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Using pulse electric fields (PEF) for selective inactivation of coliform bacteria

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Coliform bacteria usually originate from the intestine, and the contamination typically occurs when there is a lack of sterility in food industry. PEF-induced selective nonthermal pasteurisation method might pasteurise coliform bacteria while leaving the needed bacteria intact. To evaluate this hypothesis, we chose *Escherichia coli dh5α* (*E. coli*) strain as a representation of coliform bacteria for this study. We also used *Streptococcus thermophilus* bacteria strain as a representation of lactobacteria used in milk by-products. The obtained results of PEF application showed that selective death of bacteria after PEF treatment can be induced. PEF was applied on bacteria. Then the clonogenic assay, the metabolic activity, and bacterial growth in the bioreactor were evaluated. By applying PEF treatment on *E. coli* and *S. thermophilus* their survival was monitored. We found the PEF parameters under which coliform bacteria *E. coli* were killed more than 100 times effectively than *S. thermophilus*. We postulate that it is the result of the bacteria size. Here we show that a PEF-induced selective nonthermal pasteurisation method could be applied in the industry where coliform bacteria can be eliminated while leaving other needed bacteria intact. We showed this by measuring the metabolic activity, the ability to form colonies, and the change in bacteria growth rate in the bioreactor.

Keywords: electroporation, selective bacteria inactivation, pulse electric fields, coliform bacteria, bacteria inactivation

INTRODUCTION

In food preservation research, thermal pasteurisation techniques are mostly used to inactivate bacteria in a food product (Gokoglu, 2019). However, such pasteurisation is responsible for the denaturation of protein-based or other temperature-sensitive molecules; hence, a considerable loss of food quality is observed (Andreou et al., 2017; Lewis, 2003). An alternative to this is nonthermal food pasteurisation (Barba et al., 2015; Chen et al., 2010; Saldaña et al., 2014). In the field of milk nonthermal apsteurisation, two alternatives exist: milk filtration and the pulsed electric field (PEF)-based treatment. Filtration is more frequently used. However, frequent clogging of the filter leads to a significantly increased cost. Another alternative is PEF-based treatment (Barba et al., 2015; Šalaševičius et al., 2021). The mechanism of such a method is based

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on the phenomenon of electroporation. Electroporation occurs when the transmembrane potential on the membrane of the affected bacteria is increased to the threshold level and the induction of pore formation is triggered (Weaver, Chizmadzhev, 1996). If the induced number of electropores is too great for the membrane to reseal, then the lysis of the bacteria starts (de La Rosa, Kaler, 2006). According to the modelling data, the temperature of the treated solution is negligibly increased (Saldaña et al., 2014). Therefore, this can be an ideal pasteurisation method for liquid food products such as milk or its by-products (Bendicho et al., 2002; Zulueta et al., 2007).

Another possibility that the application of PEF can offer is selective pasteurisation (Wouters, PPM Smelt, 1997). The influence of the size and form of bacteria on the increased transmembrane potential is a key factor for PEF efficiency (Agarwal et al., 2007). Therefore, different parameters of bacteria can alter electroporation efficiency dependent on applied electric field duration, strength, and the number of applied pulses (Raso et al., 2016). Optimising such a technology for PEF-triggered selective pasteurisation may lead to the death of only coliform bacteria (which are bigger in size), thus leaving other bacteria intact. That would revolutionise the pasteurisation technology since fresh material could be disinfected from a specific bacterium in raw solutions for food production. For example, such a technology may help control *Listeria innocua* that inflict significant material and profit losses in the dairy industry (Nowosad et al., 2021). Another type of undesired bacteria that can be found in raw solutions for food preparation is coliform bacteria (Niyoyitungiye et al., 2020). These bacteria usually originate from the intestine, and contamination typically occurs when there is a lack of sterility in milk production facilities, logistics, or even milk processing factories (Martin et al., 2016). One of the most common coliform bacteria is *Escherichia coli*. Therefore, we chose *Escherichia coli dh5α* (*E. coli*) strain as a representation of coliform bacteria for this study. We also used *S. thermophilus* bacteria strain as a representation of lactobacteria used in milk by-products, e.g., yoghurt. The obtained results of PEF application showed that selective death of bacteria after PEF treatment can be induced.

