The effect of mild Pulsed Electric Field (PEF) conditions on acid tolerance, bile tolerance, growth and protease activity of the *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

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Summary

Pulsed electric field (PEF) processing involves the application of pulses of voltage for less than one second to fluid products placed between two electrodes. So this study was conducted for the quantitative assessments of mild PEF conditions on acid tolerance, growth, bile tolerance and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. The control and PEF treated samples were prepared by inoculating 10 ml of freshly thawed culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 into 990 ml of sterile 0.1% (wt/v) peptone water and treated in a pilot plant PEF system (OSU-4M). 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. The control was passed through the PEF system (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 was determined hourly for 25 hours of incubation at 37°C in MRS broth. The bile tolerance was determined hourly for 16 hours of incubation in MRS-Thio broth . Samples were plated in duplicates using pH modified Lactobacilli MRS agar. The petriplates were incubated anaerobically at 43°C for 72 hours. Protease activity was determined by o-phthaldialdehyde (OPA) UV- spectrophotometric assay at 0, 12, 24, 36 and 48 hours of incubation of conducted. inoculated skim milk at 40°C. Three replications were The control. The stationary phase of the bacterium was between hours 10 and 18. Moreover significant 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 viability of the control *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 was lost after 30 minutes of incubation in acidified MRS broth (pH 2), whereas, the bacterium subjected to mild PEF treatment was acid tolerant until the end of 120 minutes of incubation. Mild PEF significantly improved acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12. and the growth reached the logarithmic phase an hour earlier than the decrease in bile tolerance was also recorded.. Mild PEF treatment significantly (P<0.0001) enhanced the protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 compared to the control.

Key words:Pulsed electricfield, acid tolerance, bile tolerance, protease,lactobacillus delbrueckii spp. Bulgaricus LB-12.

تأثير المجال الكهربائي النابض المعتدل على تحمل كل من الحموضة والصفراء وكذلك النمو وفعالية انزيم البروتييز لبكتريا *Lactobacillus delbrueckii ssp. bulgaricus* LB-12.

