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Bile tolerance of *Lactobacillus acidophilus* LA-K as influenced by various pulsed electric field conditions

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ABSTRACT

***Lactobacillus acidophilus* has several health benefits and is used as an adjunct bacterium in the manufacture of cultured dairy foods. Bile tolerance is an important probiotic characteristic. Pulsed electric field (PEF) processing is non-thermal, hurdle technology which comprises of passing fluid foods between two electrodes and subjecting them to pulses of voltage for less than one second. Whether certain mild PEF conditions can enhance bile tolerance of *Lactobacillus acidophilus* is not known. The objective was to study the influence of certain PEF conditions on the, bile tolerance of *Lactobacillus acidophilus* LA-K. *Lactobacillus acidophilus* LA-K suspended in sterile peptone 0.1% w/v distilled water was treated with pulse widths of 3, 6 and 9 μs, pulse periods of 10,000; 20,000 and 30,000 μs and electric field strengths of 5, 15 and 25 kV/cm. The control did not receive any pulsed electric field condition. Bile tolerance was determined hourly for 16 hours. PROC GLM of the Statistical Analysis Systems (SAS) was used for data analysis. Significant differences were determined at $p < 0.05$. Three replications were conducted. Bipolar pulse width effect had a significant ($p < 0.0001$) influence on the bile tolerance. Bile tolerance of the control was significantly higher than the bile tolerance subjected to any of the bipolar pulse widths studied. There were no significant differences among the three different bipolar pulse widths. Pulse period had a significant ($p < 0.0001$) influence on the bile tolerance. 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 respectively. The bile tolerance subjected to 10,000 μs was significantly the lowest. Electric field strength had a significant ($p < 0.0001$) influence on the bile tolerance. Bile tolerance of the control and bile tolerance of *Lactobacillus acidophilus* LA-K subjected to 5 kV/cm were significantly the highest while the bile tolerance when subjected to 25 kV/cm was significantly the lowest.**

Keywords: Bile Tolerance; *Lactobacillus acidophilus*; Pulsed Electric Field; Probiotic

1. INTRODUCTION

Application of high electric field strengths (typically 20 - 80 kV/cm) for short time periods (less than 1 second) to fluid foods places between two electrodes is what high intensity pulsed electric field (PEF) processing is [1]. There are several PEF factors that influence microbial inactivation, they are electric field strength, pulse wave shape, treatment time and treatment temperature.

Electric field strength, is one of the main factors influencing microbial inactivation, and 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 [2]. The microbial inactivation increases with an increase in the electric field intensity, above the critical transmembrane potential [3]. 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 [4]. To achieve this transmembrane potential an electric field strength above 30 kV/cm is required for most bacteria in liquid systems [5].

Pulse Waves shape can be in the form of exponential decaying pulses, square wave pulses, oscillatory pulses or bipolar pulses of electric fields. In terms of pulse shape, square wave pulses are more energy and lethally efficient than exponential decaying pulses [6]. 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 [7]. In terms of pulse polarization, bipolar pulses are more lethal than monopolar

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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 [8].

Treatment time has been earlier defined as the product of the number of pulses and the pulse width [2]. 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.* [9] developed a mathematical model that relates microbial survival fraction with PEF treatment time. The inactivation of microorganisms increases with an increase in treatment time [10]. 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 chambers*pulse width [11].

Treatment Temperature is influenced by constant electric field strength. 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. Vega-Mercado *et al.* [12] reported that *E. coli* reduction was observed to increase from 1 to 6.5 log reduction cycles with a temperature change from 32˚C to 55˚C.

Several health benefits of *Lactobacillus acidophilus* have been reported earlier [13]. Any probiotic bacterium needs to survive the bile conditions in the gastrointestinal (GI) tract before establishing in the lower GI tract to confer the health benefits upon the host. It is not known if mild PEF conditions can enhance the bile tolerance of this health beneficial bacterium. The objective was to study the influence of pulsed width, pulse period and kV on the bile tolerance *Lactobacillus acidophilus* LA-K.

2. MATERIALS AND METHODS

2.1. Experimental Design

Freshly thawed 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˚C) was subjected to various PEF treatment conditions. The treatments were pulse widths of 3, 6, and 9 µs, pulse periods of 10,000, 20,000, and 30,000 µs, electric field strengths of 5, 10, and 15 kV/cm. Control was run through the PEF equipment at 60 mL/min without receiving any pulsed electric field treatment. Bile tolerances, were determined in the control and PEF treatment samples hourly over 16 hours of incubation. All experiments were repeated three times.

2.2. Bile Tolerance Test

Bile tolerance of *Lactobacillus acidophilus* LA-K was conducted as described earlier [14] 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 OD650 of 0.200 ± 0.005, and incubated under anaerobic conditions at 37˚C 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 OD650 readings using a standard curve (**Figure 1**).

