) through an UV- Vis Spectrophotometer (Nicolet Evolution 100, Thermo

Scientific; Madison, WI, USA) at 600 nm. Control and PEF treated samples were inoculated (10% [v/v]) into MRS broth (Criterion™, Hardy Diagnostics, Santa Maria, CA), which was previously autoclaved at 121 ⁰C for 15 min with pH 6.5 ± 0.2. The inoculated MRS broth had an initial OD_{600} of 0.200 ± 0.005, and was incubated under anaerobic conditions at 37 ⁰C for 16 hours. The OD values were collected hourly. The spectrophotometer was calibrated by using MRS broth as blank. An average of two values per treatment was taken, that is two cuvettes per treatment. An estimate of bacterial counts (CFU/mL) was calculated from OD_{600} readings using a standard curve (Figure 5).
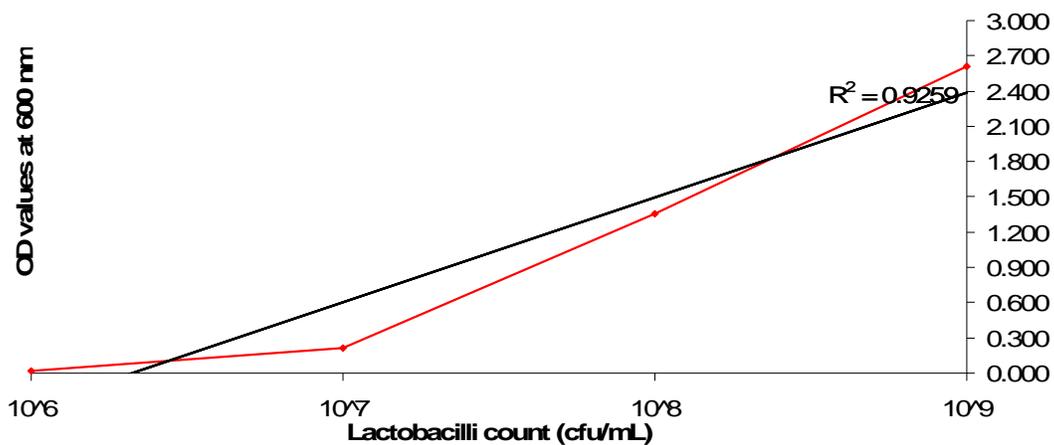
Figure 5. Standard curve for growth of LA-K in MRS broth

2.5.2 Bile Tolerance Test

Bile tolerance of *Lactobacillus acidophilus* was analyzed according to Pereira and Gibson (2002) with slight modifications. *Lactobacillus acidophilus* LA-K was evaluated for its ability to grow in MRS-THIO broth [MRS broth (Criterion™, Hardy Diagnostics, Santa Maria, CA) supplemented with 0.2% (wt/v) of sodium thioglycolate

(Acros Organics, Fair Lawn, NJ)] with bile acids. Sodium thioglycolate was used in MRS broth as an oxygen scavenger to achieve microaerophilic conditions. Control and PEF treated samples were inoculated (10% [v/v]) into MRS-THIO broth with 0.3% (wt/v) oxgall (bovine bile) (USBiological, Swampscott, MA) at an initial OD_{650} of 0.200 ± 0.005, and incubated under anaerobic conditions at 37 ⁰C for 16 hours. Absorbance in samples was measured hourly with an UV-Vis Spectrophotometer (Nicolet Evolution 100, Thermo Scientific; Madison, WI, USA) at 650 nm. The spectrophotometer was calibrated by using MRS-THIO broth with 0.3% oxgall as blank. An average of two readings per treatment was taken, that is two cuvettes per treatment. An estimate of bacterial counts (CFU/mL) was calculated from OD_{650} readings using a standard curve (Figure 6).
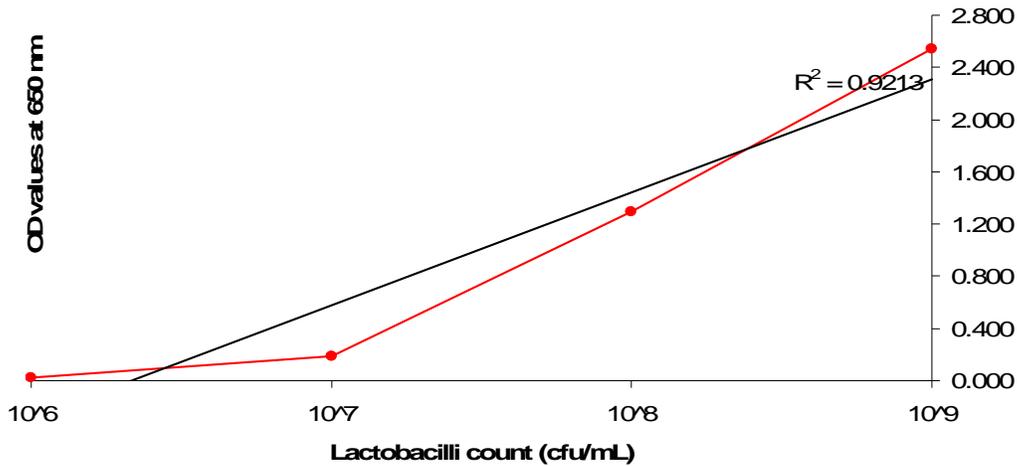
Figure 6. Standard curve for growth of LA-K in MRS-THIO broth with 0.3% oxgall

2.5.3 Acid Tolerance Test

The acid tolerance of *Lactobacillus acidophilus* LA-K was evaluated according to Pereira and Gibson (2002) with slight modifications. Control and PEF treated samples were inoculated (10% [v/v]) into acidified MRS broth (Criterion™, Hardy Diagnostics, Santa Maria, CA) previously adjusted to pH 2.0 with 1N HCl. The acidified MRS broth mixtures were incubated in a water bath at 37 $^{\circ}$C for 15 minutes. One milliliter samples were taken at various times (0, 5, 10, and 15 min), serially 10-fold diluted in peptone water, and plated in duplicate onto MRS agar (Difco, Detroit, MI). The plates were incubated at 37 $^{\circ}$C for 24 hours under anaerobic conditions before enumeration.

2.5.4 Protease Activity

The extracellular protease activity of *Lactobacillus acidophilus* LA-K was determined by the *o*-phthaldialdehyde (OPA) spectrophotometric assay according to the method described by Oberg *et al.,* (1991). *Lactobacillus acidophilus* LA-K in control and PEF treated samples was inoculated (10% [v/v]) into sterile skim milk (autoclaved at 121 $^{\circ}$C for 15 min), and incubated at 40 $^{\circ}$C for 0, 12 and 24 hours. After incubation, 2.5 ml from each sample was mixed with 1 ml distilled water and transferred into test tubes containing 5 ml of 0.75N trichloroacetic acid (TCA) (Fisher Scientific) and the test tubes were vortexed at the same time. After setting at room temperature for 10 minutes the acidified samples were filtered through a Whatman Number 2 filter paper (Clifton, NJ). Non inoculated sterile skim milk was prepared similarly to use as a reference in the assay. Duplicate aliquots from each TCA filtrate were analyzed by the *o*-phthaldialdehyde (OPA) spectrophotometric assay using an UV-Vis spectrophotometer (Nicolet Evolution 100, Thermo Scientific; Madison, WI, USA). The *o*-phthaldialdehyde 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 20%

(wt/wt) SDS (Fisher Scientific); 40 mg of *o*-phthaldialdehyde reagent (Alfa Aesar, Ward

Hill, MA) dissolved in 1 ml methanol (Sigma); and 100 µl of β-mercaptoethanol (Sigma).

One hundred and fifty µl of each TCA filtrate were mixed with 3 ml of *o*-

phthaldialdehyde final solution in a 3 ml cuvette, and the absorbance at 340 nm was read.

Absorbance of the *o*-phthaldialdehyde final solution with the non inoculated sterile skim

milk (reference) was subtracted from each sample reading. The *o*-phthaldialdehyde final

solution was used as a blank to calibrate the spectrophotometer.