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الخلاصة

تتضمن عملية المجال الكهربائي النابض توجيه موجات كهربائية نابضة ، وبجهد كهربائي معين لمدة زمنية اقل من ثانية على المنتجات الغذائية السائلة اثناء مرورها بين قطبين . ان تأثير المجال الكهربائي النابض المعتدل على خصائص المعزز الحيوي لهذه البكتريا النافعة غير مفهوم بشكل جيد ؛ لذلك كان الهدف من هذه الدراسة تحديد مدى تأثير المجال الكهربائي النابض المعتدل في قابلية تحمل بكتريا ***Lactobacillus delbrueckii ssp. bulgaricus*** **LB-12.** لكل من الحموضة واملاح الصفراء وكذلك نموها وفعالية انزيم البروتييز لها . حضرت نماذج كل من السيطرة والاخرى المعالجة بالمجال الكهربائي النابض بزرع **10** مل من المستنبت الذائب لهذه البكتريا في **990** مل من(**0.1** %) مرق الببتون المعقم وامرارها داخل منظومة المجال الكهربائي النابض (**OSU- 4M**) . تضمنت المعالجة تسليط مجال كهربائي نابض معتدل بسعة **3** مايكرو ثانية (عرض النبضة) من نوع احادي القطب الموجب ، ولمدة نبضية **0.5** ثانية وبجهد كهربائي **1** كيلو فولت / سم ، وكانت سرعة جريان مرق الببتون الملقح بالبكتريا لكل من نماذج السيطرة والاخرى المعالجة بالمجال الكهربائي هي **60** مل / دقيقة عند درجة حرارة **40.5** مْ . مررت نماذج السيطرة داخل منظومة المجال الكهربائي النابض وبدون توجيه اي جهد كهربائي عليها . حدد مدى تحمل هذه البكتريا للوسط الحامضي (**pH 2**) بزرعها كل **30** دقيقة ، ولمدة **120** دقيقة من حضنها في مرق **MRS** الحامضي . عُيّن نمو هذه البكتريا بعد كل ساعة ولمدة **25** ساعة من حضنها في مرق **MRS** عند درجة حرارة **37** مْ . حدد مدى تحمل هذه البكتريا لاملاح الصفراء بزرعها كل ساعة ، ولمدة **16** 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 اعلاه باطباق مزدوجة لكل تخفيف وباستعمال ***Lactobacilli*** **MRS agar** المحوره وحضنت في جو لاهوائي عند درجة **43** مْ ولمدة **72** ساعة .عينت فعالية انزيم البروتييز لهذه البكتريا بطريقة **o - phthaldialdehyde** للتحليل الطيفي بعد مرور **0 , 12 ,24 , 36** و **48** ساعة من حضنها في الحليب الفرز عند درجة حرارة **40** مْ . كررت كافة المعاملات اعلاه لثلاث مرات لكل تجربة و حللت النتائج احصائيا باستخدام **Proc mixed model of statistical analysis system (SAS)**. فقدت هذه البكتريا فعاليتها للنمو في نماذج السيطرة بعد مرور **30** دقيقة من حضنها في مرق **MRS** الحامضي بينما لم تفقد النماذج التي تعرضت للمجال الكهربائي النابض المعتدل فعاليتها وحيويتها في الوسط نفسه حتى بعد مرور **120** دقيقة من حضنها في مرق **MRS** الحامضي . شارك المجال الكهربائي النابض المعتدل وبصورة معنوية في تحسين قابلية هذه البكتريا لتحمل الوسط الحامضي . وصلت البكتريا في النماذج التي عوملت بموجات المجال الكهربائي النابض المعتدل الى الطور اللوغارتمي بساعة مبكرة مقارنة بمثيلتها لنماذج السيطرة. ظهر الطور الثابت للنمو بعد مرور **10** ساعات واستمر الى **18** ساعة من حضنها في مرق **MRS** . انخفضت قدرة هذة البكتريا لتحمل املاح الصفراء وبصورة معنوية عندما تعرضت النماذج لموجات المجال الكهربائي النابض المعتدل. لقد عزز المجال الكهربائي النابض المعتدل في تحسين فعالية انزيم البروتييز لهذه البكتريا مقارنة بمثيلتها لنماذج السيطرة نستنتج من دراستنا هذه بان المجال الكهربائي النابض المعتدل قد اسهم وبصورة معنويه في تحسين نمو هذه البكتريا وفي قدرتها على تحمل الوسط الحامضي وتنشيط فعالية انزيم البروتييز لها .

Introduction

Lactobacillus delbrueckii ssp. *bulgaricus* is a representative lactic acid bacterium that is used as a starter culture extensively in the production of the most popular types of fermented milk (1). Chandan (2) explained that the consumption of yogurt was enhanced in recent years, mainly because of its nutritional value and the beneficial health effects of yogurt cultures. Survival of the gastrointestinal tract transit is one of the preconditions for microorganisms to develop any beneficial effect after consumption (3). It is demanded that probiotic bacteria should be able to survive the low pH values of the stomach for at least 90 minutes and to tolerate the bile salts in the duodenum (3 , 4). The yogurt cultures have been regarded as the lesser value as probiotics, due to their inadequate survival in in-vitro acid resistance studies. However, recent studies have shown that yogurt cultures are able to survive gastrointestinal passage in vivo (5, 6) this, in addition to accumulating evidence of positive health effects of these cultures . Piaia et al.,(7) have further highlighted the importance of viability and the probiotic potential of traditional yogurt cultures. the International Scientific Association for Probiotics and Prebiotics workshop consensus document has acknowledged the probiotic nature of yogurt cultures (8). Guarner et al., (9) concluded that the yogurt starter culture clearly fulfill the current concept of probiotics. Balansky et al., (10) reported that the acetone extracts of the milk after fermentation with *Lactobacillus bulgaricus* exhibited antimutagenic activity. immuno compromised *Streptococcus thermophilus* and *Lactobacillus bulgaricus* were reported to improve symptoms of lactose intolerance (11 , 12), reduce antibiotic-associated diarrhea, produce anti ulcer effects, prevent chronic gastritis, reduce the incidence rate of diseases like colorectal cancer and necrotizing entero colitis (9 ,13). Yogurt containing these two strains was used in the management of acute diarrheal disorders as recommended by the World Health Organization (14) also enhanced the immune system in the people (9 , 15). Ingestion of *Lactobacillus bulgaricus* with *Lactobacillus acidophilus* has been reported to be beneficial in diseases such as hyperglycemia and hypertension (16). The application of moderate electric field (MEF) (1 V/cm) at frequency of 60 Hz have been shown to alter the metabolic activity and some of the growth kinetics of *Lactobacillus acidophilus* (17). The effect of mild pulsed electric fields on the probiotic characteristics of the beneficial bacteria *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 is not well understood. The objective of this study was to determine the effect of mild PEF conditions on acid tolerance, growth, bile tolerance and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12.