MATERIALS AND METHODS

Bacterial culture

Bacterial isolates of *E. coli DH5α* (Thermo Fisher Scientific, Waltham, MA, USA) and *S. thermophilus* were used for experiments. *E. coli* was grown in Luria broth (LB) medium in a shaker/incubator (Biosan ES-20, Latvia) at 37°C at 220 rpm. In the case of *S. thermophilus* the MRS growth media for lactobacteria was used. *S. thermophilus* was also grown in a shaker/incubator (Biosan ES-20, Latvia), but at different parameters: 41°C at 110 rpm.

Preparation of bacterial culture for experiments

Before the experiment, the overnight bacterial culture was diluted to the concentration of 0.05 OD. The optical density of all experiments was measured with a photometer (Den-600, Biosan, Latvia). The *E. coli* or *S. thermophilus* was grown up to 0.5 OD. Then the bacteria were centrifuged (Velocity Minifuge 13 μ, Dynamica Scientific Ltd., Livingston, UK) at 5000 rpm for 15 min and suspended in the electroporation medium in a concentration to reach 1 OD.

Equipment for PEF treatment and electroporation media

The PEF treatment was performed with an electrical pulse generator (BTX T 820, Holliston, MA, USA). The amplitude of the electric pulse was between 1000 and 2600 V, the duration between 10 and 60 μs, the number of pulses 1-99. The electric field strength was calculated by equation (1):

$$E = U/d, \quad (1)$$

where E is the electric field strength (V/cm), U is the voltage (V), and d is the distance between the electrodes (cm). The applied electric pulse

parameters were measured with a digital oscilloscope (Rigol DS2072A, Rigol Technologies Inc., Bedford, OH, USA) and a commercially available treatment chamber (BTX). Electroporation was done in cuvettes with 1 mm gap (Thermo Fisher Molecular BioProducts, USA).

The electroporation medium was composed of sucrose (242 mM), Na_2HPO_4 (5.5 mM), NaH_2PO_4 (3 mM), and $MgCl_2$ (1.7 mM). The measured specific conductivity of the electroporation medium was 0.1 S/m at 25°C. The pH was 7.1.

PEF treatment

The amount of 125 µl bacteria suspension was placed in the electroporation cuvette, and the electric fields were applied. After the treatment, the affected bacteria were incubated for 10 min. Afterwards, the bacteria were diluted in their growth media and plated on the agarose MRS or LB media in a Petri dish for a colony-forming assay or placed in the 96 well plates with growth media for *E. coli* or *S. thermophilus* bacteria. When the cell growth after electroporation was measured in a bioreactor (Biosan RTS-1C, Latvia), then the electroporated bacteria suspension was placed into 15 ml of appropriate growth media in a 50 ml tube with cap filters.

Bacteria survival measurements after electroporation

Bacteria viability measurements were done by using a colony-forming assay. Then, after electroporation, bacteria (*E. coli* and *S. thermophilus*) were diluted appropriately (from 1×10^2 to 1×10^6 times) to differentiate colonies on a Petri dish with growth media with agar. In the case of *E. coli*, the colonies were measured after 24 h. If the *S. thermophilus* bacteria was electroporated, then the measurements were done after 48 hours since the growth of *S. thermophilus* is slower. The images of the plates were taken with the laboratory imaging device (Vytautas Magnus University, Lithuania). Then the number of colonies was evaluated with open source imaging software ImageJ.

Also, bacteria metabolic activity was measured at the time of 24 hours after the application of the electric field. For such measurement, both *E. coli* and *S. thermophilus* bacteria were put in 96 well plates supplemented with 100 µl of appropriate growth media 10 min after applying the electric field. After 24 h, bacteria were supplemented with 20 µl of Alamar blue reagent and incubated at 37°C (*E. coli*) or 41°C (*S. thermophilus*). Afterwards, the fluorescence was measured with a GeniosPro (TECAN, Switzerland) fluorimeter. The absorption filter used was 535 nm, the emission filter was 590 nm.