2.5.5 Statistical Analysis

Data were analyzed using the General Linear Model (PROC GLM) of the

Statistical Analysis Systems (SAS). Differences of least square means were used to

determine significant differences at $P < 0.05$ for main effects (pulse width, pulse period,

voltage, flow rate) and interaction effects (pulse width * time, pulse period * time,

voltage * time, and flow rate * time). Data are presented as mean ± standard error of

means. Significant differences were determined at $\alpha = 0.05$.

CHAPTER 3: RESULTS AND DISCUSSION

The effects of four separate pulsed electric field (PEF) parameters namely pulse width, pulse period, voltage and flow rate on the characteristics of probiotic bacterium *Lactobacillus acidophilus* LA-K were studied. These four different PEF parameters were applied, studied and selected sequentially in four separate steps. The first pulsed electric field parameter studied was the pulse width, secondly the pulse period, followed by the voltage and finally the flow rate. Since this study had a sequential order in the application of the different pulsed electric field parameters, an initial setting of pulsed electric field conditions (pulse width of 3μs, voltage of 25 kV/cm, delay time of 20 μs, pulse period of 10,000 μs, and a flow rate of 60 mL/min) was selected and left constant to be used at the beginning of the study. The selection of these was according to earlier studies on microbial inactivation by PEF (Reina *et al.,* 1998; Rivas *et al.,* 2006; Sobrino-López *et al.,* 2006; Rowan, *et al.,* 2001). Delay time was the only pulsed electric field condition that was left constant throughout the study. The best level of each PEF parameter was selected for the subsequent step according to its effects on the growth, bile tolerance, acid tolerance and protease activity of on *Lactobacillus acidophilus* LA-K.

3.1 Pulse Width

In the first of the four separate steps the effect of three different bipolar pulse widths namely 3 μs, 6 μs and 9 μs on *Lactobacillus acidophilus* LA-K was studied. The PEF treatment conditions for the study of this first step are shown in Table 1.

3.1.1 Growth Characteristics

The OD at different bipolar pulse widths over the growth curve period of 16 hours are shown in Figure 7. Bipolar pulse width * hour interaction effect was significant ($p = 0.0155$) (Table 2). From hours 5 to 10 there were significant differences between the

Table 1. Pulsed electric field (PEF) treatment conditions applied during the study of the influence of various pulse widths on *Lactobacillus acidophilus* LA-K

Treatment parameter	Condition
Bipolar pulse width (μs)	3, 6, 9
Electric field strength (kV/cm)	25
Pulse period (μs)	10,000
Delay time (μs)	20
Flow rate (mL/min)	60

control and the different bipolar pulse widths. Bipolar pulse width effect had a significant ($p < 0.0001$) influence on the growth curve (Table 2). The growth curve of the control was significantly higher than the growth curves subjected at any of the bipolar pulse widths studied. There were no significant differences among the three different bipolar pulse widths (Table 3).

The logarithmic phase of *Lactobacillus* LA-K in control was reached faster than when treated at different bipolar pulse widths. The slope in the growth curve of the control was higher than the slope of the curve of the different bipolar pulse widths. From hours 5 to 10 the control was an average of OD 0.300 higher than the different bipolar pulse widths. According to Hülsheger (1983) bacteria cells in the stationary and lag growth are more resistant to PEF treatments than exponentially growing cells. The reason of this is because microbial growth in logarithmic phase is characterized by a high proportion of cells undergoing division, during which the cell membrane is more

Table 2. Mean square (MS) and Pr > F of pulse width, hour and their interaction for growth characteristics, bile tolerance and protease activity

Source	Growth		Bile tolerance		Protease activity	
	MS	Pr > F	MS	Pr > F	MS	Pr > F
Pulse width	0.278	<0.0001	1.018	<0.0001	0.002	0.3201
Hour	6.384	<0.0001	2.445	<0.0001	0.154	<0.0001
Pulse width * hour	0.015	0.0155	0.030	<0.0001	0.005	0.0487
Error	0.009		0.003		0.002	
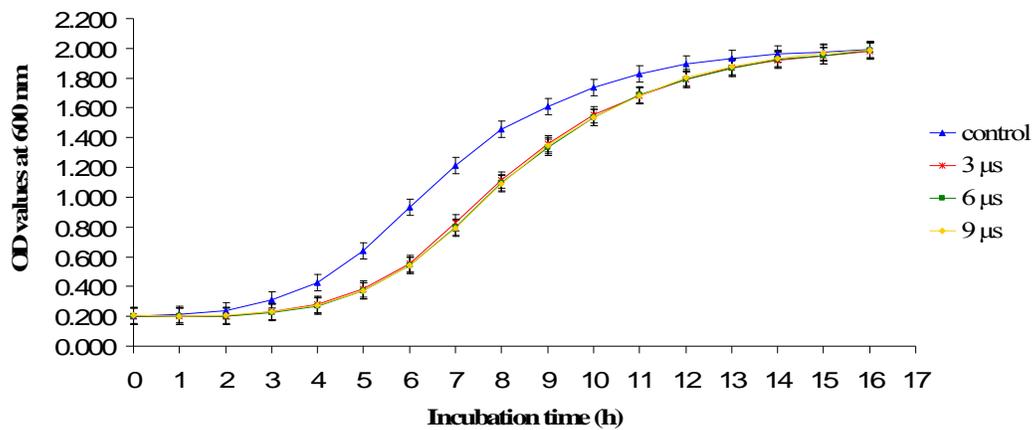
Figure 7. Pulse width influence on growth of LA-K

Table 3. Least square means for growth characteristics, bile tolerance and acid tolerance as influenced by pulse width

Treatment	Growth LSMean	Bile tolerance LSMean	Acid tolerance LSMean
Control	1.210 ^{A}	0.825 ^{A}	3.933 ^{A}
3 μs	1.066 ^{B}	0.546 ^{B}	2.924 ^{B}
6 μs	1.059 ^{B}	0.542 ^{B}	2.754 ^{C}
9 μs	1.062 ^{B}	0.540 ^{B}	2.575 ^{D}

LSMeans with same letter are not significantly different ($p < 0.05$)

susceptible to the applied electric field. The concentration of microorganisms may have an effect on their inactivation with PEF. The initial concentration of *Lactobacillus acidophilus* LA-K in this study was 10^7. Barbosa-Canovas *et al.,* (1999) reported than inactivation of *E. coli* in simulated milk ultrafiltrate was not affected when the concentration of microorganisms was varied from 10^3 to 10^8 cfu/mL after being subjected to 70 kV/cm, 16 pulses, and a pulse width of 2 μs.

3.1.2 Bile Tolerance

The OD values at different bipolar pulse widths over the bile tolerance period of 16 hours are shown in Figure 8. Bipolar pulse width * hour interaction effect was significant ($p < 0.0001$) (Table 2). From hours 4 to 16 there were significant differences between the control and the three different bipolar pulse widths. Bipolar pulse width effect had a significant ($p < 0.0001$) influence on the bile tolerance (Table 2). The bile tolerance of the control was significantly higher than the bile tolerance subjected at any of the bipolar pulse widths studied. There were no significant differences among the three different bipolar pulse widths (Table 3).