Materials and Methods

Any pulsed The control and PEF treated samples were prepared by inoculating 10 ml of freshly thawed pure frozen concentrated culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 (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 40.5^0C PEF treatment temperature. The control was the sample passed through the PEF equipment at 60ml/min without receiving electric field treatment . 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 bile tolerance model of Statistical Analysis System (SAS).

The acid tolerance of the culture was determined by the method proposed by Pereira and Gibson, (18) with slight modifications. The control and PEF treated samples were inoculated 10% (v/v) in acidified MRS broth previously adjusted to pH 2 using 1N HCl. The inoculated acidified MRS broth were incubated at 43°C and plating for every 30 minutes up to 120 minutes. Growth was determined by the method proposed by Lin and Young, (19) with slight modifications. Control and PEF treated samples were inoculated 10% (v/v) separately into MRS broth. Growth was determined hourly for 25 hours of incubation at 43°C. The was determined according to method proposed by Pereira and Gibson, (18) with slight modifications. The bile tolerance of the culture was analyzed in MRS-THIO broth supplemented with 0.3% (wt/v) Oxgall (bovine bile) and 0.2 % (wt/v) sodium thioglycolate. Control and PEF treated samples were inoculated 10% (v/v) separately in MRS-THIO broth and incubated at 43°C for 16 hours. 1 ml of the inoculated broth was serially diluted in peptone water (0.1% wt/v) and plated in duplicates using pH modified Lactobacilli MRS agar. The petriplates were incubated anaerobically at 43°C for 72 hours befor enumeration. The protease activity of the culture was determined by o-phthaldialdehyde (OPA) UV-spectrophotometric method proposed by Oberg et al., (20) 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. The viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 in the control

samples was lost after 30 minutes of incubation in acidified MRS broth at pH 2. The mild PEF treatment had a significant (P<0.0001) effect on acid tolerance (Table 1). The viable counts of the bacterium subjected to mild PEF conditions were significantly (P<0.0001) higher at every 30 minutes of incubation when compared to the control and this trend was observed throughout 120 minutes of incubation. The mild PEF treatment significantly (P<0.0001) increased the acid tolerance of the bacterium compared to the control (Table 2). 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.