The growth rate after electroporation was measured with a bioreactor (Biosan RTS-1C, Latvia). The electroporated bacteria suspension was placed into 15 ml of appropriate growth media in a 50 ml tube with cap filters. The shaking in the bioreactor for *E. coli* was 220 rpm, temperature 37°C, and the frequency of measurement was every 15 min. The shaking in the bioreactor for *S. thermophilus* was 100 rpm, temperature 41°C, and the frequency of OD measurement was also every 15 min.

RESULTS

For the proof of the PEF technique as a selective nonthermal pasteurisation method, two types of bacteria were chosen: *E. coli* and *S. thermophilus*. As previously stated, *E. coli* represent a coliform bacterium that negatively affects raw material. The other bacteria, *S. thermophilus*, which is non-toxic to humans, is naturally found in raw milk and is a key component for the production of milk by-products. In the industry, sometimes there is a need to disinfect raw material from coliform bacteria whilst keeping the naturally occurring bacteria (like probiotics) intact. In this case, selective nonthermal pasteurisation is a key component.

First we tested PEF-treatment efficiency depending on both cell lines and applied electric field intensity. The results are presented in Fig. 1, part A. The intensity range was chosen from 10 kV/cm to 26 kV/cm. According to the equation of electroporation models by Schwan, 10 kV/cm should start the electroporation process. The highest value was measured empirically since higher amplitudes were starting to

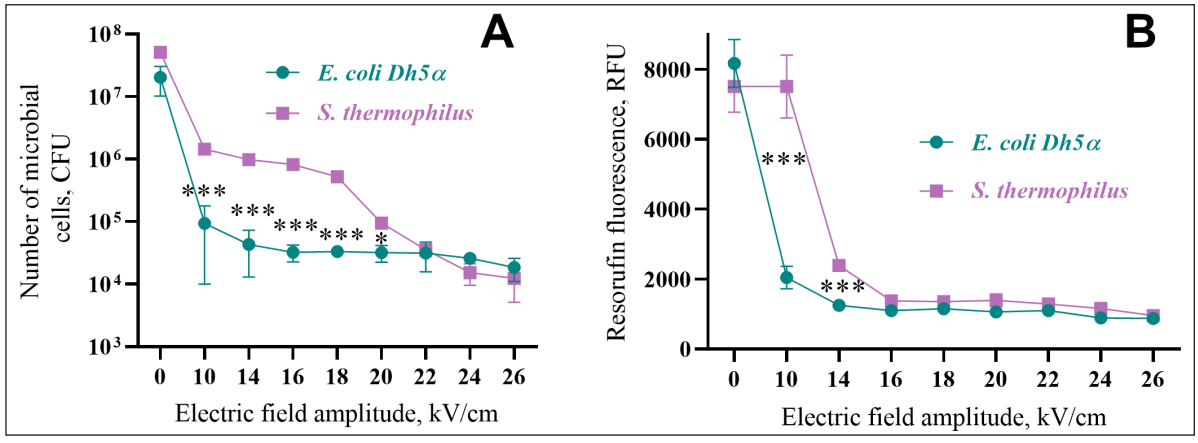

Fig. 1. The dependence of *E. coli* and *S. thermophilus* colony formation (A) and metabolic activity (B) on the applied strength of the electric field. Whiskers represent standard deviation (SD). Significance was measured by using a T-test. *** represents 0.001 significance

induce electric discharge. As this is a different and less controllable phenomenon, we decided to avoid it by choosing the maximal amplitude value of 26 kV/cm. Immediately, we observed the differences in viability after PEF treatment. Highly significant ($p < 0.001$) changes between cell lines were found from the applied electric field amplitude from 10 to 18 kV/cm. In this range, *S. thermophilus* viability was more than ten times higher than *E. coli*. Also, some significance ($p < 0.05$) was found at 20 kV/cm, when the *E. coli* had two times less viability.