The bile tolerance of different strains of *Lactobacillus acidophilus* isolated from human intestinal were studied by Buck and Gilliand (1994). They found that *Lactobacillus acidophilus* ATCC 43121 was significantly more bile tolerant than isolates C14, G20, G5, H13, H11, J18 and J12. This strain required only 2 hours for the optical density to increase by 0.3 units, whereas strains J18 and J12 required 7 hours to increase.
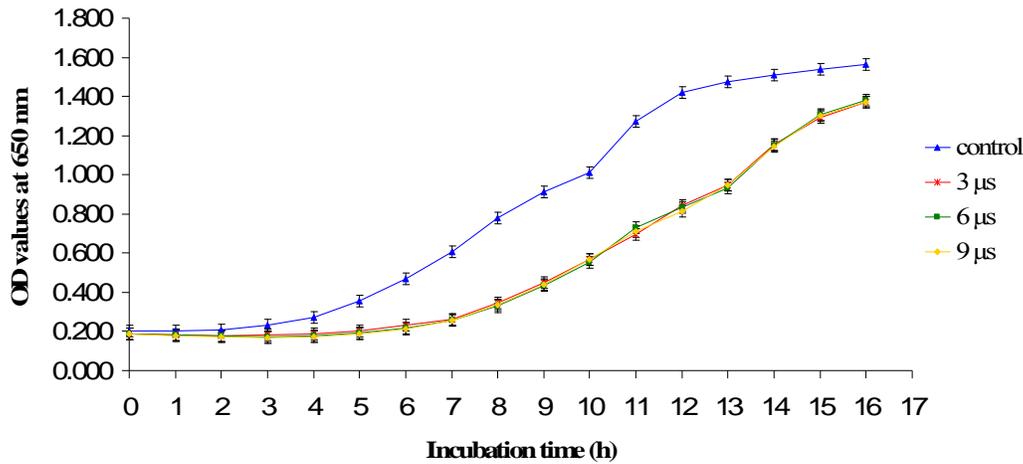
Figure 8. Pulse width influence on bile tolerance of LA-K

3.1.3 Acid Tolerance

The acid tolerance at different bipolar pulse widths over the four time points of 0, 5, 10 and 15 minutes are shown in Figure 9. Bipolar pulse width * minute interaction effect was significant ($p < 0.0001$) (Table 4). From minutes 0 to 15 there were significant differences between the control and the different bipolar pulse widths. At minute 0, among the three different bipolar pulse widths, the acid tolerance subjected to bipolar pulse widths of 3 µs was significantly higher than 6 µs and 9 µs. At minute 5 the acid tolerances subjected to bipolar pulse widths of 3 µs and 6 µs were significantly higher compared to 9 µs. The acid tolerance subjected to 3 µs was significantly the highest at minute 10 followed by 6 µs and 9 µs consecutively. There were no significant differences among the three different bipolar pulse widths at 15 minutes. Bipolar pulse width effect had a significant ($p < 0.0001$) influence on the acid tolerance (Table 4). According to Table 3 the control and the three different bipolar pulse widths studied were significantly different from each other. The acid tolerance of the control was significantly the highest,

followed by the acid tolerance subjected to 3 µs and 6 µs. The acid tolerance subjected to

9 µs was the lowest.
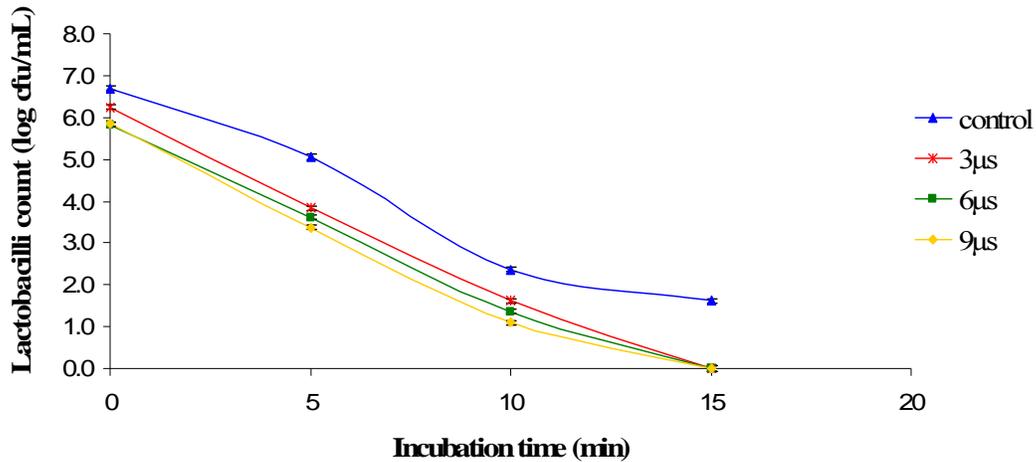
Figure 9. Pulse width influence on acid tolerance of LA-K

Table 4. Mean square (MS) and Pr > F of pulse width, minute and their interaction for acid tolerance

	Acid tolerance	
Source	MS	Pr > F
Pulse width	4.436	<0.0001
Minute	78.519	<0.0001
Pulse width * minute	0.169	<0.0001
Error	0.009	

The majority of studies have used exponential decay pulses when studying

microbial inactivation by PEF. However, square wave pulses are more energy and

lethally efficient as well as more accurate for calculation of treatment time at a given

electric field strength (Wouters *et al.,* 2001b). The bipolar pulse widths applied in this

study were square wave pulses. According to Qin *et al.,* (1994) bipolar pulse widths are

more lethal than monopolar pulses because PEF causes a movement of charged

molecules in the cell membranes of microorganisms, and reversal in the orientation or polarity of the electric field causes a corresponding change in the direction of charged molecules.

3.1.4 Protease Activity

The protease activity at different bipolar pulse widths over three time points of 0, 12 and 24 hours are shown in Figure 10. Bipolar pulse width * hour interaction was significant ($p = 0.0487$) (Table 2). At hours 0 and 12 there were no significant differences among the control and the three different bipolar pulse widths. The protease activity subjected to bipolar pulse widths of 6 and 9 µs were significantly higher compared to the control and 3 µs at hour 24. There were no significant differences between the control and 3 µs at hour 24. Likewise there were no significant differences between 6 and 9 µs at hour 24. Bipolar pulse width effect had no significant ($p < 0.3201$) influence on the protease activity (Table 2).
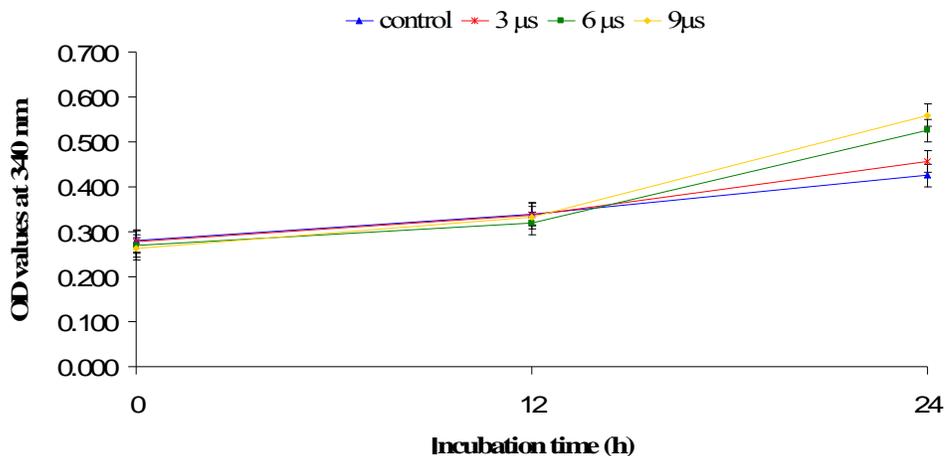
Figure 10. Pulse width influence on protease activity of LA-K

Pulse width affects enzyme activity. If the number of pulses and the electric field strength are kept constant, an increase in pulse width will lead to higher levels of enzyme inactivation (Martin-Belloso and Elez-Martinez, 2005). In a study conducted by Bendicho *et al*., (2002) no difference was found in protease activity from *Bacillus subtilis* inoculated in milk when using pulse widths between 4 and 7 µs. However, with a greater pulse with fewer pulses were needed to achieve the same inactivation.

According to the growth and bile tolerance results of *Lactobacillus acidophilus* LA-K, there were no significant differences among the three different bipolar pulsed widths applied on it. However, *Lactobacillus acidophilus* LA-K subjected to bipolar pulse widths of 3µs showed to be the most acid tolerant up to 10 minutes at pH 2.0. To provide health benefits, probiotics must first overcome the acid in the stomach. Therefore the bipolar pulse width of 3µs was selected and left constant to be used in the second step when studying different pulse periods.