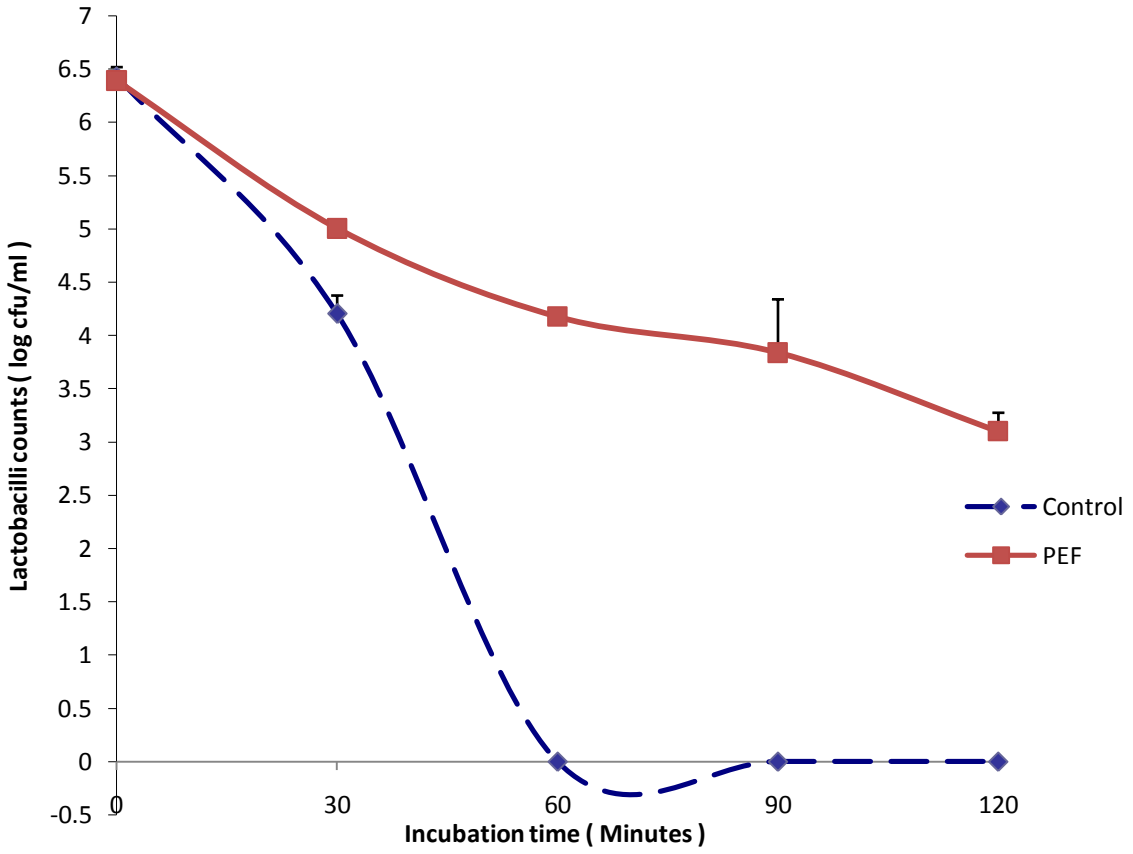

Figure 1 : Influence of mild pulsed electric field (PEF) conditions on the acid tolerance of *Lactobacillus delbrueckii ssp bulgaricus LB-12.*

Table 1: Mean square (MS) and Pr > F of mild pulsed electric field (PEF), minute and their interaction for acid tolerance of Lactobacillus delbrueckii spp. bulgaricus LB-12.

Source	Acid tolerance	
	MS	Pr > F
Pulsed Electric Field (PEF)	42.2933	< 0.0001
Minute	26.6712	< 0.0001
Pulsed Electric Field (PEF)* Minute	5. The growth of the bacterium subjected to positive square unipolar pulse width of 3 µs for pulse period of 0.5 sec. and voltage of 1 kV/cm at 40.5°C PEF treatment temperature over the growth period of 25 hours is shown in 3245 < 0.0001	
Error	0.0329	

Table 2: Least square means for acid tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 as influenced by mild pulsed electric field (PEF).

Treatment	Acid tolerance LS Mean
Pulsed Electric Field (PEF)	4.49971 A
Control	2.12503 B

LS Means with same letter are not significantly different (*P* > 0.05)

Hour 25 the mild Figure 2. The mild PEF treatment had a significant (*P*<0.0001) influence on the growth of the bacterium (Table 3). The mild PEF treated cultures reached the logarithmic phase an hour earlier than the control. The control and the mild PEF treatment studied were significantly different from each other (Table 4). In exponential phase of the growth (from hours 3-10 for PEF treated culture and from 4-10 hours for the control) the viable bacterial counts of the bacterium subjected to mild PEF conditions were significantly (*P*<0.001) higher than those of the control at hours 4 and 5. During the stationary phase of the growth (from hours 10 to 18) the PEF treated culture had no significant (*P*>0.05) difference in the viable bacterial counts compared to the control. At PEF treated culture had significantly (*P*<0.0001) higher viable bacterial counts than the control. Mild PEF treatment significantly increased the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 compared to the control. Simova, et al., (21) analyzed the growth profile of *Lactobacillus bulgaricus* HP1 inoculated in autoclaved reconstituted skim milk and reported that the growth reached exponential phase in the first 5 hours and reached stationary phase in 8-12 hours.

