The Influence of Mild Pulsed Electric Field (PEF) on Acid Tolerance, Bile Tolerance, Growth and Protease Activity of the Dairy Culture Bacteria *Lactobacillus acidophilus* LA-K.

**Najim H. Najim**
Department of Veterinary Public Health, College of Veterinary Medicine, Baghdad University, Iraq

**nnhh52@yahoo.com**

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**Summary**

The objective of this study was to determine the influence of mild PEF conditions on acid tolerance, growth, bile tolerance and protease activity of *L. acidophilus LA-K*. The treatments were positive square unipolar pulse width of 3 µs, pulse period of 0.5 second, voltage of 1 kV/cm, delay time of 20 µs and flow rate of 60 ml/min at 40.5°C PEF treatment temperature. The control was passed through the PEF system at the same flow rate (60 ml/min) without receiving any pulsed electric field condition. The acid tolerance was determined every 30 minutes for 120 minutes of incubation in acidified MRS broth at pH 2. Growth of the culture was determined hourly for 32 hours of incubation at 37°C in MRS broth. The bile tolerance was determined hourly for 16 hours of incubation in MRS-Thio broth supplemented with 0.3% (w/v) Oxgall (bovine bile) and 0.2% sodium thioglycolate. Samples were plated in duplicates using Lactobacilli MRS agar. The petriplates were incubated anaerobically at 37°C for 48 hours. Protease activity was determined by o-phthaldialdehyde (OPA) spectrophotometric assay at 0, 12, 24, 36 and 48 hours of incubation of inoculated skim milk at 40°C three replications were conducted. The experimental design was repeated measurements on complete randomized block, replications were the blocks. *Lactobacillus acidophilus* subjected to mild PEF conditions as well as the control were acid tolerant until the end of the 120 minutes of incubation but there was a significant (P<0.0001) decrease in the viable bacterial counts after each incubation time of 30 minutes. Mild PEF conditions studied significantly improved acid tolerance of the bacterium. The mild PEF treated culture reached the logarithmic phase of the growth an hour earlier than the control. *Lactobacillus acidophilus* LA-K exhibited tolerance to the bile conditions and exhibited similar growth patterns in the presence or absence of bile acids but the bacterium reached the stationary phase after 16 hours of incubation in bile conditions compared to 16 hours of incubation in the absence of bile conditions. Mild Pulsed Electric Field conditions had insignificant effect on bile tolerance of *L. acidophilus LA-K*. Mild Pulsed Electric Field treatment significantly (P<0.0001) enhanced the protease activity of the bacterium compared to the control.

**Keywords:** pulsed, electric field, *Lactobacillus acidophilus*, protease, bile, tolerance.

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**Introduction**

High-intensity pulsed electric field (HIPEF) has been proven to inactivate microorganisms and have the potential to replace thermal processing for liquid foods (1) with minimal impact on nutritional and organoleptic qualities (2). 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 (3). It is well known that most if not all microorganisms are practically unaffected by electric fields of less than about 4-8 kV/cm (4). It was reported that electrical treatments could affect cell physiology (5). Other researchers (6) have shown that the presence of a moderate electric field (MEF) (1 V/cm) had interesting effects on biological materials. In addition to that (7) 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 (1V/cm) at frequency of 60 Hz has been shown to alter the metabolic activity of the microbial cells (8). *L. acidophilus* is known to be a probiotic microorganism and used as a starter for dairy products. The presence of *L. acidophilus* can increase the desirable flavor, texture, and nutritional value. In addition, *L. acidophilus* possesses a number of beneficial properties like the production of probiotic metabolites, the acidification of milk, production of enzymes, and the production of healthy LAB (8). In order to preserve the probiotic and enzymatic activity of *L. acidophilus*, this study was conducted to determine the influence of mild PEF conditions on acid tolerance, growth, bile tolerance, and protease activity of *L. acidophilus* LA-K.
acidophilus is a probiotic bacterium widely used as an adjunct culture in approximately 80% of the yogurts in USA (9). Several studies reported yogurt cultures and Lactobacillus acidophilus as probiotics (10, 11, 12 and 13). To provide health benefits, probiotics must overcome physical and chemical barriers such as acid and bile in the gastrointestinal tract (14). Cellular stress begins in the stomach, which has a pH as low as 1.5-2 (15).

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 minutes (16). L. acidophilus is a probiotic bacterium with several health benefits, including: Enhancement of the immune system in the immuno compromised people (17), reduction of various types of diarrhea in human (18), replace various pathogenic organisms in the intestine such as S. aureus and E. coli leading to a healthy microbiological balance (19), reduce serum cholesterol levels in human (18), improve symptoms of lactose intolerance (20), balancing of intestinal microflora (18), reduce the incidence rate of colon cancer (20), has anti-hyperglycemic and anti-hypertensive effects (11), treatment of urogenital infections and vaginitis in women (21) and alleviation of Crohn’s disease (18).