3.2 Pulse Period

In the second step the effect of three different pulse periods namely 10,000 µs, 20,000 µs and 30,000 µs on the characteristics of *Lactobacillus acidophilus* LA-K was studied. The PEF treatment conditions applied for the study of this second step are shown in Table 5.

Table 5. Pulsed electric field (PEF) treatment conditions applied during the study of the influence of various pulse periods on *Lactobacillus acidophilus* LA-K

Treatment parameter	Condition
Bipolar pulse width (µs)	3
Electric field strength (kV/cm)	25
Pulse period (µs)	10,000; 20,000; 30,000
Delay time (µs)	20
Flow rate (mL/min)	60

3.2.1 Growth Characteristics

The OD values at different pulse periods over the growth period of 16 hours are shown in Figure 11. Pulse period * hour interaction effect was not significant ($p = 1.0000$) (Table 6). Pulse period had a significant ($p = 0.0017$) influence on the growth curve (Table 6). According to Table 7 there were no significant differences among the control, 30,000 μs and 20,000 μs. The growth curve subjected to the pulse period of 10,000 μs was significantly lower than the growth curve of control and the growth curve subjected at 30,000 μs.
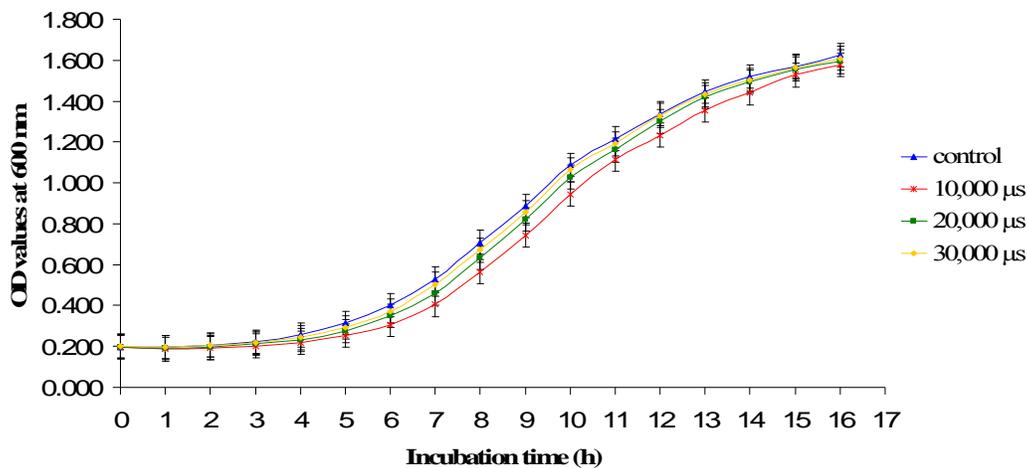
Figure 11. Pulse period effect on the growth of LA-K

Table 6. Mean square (MS) and Pr > F of pulse period, hour and their interaction for growth characteristics, bile tolerance and protease activity

	Growth		Bile tolerance		Protease activity	
Source	MS	Pr > F	MS	Pr > F	MS	Pr > F
Pulse period	0.070	0.0017	0.338	<0.0001	0.0002	0.8764
Hour	4.840	<0.0001	0.473	<0.0001	0.394	<0.0001
Pulse period * hour	0.002	1.0000	0.026	<0.0001	0.008	<0.0001
Error	0.013		0.005		0.0007	

Table 7. Least square means for growth characteristics, bile tolerance and acid tolerance as influenced by pulse period

Treatment	Growth LSMean	Bile tolerance LSMean	Acid tolerance LSMean
Control	0.808 [A]	0.388 [A]	3.852 [A]
10,000 μs	0.733 [B]	0.223 [D]	2.782 [D]
20,000 μs	0.772 [A B]	0.264 [C]	2.916 [C]
30,000 μs	0.792 [A]	0.308 [B]	2.993 [B]

LSMeans with same letter are not significantly different ($p < 0.05$)

3.2.2 Bile Tolerance

The OD values at different pulse periods over the bile tolerance period of 16 hours are shown in Figure 12. Pulse period * hour interaction effect was significant ($p < 0.0001$) (Table 6). From hours 11 to 16 there were significant differences between the control and the three different pulse periods. However, the 10,000 μs and 20,000 μs pulse periods compared to the control, showed significant differences from hours 9 and 10 respectively. From hours 12 to 16, among the different pulse periods, the 30,000 μs pulse period was significantly higher compared to 20,000 μs which in turn was significantly higher compared to 10,000 μs. Bile tolerances at all different pulse periods were significantly different from each other from hours 14 to 16. Pulse period had a significant ($p < 0.0001$) influence on the bile tolerance (Table 6). According to Table 7 the control and the three different pulse periods studied were significantly different from each other. The bile tolerance of the control was significantly the highest, followed by the bile tolerances subjected to 30,000 μs and 20,000 μs consecutively. The bile tolerance subjected to 10,000 μs was significantly the lowest.
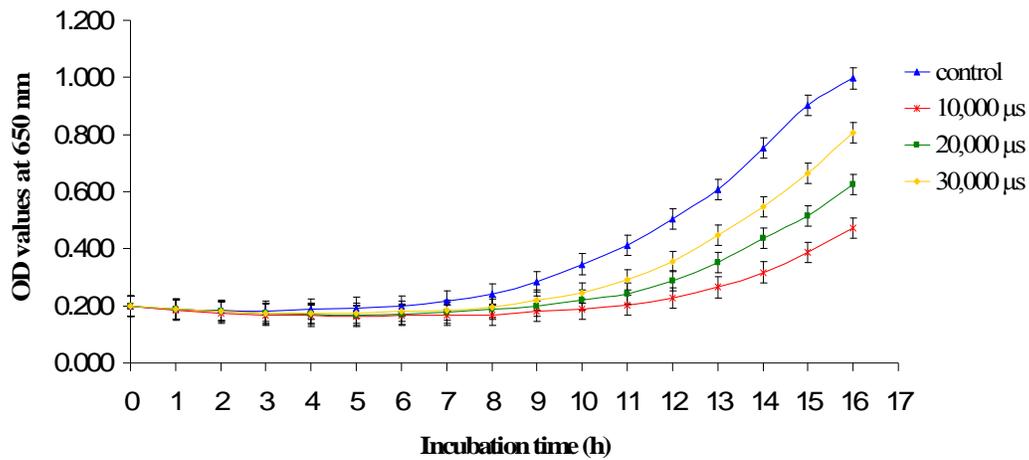
Figure 12. Pulse period influence on bile tolerance of LA-K

Chateau *et al.,* (1994) arbitrarily classified bile resistance of *Lactobacillus* into four groups according to the delay of growth. These groups are: resistant strains (delay of growth d ≤ 15 min), tolerant strains (15 min < d ≤ 40 min), weakly tolerant strains (40 min < d < 60 min) and sensitive strains (d ≥ 60 min).