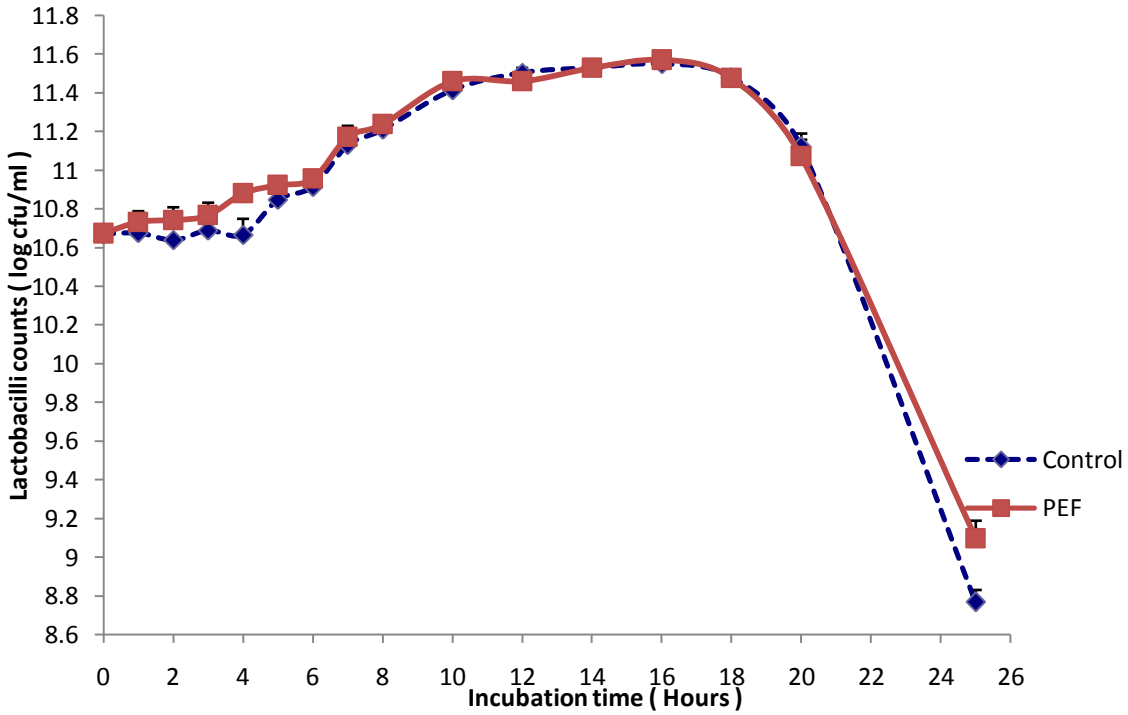

Figure 2 : The influence of mild pulsed electric field (PEF) conditions on the growth characteristics of *Lactobacillus delbrueckii ssp bulgaricus LB-12*.

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 delbrueckii* ssp. *bulgaricus* LB-12.

Source	Growth		Bile tolerance		Protease activity	
	MS	Pr > F	MS	Pr > F	MS	Pr > F
PEF	0.1515	<0.0001	8.9489	<0.0001	0.0459	<0.0001
Hour	6.8889	<0.0001	3.1581	<0.0001	0.4213	<0.0001
PEF * hour	0.0206	<0.0001	0.0879	<0.0001	0.0027	<0.0001
Error	0.0049		0.0133		0.00020	

Table 4: Least square means for growth, bile tolerance and protease activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 as influenced by mild pulsed electric field.