2.3. Statistical Analysis

The General Linear Model (PROC GLM) of the Statistical Analysis Systems (SAS) was used to analyze the data. Differences of least square means were used to determine significant differences at $p < 0.05$ for main effects and interaction effects. Significant differences were determined at $\alpha = 0.05$.

3. RESULTS AND DISCUSSION

3.1. Pulse Width

The OD values at different bipolar pulse widths over the bile tolerance period of 16 hours are shown in **Figure 2**. Various treatments applied are shown in **Table 1**. conditions are in 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

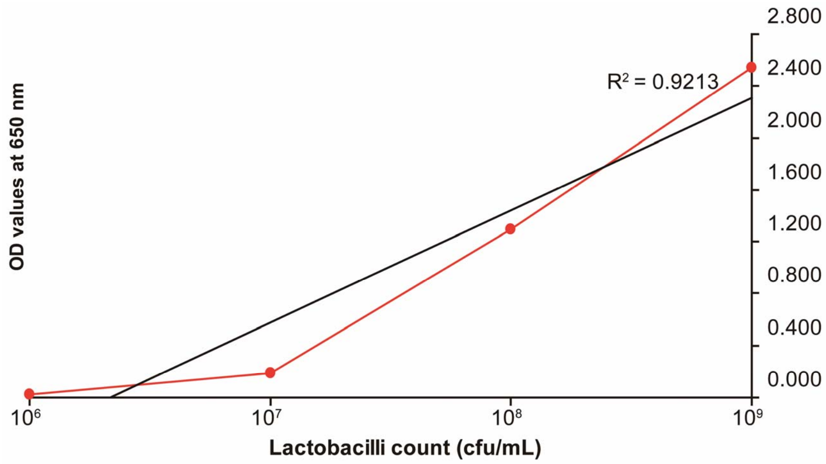

Figure 1. Standard curve for growth of LA-K in MRS-THIO broth with 0.3% oxgall.

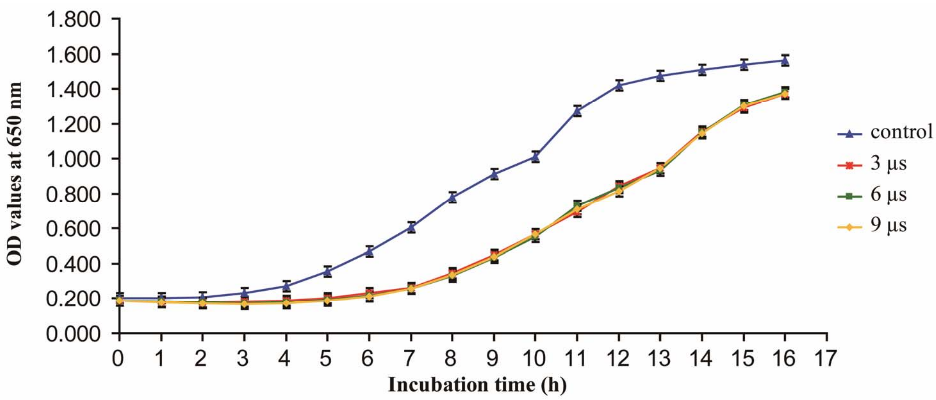

Figure 2. Pulse width effect on bile tolerance of *Lactobacillus acidophilus* LA-K, means ± SE.

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

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

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

Source	Bile tolerance	
	MS	Pr > F
Pulse width	1.018	<0.0001
Hour	2.445	<0.0001
Pulse width*hour	0.030	<0.0001
Error	0.003	

Table 3. Least square means for bile tolerance as influenced by pulse width.

Treatment	Bile tolerance
	LS Mean
Control	0.825^{A}
3 µs	0.546^{B}
6 µs	0.542^{B}
9 µs	0.540^{B}

tolerance of different strains of *Lactobacillus acidophilus* isolated from human intestinal were studied by Buck and Gilliand [15]. 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.

3.2. Pulse Period

The OD values at different pulse periods over the bile tolerance period of 16 hours are shown in **Figure 3**. Various treatments applied are shown in **Table 4**. Pulse period*hour interaction effect was significant ($p < 0.0001$) (**Table 5**). 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 5**). According to **Table 6** 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. Bile resistance of *Lactobacillus* was arbitrarily classified into four groups according to the delay of growth [10]. 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) [16].

3.3. Electric Field Strength

The OD values at different electric field strengths over the bile tolerance period of 16 hours are shown in **Figure 4**. Various treatments applied are shown in **Table 7**. Electric field strength*hour interaction effect was significant ($p < 0.0001$) (**Table 8**). 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 5 kV/cm during all 16 hours period. Electric field strength had a significant ($p < 0.0001$) influence on the bile tolerance (**Table 8**). 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 (**Table 9**). The bile tolerance subjected to 25 kV/cm was significantly the lowest.