3.2.3 Acid Tolerance

The acid tolerance at different pulse periods over the four time points of 0, 5, 10 and 15 minutes are shown in Figure 13. Pulse period * minute interaction effect was significant ($p < 0.0001$) (Table 8). At minute 0 there was a significant difference between the control and 10,000 μs. From minutes 5 to 15 there were significant differences between the control and the different pulse periods. From minutes 0 to 15 there were no significant differences between the acid tolerance subjected to 20,000 μs and the acid tolerance subjected to 30,000 μs. From minutes 0 to 10 the acid tolerance subjected to pulse periods of 10,000 μs was significantly lower than the acid tolerance subjected to pulse periods of 30,000 μs. At minute 15 there were no significant differences among the

three different pulse periods. Pulse period had a significant ($p < 0.0001$) influence on the

acid tolerance (Table 8). According to Table 7 the control and the three different pulse

periods evaluated were significantly different from each other. The acid tolerance of the

control was significantly the highest, followed by the acid tolerances subjected to 30,000

µs and 20,000 µs consecutively. The acid tolerance subjected to 10,000 µs was

significantly the lowest.
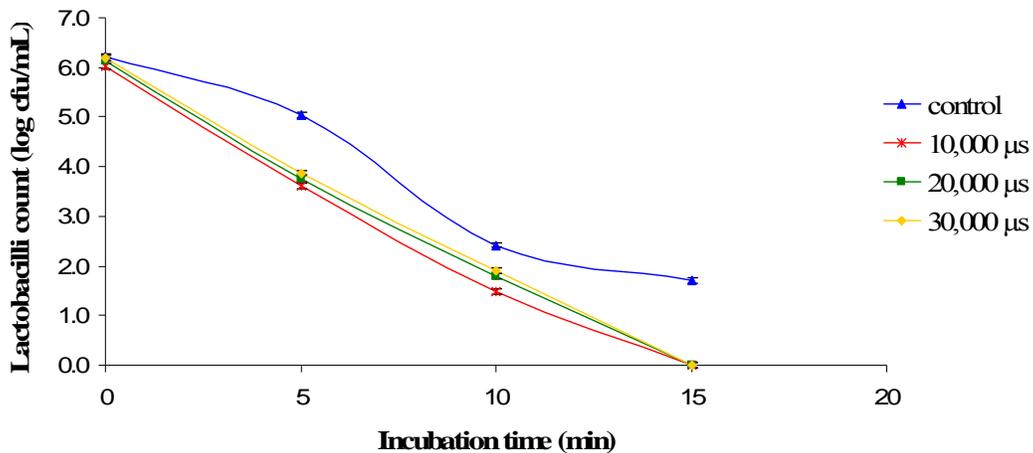
Figure 13. Pulse period effect on acid tolerance of LA-K

Table 8. Mean square (MS) and Pr > F of pulse period, minute and their interaction for acid tolerance

	Acid tolerance	
Source	MS	Pr > F
Pulse period	2.827	<0.0001
Minute	75.104	<0.0001
Pulse period * minute	0.383	<0.0001
Error	0.007	

In this study *Lactobacillus acidophilus* LA-K subjected at any of the different

pulse widths and pulse periods studied did not survive after 15 minutes at pH 2.0. In a

study carried out by Pereira and Gibson (2002) it was shown that the viability of

Lactobacillus pentosus (B) and *Streptococcus thermophilus* DSM 20617 was lost in less

than 15 minutes at pH 2.0. They also found that *Lactobacillus fermentun* KC5b,

Lactobacillus delbrueckii JCM 1002, and *Lactobacillus acidophilus* johnsonii were the

most acid tolerant strains by retaining around 100% viability for up to 2 hours at pH 2.0.

3.2.4 Protease Activity

The protease activity at different pulse periods over three time points of 0, 12 and

24 hours are shown in Figure 14. Pulse period * hour interaction effect was significant (p

< 0.0001) (Table 6). At hours 0 and 12 there were no significant differences among the

control and the three different pulse periods. At hour 24 the protease activities subjected

to pulse periods of 30,000 µs and 20,000 µs were significantly lower compared to the

control and 10,000 µs. At this time 30,000 µs showed significantly the lowest protease

activity followed by 20,000 µs. There were no significant differences between the

protease activity of the control and 10,000 µs at hour 24. Pulse period had no significant

(p = 0.8764) influence on the protease activity (Table 6).

Although *L. acidophilus* has been reported to posses many advantages and healthy

giving properties, its growth in milk is slow compared to *Bifidobacteria* (Itoh *et al.,*

1991). Milk contains sufficient growth factors; however, the concentration of free amino

acids in milk is too low to support growth of lactobacilli to high populations or for rapid

acid productions, both of which are essential to manufacture fermented milk products

(Thomas and Pritchar, 1987). For this reason many commercialized fermented milk

products containing *L. acidophilus* are made in combination with yogurt starters or by

adding a concentrate of live *L. acidophilus* cells propagated and collected in advance

from broth (Masuda *et al.,* 2005). Degraded proteins generated from high protease activity may affect the sensory properties of the fermented products during further fermentation and storage. Intracellular enzymes exuded via autolytic rupture of the cells may also cause such flavor alterations (Masuda *et al.,* 2005).
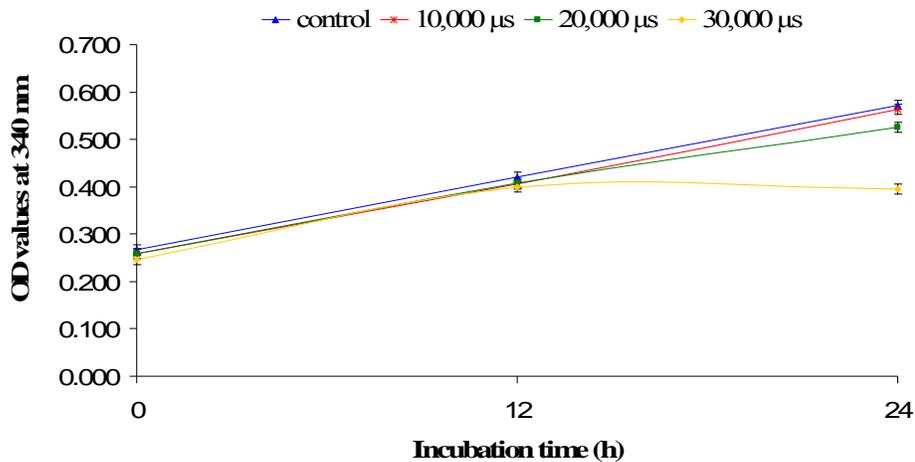
Figure 14. Pulse period effect on protease activity of LA-K

In this second step of the study it was shown that there were no differences in the acid tolerance and growth of *Lactobacillus acidophilus* LA-K subjected to pulse periods of 20,000 and 30,000 µs. However, the bile tolerance of *Lactobacillus acidophilus* LA-K subjected to pulse periods of 30,000 µs showed to be the highest compared to 20,000 and 10,000 µs. Bile is the second obstacle that probiotics have to defeat in the upper part of the small intestine after acid tolerance in stomach. Therefore the pulse period of 30,000 µs was selected and left constant to be used in the third step of the study.

3.3 Voltage (kV/cm)

After studying the effect of pulse width and pulse period in the steps before, the effect of three different voltages namely 5 kV/cm, 15 kV/cm, and 25 kV/cm on the characteristics of *Lactobacillus acidophilus* LA-K was studied in the third step. The PEF treatment conditions applied for the study of this third step are shown in Table 9.

Table 9. Pulsed electric field (PEF) treatment conditions applied during the study of the influence of various voltages on *Lactobacillus acidophilus* LA-K

Treatment parameter	Condition
Bipolar pulse width (μs)	3
Electric field strength (kV/cm)	5, 15, 25
Pulse period (μs)	30,000
Delay time (μs)	20
Flow rate (mL/min)	60

3.3.1 Growth Characteristics

The OD values at different voltages over the growth period of 16 hours are shown in Figure 15. Voltage * hour interaction effect was not significant ($p = 0.2706$) (Table 10). Voltage had a significant ($p < 0.0001$) influence on the growth curve (Table 10). According to Table 11 the growth curves subjected at 15 and 25 kV/cm were significantly lower than the control and 5 kV/cm. There were no significant differences between the control and 5 kV/cm. Furthermore, there were no significant differences between the growth curves at 15 and 25 kV/cm.