Treatment	Growth	Bile tolerance	Protease activity
	LS Mean	LS Mean	LS Mean
PEF	10.7816 ^{A}	7.9756 ^{B}	0.4357 ^{A}
Control	10.7046 ^{B}	8.6531 ^{A}	0.3574 ^{B}

LS Means with the same letter are not significantly different ($P > 0.05$)

The bile tolerance expressed as log cfu/ml for the *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 subjected to positive square unipolar pulse width of 3 µs for pulse period of 0.5 sec. and voltage of 1 kV/cm at 40.5°C PEF treatment temperature over the bile tolerance period of 16 hours is shown in Figure 3. From hours 1 to 16 the bile tolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 subjected to mild PEF treatment was significantly (P<0.0001) lower than the control (Figure 3). The mild PEF treatment effect was significant (Table 3). The mild PEF treatment significantly (P<0.0001) decreased bile tolerance of the bacterium (Table 4). The hour effect was significant (Table 3). There were significant (P<0.0001) reduction in the Lactobacilli counts (log cfu/ml) in both the control and the PEF treated cultures over the bile tolerance periods of 16 hours (Figure 3). Recent studies have shown that *Lactobacillus bulgaricus* strains are able to survive gastrointestinal passage in vivo (22).

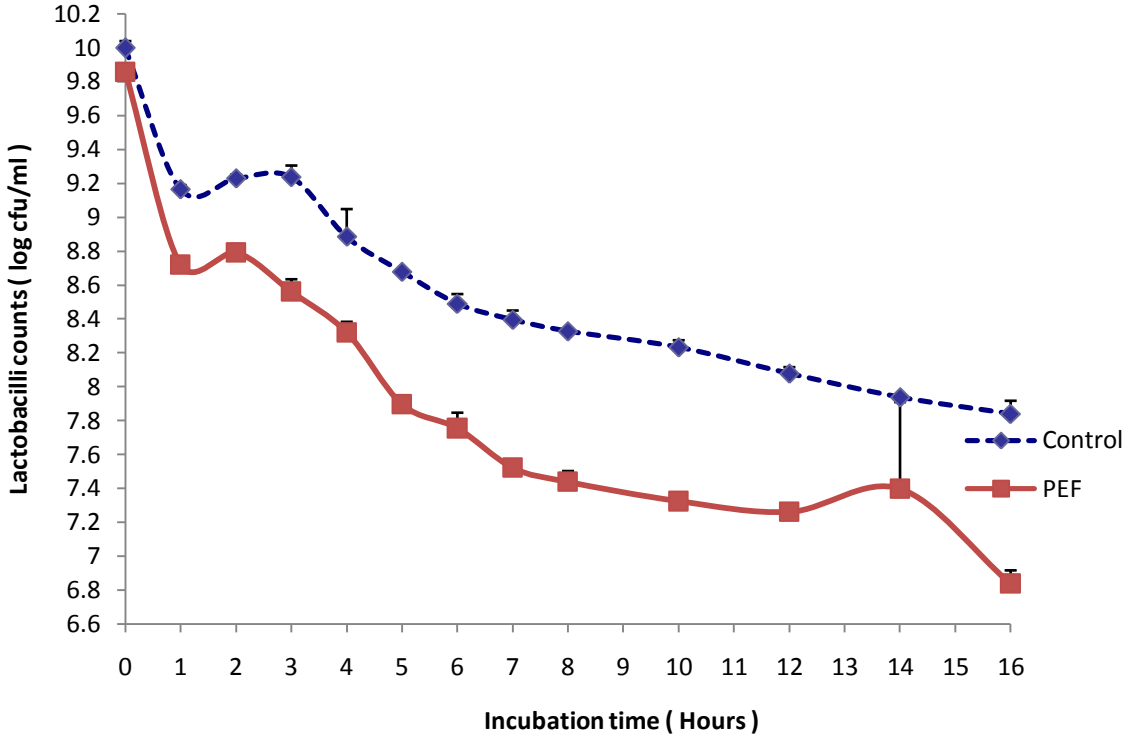

Figure 3 : Influence of mild pulsed electric field (PEF) conditions on the bile tolerance of *Lactobacillus delbrueckii ssp bulgaricus LB-12.*