Table 4. Pulsed electric field (PEF) treatment conditions applied during the study of the influence of various pulse periods on *Lactobacillus acidophilus* LA-K.

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

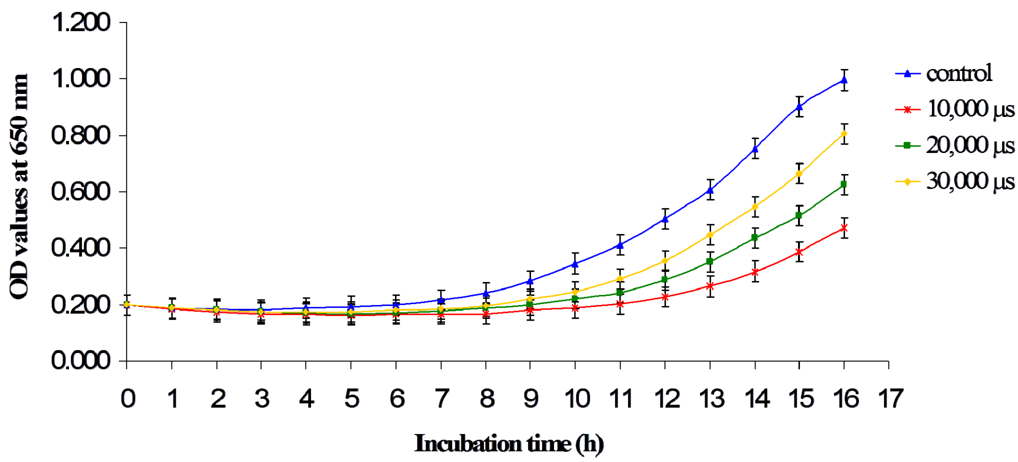

Figure 3. Pulse period effect on bile tolerance of *Lactobacillus acidophilus* LA-K, means ± SE.

Table 5. Mean square (MS) and Pr > F of pulse period, hour and their interaction for bile tolerance.

Source	Bile tolerance	
	MS	Pr > F
Pulse period	0.338	<0.0001
Hour	0.473	<0.0001
Pulse width*hour	0.026	<0.0001
Error	0.005	

Table 6. Least square means for bile tolerance as influenced by pulse period.

Treatment	Bile tolerance
	LS Mean
Control	0.338^{A}
10,000 µs	0.223^{D}
20,000 µs	0.264^{C}
30,000 µs	0.308^{B}

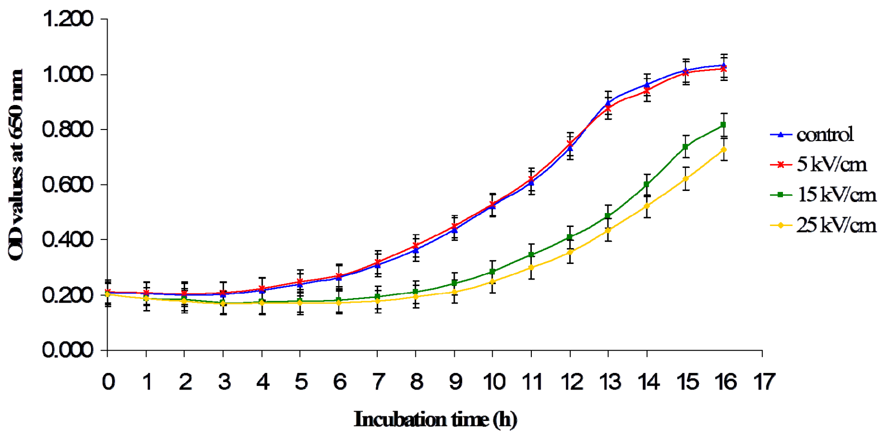

Figure 4. Electric field strength influence on bile tolerance of *Lactobacillus acidophilus* LA-K, means ± SE.

Table 7. Pulsed electric field (PEF) treatment conditions applied during the study of the influence of various electric field strengths on *Lactobacillus acidophilus* LA-K.

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

Table 8. Mean square (MS) and Pr > F of electric field strength, hour and their interaction for bile tolerance.

Source	Bile tolerance	
	MS	Pr > F
Electric field strength	0.582	<0.0001
Hour	0.746	<0.0001
Electric field strength*hour	0.021	<0.0001
Error	0.005	

Table 9. Least square means for bile tolerance as influenced by electric field strength.

Treatment	Bile tolerance
	LS Mean
Control	0.494^{A}
5 kV/cm	0.497^{A}
15 kV/cm	0.329^{B}
25 kV/cm	0.296^{C}

4. CONCLUSION

Bipolar pulse width and pulse period significantly lowered bile tolerance. Electric field strength significantly influenced bile tolerance. Bile tolerance of the control LA-K and LA-K subjected to 5 kV/cm were the highest while bile tolerance LA-K subjected to 25 kV/cm was the lowest.

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