Table 10. Mean square (MS) and Pr > F of voltage, hour and their interaction for growth characteristics, bile tolerance and protease activity

	Growth		Bile tolerance		Protease activity	
Source	MS	Pr > F	MS	Pr > F	MS	Pr > F
Voltage	0.044	<0.0001	0.582	<0.0001	0.0003	0.7977
Hour	3.698	<0.0001	0.746	<0.0001	0.209	<0.0001
Voltage * hour	0.001	0.2706	0.021	<0.0001	0.0008	0.5129
Error	0.001		0.005		0.0009	
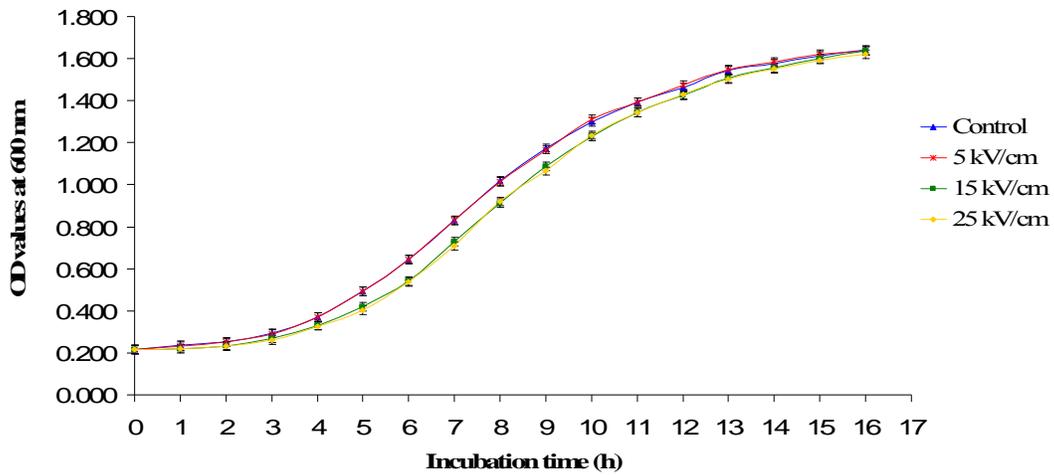
Figure 15. Voltage effect on the growth of LA-K

Table 11. Least square means for growth characteristics, bile tolerance and acid tolerance as influenced by voltage.

	Growth	Bile tolerance	Acid tolerance
Treatment	LSMean	LSMean	LSMean
Control	0.945 ^{A}	0.494 ^{A}	3.871 ^{A}
5 kV/cm	0.946 ^{A}	0.497 ^{A}	3.756 ^{B}
15 kV/cm	0.897 ^{B}	0.329 ^{B}	2.952 ^{C}
25 kV/cm	0.892 ^{B}	0.296 ^{C}	2.716 ^{D}

LSMeans with same letter are not significantly different ($p < 0.05$)

3.3.2 Bile Tolerance

The OD values at different voltages over the bile tolerance period of 16 hours are shown in Figure 16. Voltage * hour interaction effect was significant ($p < 0.0001$) (Table 10). The bile tolerance of the control and the bile tolerance subjected to 5 kV/cm were significantly different than the bile tolerances subjected to 15 and 25 kV/cm throughout the entire incubation time period. There were no significant differences between the

control and 5kV/cm during all 16 hours period. Voltage had a significant ($p < 0.0001$) influence on the bile tolerance (Table 10). According to Table 11 the bile tolerance of the control and the bile tolerance subjected to 5 kV/cm were significantly the highest compared to 15 and 25 kV/cm. The bile tolerance subjected to 25 kV/cm was significantly the lowest.
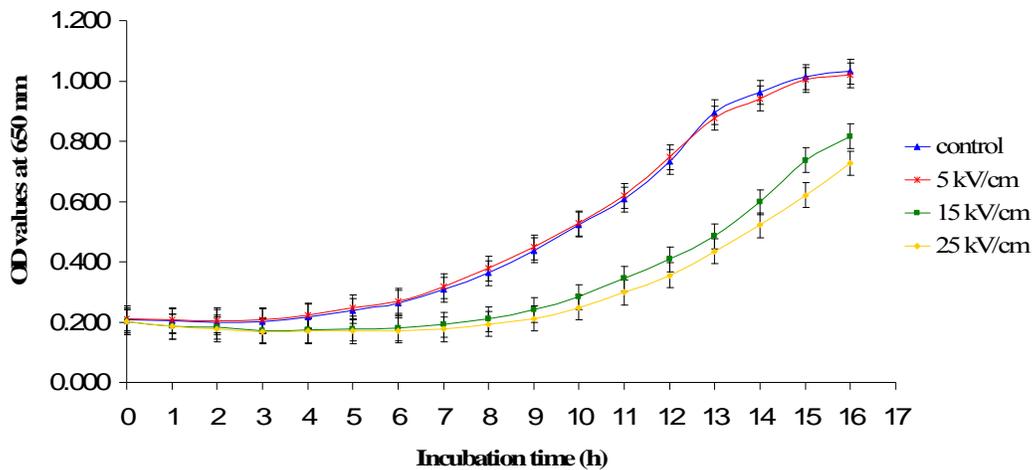
Figure 16. Voltage influence on the bile tolerance of LA-K

3.3.3 Acid Tolerance

The acid tolerance at different voltages over the four time points of 0, 5, 10 and 15 minutes are shown in Figure 17. Voltage * minute interaction effect was significant ($p < 0.0001$) (Table 12). From minutes 0 to 10 the acid tolerance of the control along with the acid tolerance subjected at 5 kV/cm were significantly higher than the acid tolerances subjected at 15 kV/cm and 25 kV/cm. Moreover, at this same time interval, the acid tolerance subjected at 25 kV/cm was significantly the lowest followed by the acid tolerance subjected at 15 kV/cm. At minute 15 the acid tolerance of the control was significantly the highest compared to the other voltages followed by the acid tolerance

subjected at 5 kV/cm. At this same minute there were no significant differences between the acid tolerances subjected at 15 kV/cm and 25 kV/cm. Voltage had a significant ($p < 0.0001$) influence on the acid tolerance (Table 11). According to Table 11 the control and the three different voltages studied were significantly different from each other. The acid tolerance of the control was significantly the highest followed by the acid tolerances subjected to 5. The acid tolerance subjected to 25 kV/cm was the lowest followed by the acid tolerance subjected to 15 kV/cm.

Table 12. Mean square (MS) and Pr > F of voltage, minute and their interaction for acid tolerance

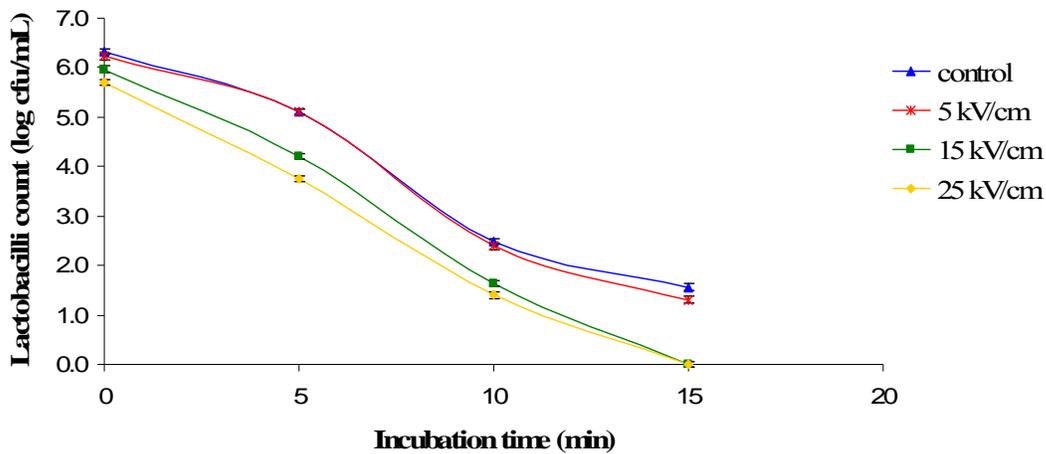|	Acid tolerance	
Source	MS	Pr > F
Voltage	3.981	<0.0001
Minute	70.209	<0.0001
Voltage * minute	0.198	<0.0001
Error	0.012	

Figure 17. Voltage effect on acid tolerance of LA-K

Different lactobacilli strains were studied for their ability to grow on MRS broth at pH 2.0 (Liong and Shah, 2005). In this study it was found that *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 292 were the most acid tolerant strains with more than 10^7 cfu/mL after incubation for 2 hours at pH 2.0, while *L. casei* ASCC 1520, *L. casei* ASCC 1521, *L. casei* ASCC 279, *L. casei* 15820 and *L. casei* CSCC 2607 were the least acid tolerant with only 10^4 total cfu/mL after the 2 hours of incubation. They also found that strains of *L. acidophilus* showed greater acid tolerance over the entire incubation period, and theirs counts decreased by 2.66 to 4.38 log cycles, compared with 3.16 and 6.20 log cycles for *L. casei.*