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 1 kV/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. At hours 0, 12, 24, 36 and 48, the protease activities of the bacterium subjected to mild PEF treatment were significantly (P<0.0001) higher compared to the control. The hour effect was significant (Table 3). There were a significant (P<0.0001) differences in the protease activity of the control between 0, 12 and 24 hours, whereas, that subjected to mild PEF treatment between 0, 12, 24, 36

and 48 hours of incubation at 40°C. The mild PEF treatment effect was significant (Table 3). Mild PEF treatment significantly (P<0.0001) improved the protease activity of the *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 (Table 4). *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 in both the control and the mild PEF treated cultures exhibited significantly (P<0.0001) the highest proteolytic activity at 24 hours of incubation compared to 36 and 48 hours of incubation at 40°C. In general, enzymes require more severe HIPEF treatment than microorganisms to obtain significant inactivation (23). Bendicho, et al., (24) studied PEF treatment on *Bacillus subtilis* protease and reported that an enhancement in proteolytic activity was found when the PEF treatment was carried out in milk.

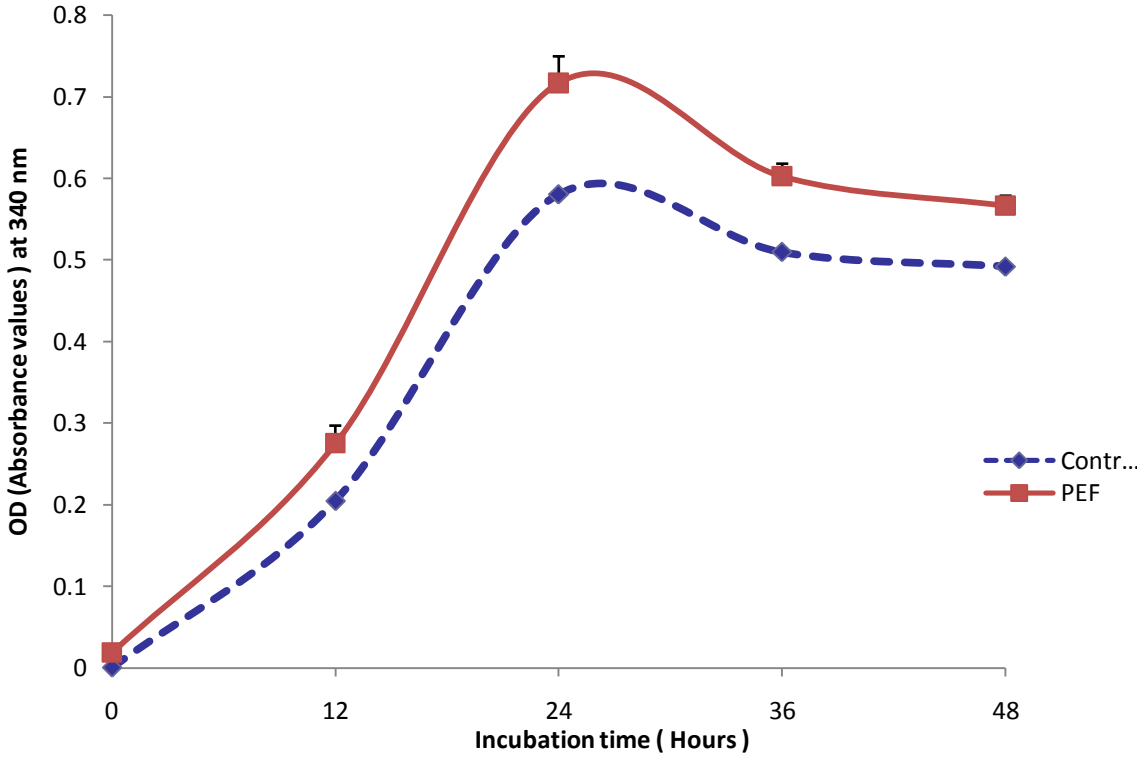

Figure 4: The influence of mild pulsed electric field (PEF) conditions on the protease activity of *Lactobacillus delbrueckii ssp bulgaricus LB-12.*

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