Materials and Methods

The control and Pulsed Electric Field (PEF) treated samples were prepared by inoculating 10 ml of freshly thawed pure frozen concentrated culture of Lactobacillus acidophilus LA-K (Chr. Hansen’s Laboratory, WI, USA) into 990 ml of sterile 0.1% peptone water that make it 1% (v/v) and treated in a pilot plant PEF system (OSU-4M). The mild PEF treatment conditions were positive square unipolar pulse widths of 3 µs, pulse periods of 0.5 sec., voltage of 1kV/cm, the delay time of 20 µs, the flow rate of 60 ml/min with 40.5°C PEF treatment temperature. The control was passed through the PEF equipment at 60ml/min without receiving any pulsed electric field treatment. Prior to testing the PEF equipment was cleaned and sanitized with 5% Sodium hypochlorite solution and then rinsed thoroughly with sterile distilled water. The control and the PEF treated samples were tested for acid tolerance, growth, bile tolerance and protease activity. Three replications were conducted. The experimental design was repeated measurements on complete randomized block. Replications were the blocks. Data were analyzed using Proc Mixed model of Statistical Analysis System (SAS).

The acid tolerance of the Lactobacillus acidophilus LA-K was determined by the method proposed by Pereira and Gibson, (22) with slight modifications. The control and PEF treated samples were inoculated 10% (v/v) in acidified MRS broth previously adjusted to pH 2 using IN HCl. The inoculated acidified MRS broth were incubated at 37°C and plating for every 30 minutes up to 120 minutes. Growth was determined by the method proposed by Lin and Young, (23) with slight modifications. Control and PEF treated samples were inoculated 10% (v/v) separately into MRS broth. Growth was determined hourly for 32 hours of incubation at 37°C. The bile tolerance was determined according to method proposed by Pereira and Gibson, (22) with slight modifications. The bile tolerance of the culture was analyzed in MRS-THIO broth supplemented with 0.3% (w/v) Oxgall (bovine bile) and 0.2 % (w/v) sodium thioglycolate. Control and PEF treated samples were inoculated 10% (v/v) separately in MRS-THIO broth and incubated at 37°C for 16 hours. 1 ml of the inoculated broth was serially diluted in peptone water (0.1% w/v) and plated in duplicates using using Lactobacilli MRS agar. The petriplates were incubated anaerobically at 37°C for 48 hours befor enumeration. The protease activity of the culture was determined by o-phthalaldehyde (OPA) spectrophotometric method (24) with slight modification. The control and the PEF treated samples were inoculated 10% (v/v) separately into sterile skim milk and incubated at 40°C for 0, 12, 24, 36 and 48 hours.
Results and Discussion

The viability of the bacterium subjected to positive square unipolar pulse width of 3 µs for pulse period of 0.5 sec. using voltage of 1 kV/cm at 40.5°C PEF treatment temperature when incubated in acid condition (pH 2) over the five time points of 0, 30, 60, 90 and 120 minutes are shown in (Figure 1).

Culture subjected to mild PEF conditions as well as the control were acid tolerant until the end of the 120 minutes of incubation. The time effect was significant (table 1). There was a significant (P<0.0001) decrease in the viable counts after each incubation time of 30 minutes. The PEF treatment effect was also significant (P<0.0001) (Table 1).

Table 1: Mean square (MS) and Pr > F of mild pulsed electric field, minute and their interaction for acid tolerance of Lactobacillus acidophilus LA-K.

<table>
<thead>
<tr>
<th>Source</th>
<th>Acid tolerance</th>
<th>Acidity</th>
<th>MS</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsed Electric Field (PEF)</td>
<td>6.8190</td>
<td></td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Minute</td>
<td>16.8445</td>
<td></td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Pulsed Electric Field (PEF)* Minute</td>
<td>0.7131</td>
<td></td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>0.0778</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From minutes 30 to 120 the acid tolerance of Lactobacillus acidophilus LA-K subjected to mild PEF conditions was significantly (P<0.001) higher than the control (Table 2).

Lactobacillus acidophilus was more acid resistant at pH 1.2 and 2.5 for 30 minutes, compared to Bifidobacterium bifidum which did not tolerate the acid conditions after 5 minutes (25). On the other hand, else researchers (26) observed that Lactobacillus acidophilus exhibited more acid tolerance than L. casei. at pH 2. Mild electrical stimulation treatment that alters the synthesis of molecular chaperones may affect bacterial resistance to acid stress. 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.

The growth of the bacterium expressed as log cfu/ml that subjected to positive square unipolar pulse width of 3 µs for pulse period of 0.5 sec. and voltage of 1kV/cm at 40.5°C PEF treatment temperature over the growth periods of 32 hours is shown in figure,2. The mild PEF treatment had a significant (P<0.0001) influence on the growth of Lactobacillus acidophilus LA-K (table, 3). The mild PEF treated culture reached the logarithmic phase of the growth an hour earlier than the control.