3.3.4 Protease Activity

The protease activity at different voltages over three time points of 0, 12 and 24 hours are shown in Figure 18. Voltage * hour interaction effect was not significant ($p = 0.5129$) neither was the voltage effect significant ($p = 0.7977$) (Table 10).
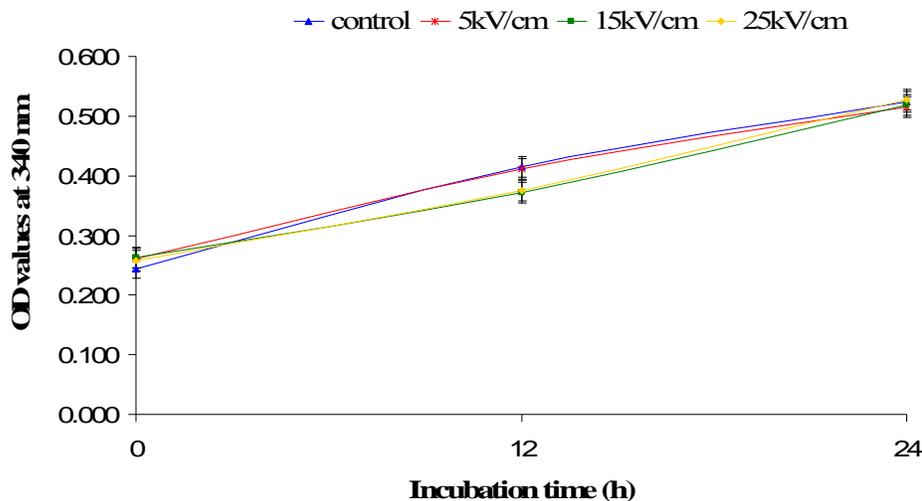

Figure 18. Voltage effect on protease activity of LA-K

According to Vega-Mercado *et al.,* (1995) inactivation of proteases varies with the intensity of electric field, the number of pulses and the presence of substrates. An 80% reduction in protease activity from *Pseudomona fluorescens* M3/6 in tryptic soy broth was achieved after 20 pulses of 2 μs and 18 kV/cm at 0.25 Hz. When the protease was treated in sterilized skim milk, pulsed electric field strengths of 14 kV/cm with 32 pulses of 2 μs achieved a 40% inactivation of the enzyme. Moreover, an inactivation of 60% was reached after applying a pulsed electric field strength of 15 kV/cm with 98 pulses of 2 μs. However, the protease treated in skim milk with a 25 kV/cm electric field strength at 0.6 Hz increased in proteolytic activity (Vega-Mercado *et al.,* 1995).

Lactobacillus acidophilus LA-K subjected to 5 kV/cm showed better results than 15 and 25 kV/cm during the growth, acid tolerance and bile tolerance. Control and 5 kV/cm were not significantly different. The protease activity at hour 24 was the same among the different voltages applied. Since 5 kV/cm showed to have the best effect on the growth, acid tolerance and bile tolerance of *Lactobacillus acidophilus* LA-K, this was selected and left constant for the final step of this study.

3.4 Flow Rate

In the fourth and final step the effect of three different flow rates namely 10 mL/min, 60 mL/min, and 110 mL/min on the characteristics of *Lactobacillus acidophilus* LA-K were studied. The PEF treatment conditions applied for the study of this last step are shown in Table 13.

3.4.1 Growth Characteristics

The OD values at different flow rates over the growth period of 16 hours are shown in Figure 19. Flow rate * hour interaction effect was significant ($p < 0.0001$) (Table 14).

Table 13. Pulsed electric field (PEF) treatment conditions applied during the study of the
influence of various flow rates on *Lactobacillus acidophilus* LA-K

Treatment parameter	Condition
Bipolar pulse width (µs)	3
Electric field strength (kV/cm)	5
Pulse period (µs)	30,000
Delay time (µs)	20
Flow rate (mL/min)	10, 60, 110

At hour 5 the growth of the control was significantly higher than the growth at
three different flow rates. At hour 8 the growth at flow rate of 60 mL/min was
significantly lower than the control, 10 mL/min and 100 mL/min. From hours 12 to 14
the growth at 110 mL/min was significantly higher than the control, 10 and 60 mL/min.
Flow rate had a significant ($p < 0.0001$) influence on the growth curve (Table 14).
According to Table 15 the control and the three different flow rates studied were
significantly different from each other. The growth curve subjected to flow rates of 110
mL/min was significantly the highest followed by the growth curve of the control. The
growth curve subjected to 60 mL/min was significantly the lowest followed by the
growth curve subjected to 10 mL/min.

3.4.2 Bile Tolerance

The OD values at different flow rates over the bile tolerance period of 16 hours
are shown in Figure 20. Flow rate * hour interaction effect was significant ($p < 0.0001$)
(Table 14). At hour 8 the bile tolerance of the control was significantly higher than the
bile tolerance at three different flow rates. At hour 11 the bile tolerance at flow rate 110
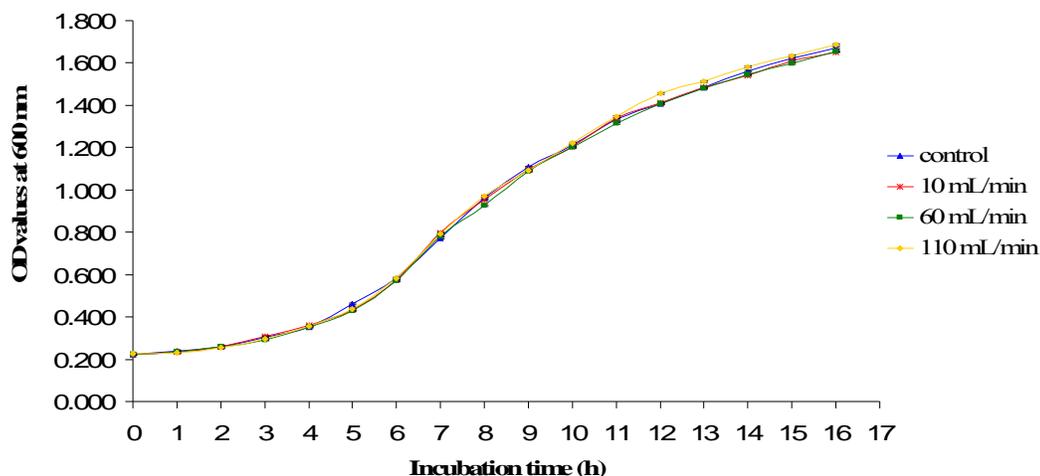mL/min was significantly higher than the control, 10 and 60 mL/min.

Figure 19. Flow rate influence on growth of LA-K

Table 14. Mean square (MS) and Pr > F of flow rate, hour and their interaction for growth characteristics, bile tolerance and protease activity

	Growth		Bile tolerance		Protease activity	
Source	MS	Pr > F	MS	Pr > F	MS	Pr > F
Flow rate	0.003	<0.0001	0.002	<0.0001	0.0004	0.2887
Hour	3.579	<0.0001	1.545	<0.0001	0.270	<0.0001
Flow rate * hour	0.0003	<0.0001	0.0004	<0.0001	0.0008	0.0358
Error	0.00005		0.00003		0.0003	

Table 15. Least square means for growth characteristics, bile tolerance and acid tolerance as influenced by voltage

	Growth	Bile tolerance
Treatment	LSMean	LSMean
Control	0.916 [B]	0.631 [B]
10 mL/min	0.911 [C]	0.624 [C]
60 mL/min	0.904 [D]	0.632 [B]
110 mL/min	0.922 [A]	0.639 [A]

LSMeans with same letter are not significantly different ($p < 0.05$)

At hour 12 the bile tolerance at 110 and 60 mL/min was significantly higher than the control and 60 mL/min. From hours 13 to 15 the bile tolerance at 110 mL/min was significantly higher than the control, 10 and 60 mL/min. At hour 16 the bile tolerances at 110 and 60 mL/min were significantly higher than the control and 10 mL/min. Flow rate had a significant ($p < 0.0001$) influence on the bile tolerance (Table 14). According to Table 15 the bile tolerance subjected to 110 mL/min was significantly the highest. The bile tolerance subjected to 10 mL/min was significantly the lowest.
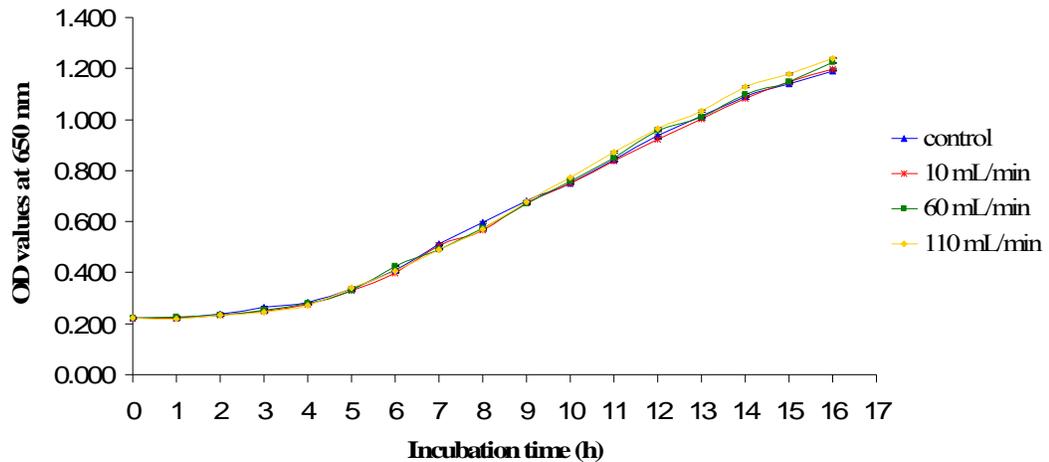
Figure 20. Flow rate effect on bile tolerance of LA-K

3.4.3 Acid Tolerance

The acid tolerance at different flow rates over the four time points of 0, 5, 10 and 15 minutes are shown in Figure 21. Flow rate * minute interaction effect was not significant ($p = 0.2419$) neither was the flow rate effect significant ($p = 0.0533$) (Table 16).

Table 16. Mean square (MS) and Pr > F of flow rate, minute and their interaction for acid tolerance

	Acid tolerance	
Source	MS	Pr > F
Flow rate	0.003	0.0533
Minute	53.727	<0.0001
Flow rate * minute	0.002	0.2419
Error	0.001	
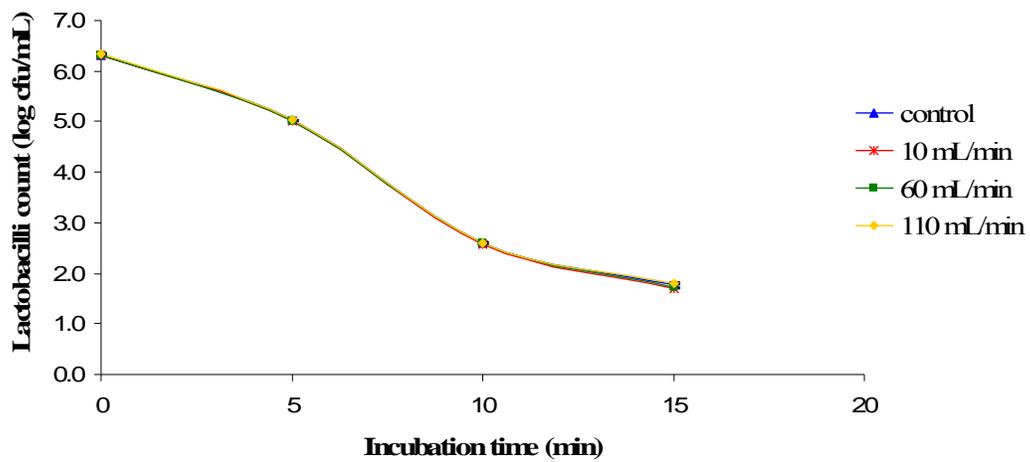
Figure 21. Flow rate influence on acid tolerance of LA-K

3.4.4 Protease Activity

The protease activity at different flow rates over the three time points of 0, 12, and 24 hours are shown in Figure 22. Flow rate * hour interaction effect was significant ($p = 0.0358$) (Table 14). At hour 12 the protease of the control was significantly higher than the protease activities of the three different flow rates. However, the protease activities of all different flow rates were not significantly different from each other at hour 12. At hour 24 the protease activity at the flow rate of 110 mL/min was significantly the highest compared to the control and 10 mL/min. Flow rate had no significant ($p = 0.2827$) influence on the protease activity (Table 14).

Figure 22. Flow rate effect on protease activity of LA-K

CHAPTER 4: CONCLUSIONS

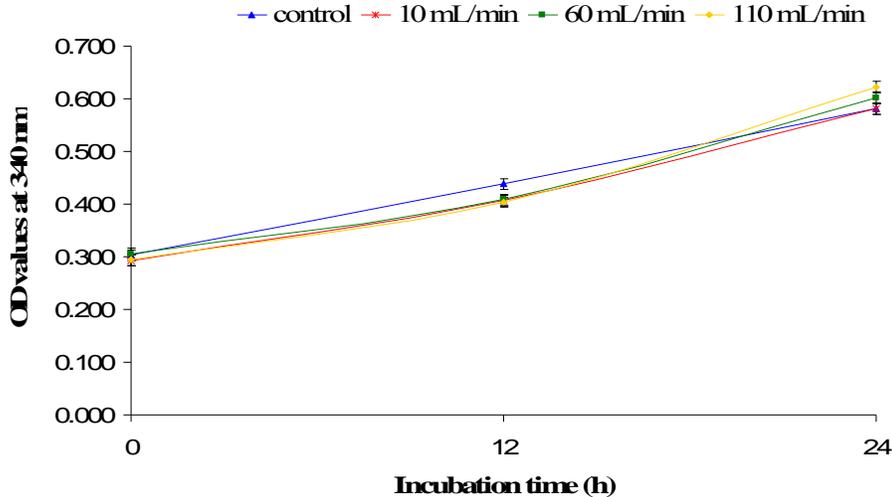Bipolar pulse width and pulse period significantly lowered acid tolerance and bile tolerance as well as slowed log stage growth of *Lactobacillus acidophilus* LA-K. Slower growth of adjunct bacteria can sometimes be good in manufacture of fermented dairy products as it results in controlled release of bacterial enzymes for improved flavor and texture development. Bipolar pulse width and pulse period did not influenced protease activity of *Lactobacillus acidophilus* LA-K.

Voltage significantly influenced acid tolerance, bile tolerance and growth of *Lactobacillus acidophilus* LA-K. Bile tolerance and growth of the control LA-K and LA-K subjected to 5 kV/cm were higher than bile tolerance and growth LA-K subjected to 15 and 25 kV/cm. Acid tolerance of the control LA-K was significantly higher than the acid tolerance of LA-K subjected at any of the three voltages studied. The highest the voltage applied the lowest the acid tolerance of LA-K. Voltage did not influenced protease activity of *Lactobacillus acidophilus* LA-K.

Flow rate significantly influenced bile tolerance and growth of *Lactobacillus acidophilus* LA-K. Bile tolerance and growth of LA-K subjected to 110 mL/min was significantly higher than LA-K of control and LA-K subjected to the other two voltages studied. Flow rate did not significantly influenced acid tolerance and protease activity of *Lactobacillus acidophilus* LA-K.

Pulsed electric field conditions studied modulated the characteristics of LA-K having an overall mixed influence on the beneficial characteristics of *Lactobacillus acidophilus* LA-K.

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