Growth of Lactobacillus acidophilus LA-K when subjected to the mild PEF treatment was significantly higher than the control (table,4). The exponential phase of the growth curve of the control was between hours 4 and 16 while that of the bacterium subjected to mild PEF treatment was between hours 3 and 16 (Figure,3). The stationary phase of the growth curve of the bacterium subjected to mild PEF treatment was between hours 16 and 26 while that of the control was between hours 16 and 24.

Table 2: Least square means for acid tolerance of Lactobacillus acidophilus LA-K as influenced by mild pulsed electric field (PEF).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acid tolerance LS Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulsed Electric Field (PEF)</td>
<td>A 6.0039</td>
</tr>
<tr>
<td>Control</td>
<td>B 5.0503</td>
</tr>
</tbody>
</table>

LS Means with different letters are significantly different (P<0.05).
The mild PEF conditions significantly (P<0.05) enhanced the growth of *Lactobacillus acidophilus* LA-K. Liang and Shah (26) studied cholesterol removal ability of Lactobacilli strains and also observed growth of the bacterium *Lactobacillus acidophilus* to be predominant in the first 9-15 hours after which it reached a stationary phase. Electrical treatment could affect cell physiology (5). The application of moderate electric field (MEF) (1 V/cm) produced a shorter lag phase than conventional (control) fermentation of *Lactobacillus acidophilus* OSU at the same temperature (27). However, other studies under controlled temperature conditions have shown that MEF accelerate growth in the early stage, but inhibits growth at the late stage of fermentation of *Lactobacillus acidophilus* (7).

The bile tolerance expressed as log cfu/ml for the bacterium subjected to positive square unipolar pulse width of 3 µs for pulse period of 0.5 sec. and voltage of 1kV/cm at 40.5°C PEF treatment temperature over the bile tolerance periods of 16 hours is shown in Figure 3. The mild PEF treatment had non-significant (p=0.7458) effect on bile tolerance of the bacterium (table,3). *Lactobacillus acidophilus* LA-K exhibited good tolerance to the bile conditions with a significant (P<0.0001) increase in the viable bacterial counts (log cfu/ml) in both the control and the mild PEF treated cultures during the 16 hours of incubation in bile conditions (figure,3). The mild PEF treated cultures did not have significantly (P>0.05) different bile tolerance compared to the control (table,4). In the present study, we found that *Lactobacillus acidophilus* LA-K exhibited similar growth patterns in the presence or absence of bile acids (figures, 2 and 3) respectively, but the bacterium reached the stationary phase after 12 hours of incubation in bile conditions (figure,3) whereas, the bacterium reached the stationary phase after 16 hours of incubation in the absence of bile conditions (figure,2). Similar findings were reported by workers (26) studied the bile tolerance of different strains of Lactobacillus species and found that *Lactobacillus acidophilus* exhibited similar growth pattern in the presence or absence of bile acid (Oxgall). Shah and Jelen (28) attributed increased bile tolerance of *Lactobacillus acidophilus* to its rigid cell wall. Other researchers (29) strongly support the hypothesis that microbial bile salt hydrolase (BSH) function in the detoxification of bile salts and in doing so, increase the intestinal survival and persistence of producing strains in the hostile environment of the gastrointestinal tract.

The Optical Density (OD) (Absorbance) values of the protease activity of the bacterium subjected to the positive square unipolar pulse width of 3 µs for pulse period of 0.5 sec. and voltage of 1kV/cm at 40.5°C PEF treatment temperature over the five time points of 0, 12, 24, 36 and 48 hours are shown in figure,4. The treatment effect was significant (table,3). The protease activity of the bacterium subjected to mild PEF treatment were significantly (P<0.05) higher than the control (table,4). The hour effect was significant (table,3). A significant (P<0.0001) increase in the protease activities were found between12, 24, 36 and 48 hours of incubation at 40°C in the control samples. Also there were a significant (P<0.0001) increase in the protease activities of the mild PEF treated culture between 0, 12, 24, 36 and 48 hours of incubation at 40°C. Mild PEF treatment significantly (P<0.0001) enhanced the protease activity of Lactobacillus acidophilus LA-K (table,4). *Lactobacillus acidophilus* LA-K subjected to mild PEF conditions exhibited significantly (P<0.0001) the highest protease activity at 36 hours of incubation at 40°C compared to the control (figure,4). In general, PEF processing affects protease activity and an enhancement in the initial activity was detected (30). Researchers (31) suggested that the PEF could originate small conformational changes leading to enhanced proteolytic activity.
Table 3: Mean square (MS) and Pr > F of mild PEF treatment, hour, and their interaction for growth, bile tolerance and protease activity of Lactobacillus acidophilus LA-K.

<table>
<thead>
<tr>
<th>Source</th>
<th>Growth</th>
<th>Bile tolerance</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
<td>Pr &gt; F</td>
<td>MS</td>
</tr>
<tr>
<td>PEF</td>
<td>0.1558</td>
<td>&lt;0.0001</td>
<td>0.0019</td>
</tr>
<tr>
<td>Hour</td>
<td>13.6507</td>
<td>&lt;0.0001</td>
<td>10.2905</td>
</tr>
<tr>
<td>PEF * hour</td>
<td>0.0035</td>
<td>0.1679</td>
<td>0.0039</td>
</tr>
<tr>
<td>Error</td>
<td>0.00258</td>
<td>0.0184</td>
<td>0.00027</td>
</tr>
</tbody>
</table>

Table 4: Least square means for growth, bile tolerance and protease activity of Lactobacillus acidophilus LA-K as influenced by mild pulsed electric field.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth</th>
<th>Bile tolerance</th>
<th>Protease activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS Mean</td>
<td>LS Mean</td>
<td>LS Mean</td>
</tr>
<tr>
<td>PEF</td>
<td>10.4891 A</td>
<td>9.5109 A</td>
<td>0.7048 A</td>
</tr>
<tr>
<td>Control</td>
<td>10.4204 B</td>
<td>9.5013 A</td>
<td>0.6313 B</td>
</tr>
</tbody>
</table>

LS Means with the different litters are significantly different (P<0.05).

Figure, 3: Influence of mild pulsed electric field conditions on the bile tolerance of Lactobacillus acidophilus LA-K.
Figure 4: The influence of mild pulsed electric field conditions on the protease activity of *Lactobacillus acidophilus* LA-K.

References


تأثير المجال الكهربائي النابض المعتدل على تحمل كل من الحموضة والصفراء والنمو وفعالية إنزيم البروتيز لبكتريا Lactobacillus acidophilus LA-K

نجم هادي نجم
فرع الصحة العامة البيطرية - كلية الطب البيطري - جامعة بغداد - العراق

الخلاصة

استهدفت الدراسة تعني مدى تأثير المجال الكهربائي النابض المعتدل على قابلية تحمل بكتريا Lactobacillus acidophilus LA-K للحموضة والصفراء وكذلك على نموها وفعالية إنزيم البروتيز لها. تضمنت المعالجة تسليط مجال كهربائي نابض معتدل سعة 3 مايكروثانية من نوع احادي القطب الموجب ول_minute نبض 0.5 ثانية وصعدة 1 كيلوفولت/سم. وكانت سرعة جريان مرق الببتون للمملح بالبكتريا لكل من نماذج السيطرة والمتاحة معالجة المجال الكهربائي هي 60 مل/دقيقة. عند درجة حرارة 40.5° C. مرت نماذج السيطرة داخل منظومة المجال الكهربائي النابض وبدون توجيه أي حلم كهربائي عليها. 

حدد مدى تحمل هذه البكتريا للوسط الحامضي (pH 2) (pH 2) بزرعها كل 30 دقيقة، ولعدة 120 دقيقة من حضنها في مرق MRS الحامضي. عنيت نمو هذه البكتريا بعد كل ساعة ولعدة 32 ساعة من حضنها في مرق MRS عند درجة حرارة 37° C. حدد مدى تحمل هذه البكتريا لاملاح الصفراء بزرعها كل ساعة، ولعدة 12 ساعة من حضنها في مرق MRS - Thio broth الذي زود بكل من املاح الصفراء بنسبة 0.3% وثايوكلايكوليت الصوديوم بنسبة 0.2%. زرعت كافة النماذج بعد 48 ساعة. عنيت فعالية إنزيم البروتيز لهذه البكتريا بطريقة

phthaldialdehyde للتحليل الطيفي بعد مرور 24، 12، 0، 36 و48 ساعة من حضنها في الحليب الفرز عند درجة حرارة 40° C. كررت كافة المعاملات اعلاها لثلاث مرات لكل تجربة. حللت النتائج احصائياً باستخدام Proc mixed model of statistical analysis system (SAS).

اظهرت بكتريا Lactobacillus acidophilus LA-K في نماذج السيطرة نموها بعد كل ساعة ولعدة 32 ساعة من حضنها في مرق MRS - Thio broth. اظهرت هذه البكتريا قابليتها لتحمل أملاح الصفراء وعزز المجال الكهربائي النابض المعتدل وبصورة معنوية (P<0.0001) في زيادة فعالية إنزيم البروتيز للبكتريا. 

 الكلمات المفتاحية: النانض، المجال الكهربائي، بكتريا Lactobacillus acidophilus، الصفراء، تحمل.