Cell metabolic activity was tested at the same PEF parameters. The results show the significance ($p < 0.001$) only in the range between 10 and 14 kV/cm. In particular, the high difference between the bacteria lines at 10 kV/cm was more than four times.

After finding the range of amplitude that can initiate selective pasteurisation by using PEF, we decided to find the appropriate number of pulses needed for the selective PEF treatment. Due to the limitation of our device, we could only perform 99 pulses. Nevertheless, it was sufficient to find the range of pulses needed for selective pasteurisation. Surprisingly, highly significant ($p < 0.001$) differences between the PEF effect of the capability the treated bacteria lines to form colonies were found (Fig. 2 A). In the range between one and

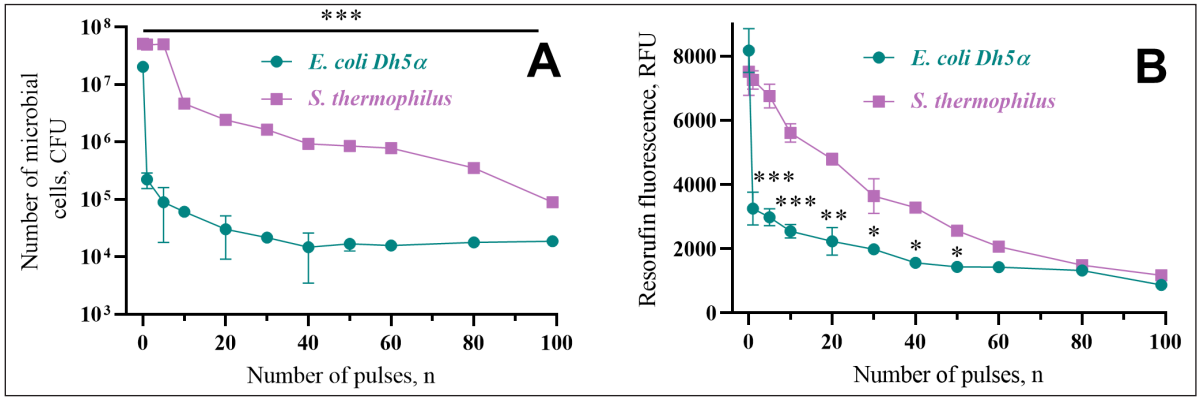

Fig. 2. The dependence of *E. coli* and *S. thermophilus* colony formation (A) and metabolic activity (B) on a number of applied electric field pulses. Whiskers represent standard deviation (SD). Significance was measured by using a T-test. *, **, *** represent significance $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

60 pulses, the difference in surviving bacteria was at around 1000 times when comparing both bacterial strains. However, when using a number of pulses between 80 and 99, the differences were considerably less. Similar ranges for selective pasteurisation were found when measuring the metabolic activity of the bacteria after PEF treatment (Fig. 2 B). The significant pulse range was between 1 and 50 pulses. However, the differences in metabolic activity of the measured bacterial lines diminished as the number of pulses increased from approximately 3.2 times (one pulse) to only 1.5 times (50 pulses).

After finding the ranges for selective pasteurisation in amplitude and number of pulses, it was also decided to investigate a similar range at the pulse wavelength applied. The pulse wavelength was between 1 and 60 µs. The minimum range was set because of the limitation of our electric field generator. The highest 60 µs pulse duration was chosen since higher pulse durations induced higher temperatures that resulted in electric discharges.

Interestingly, the reactions of both bacterial lines were similar when measuring with the colony-formation assay (Fig. 3 A). However, significance was still present when using 1 to 10 µs. The change in the viability of both bacterial lines was practically identical when a higher pulse duration was applied.

Nevertheless, a different view could be observed when investigating the same PEF parameters on bacteria metabolic activity (Fig. 3 B). There, high significance ($p < 0.001$) was found between between bacteria used when applying pulse duration in the range of 1 to 20 µs, but higher value differences were observed between 1 and 10 µs, when the values differed around three times.

After investigating the selective PEF non-thermal pasteurisation, we additionally evaluated the growth of *E. coli* (Fig. 4 A) and *S. thermophilus* (Fig. 4 B) in the bioreactor after the

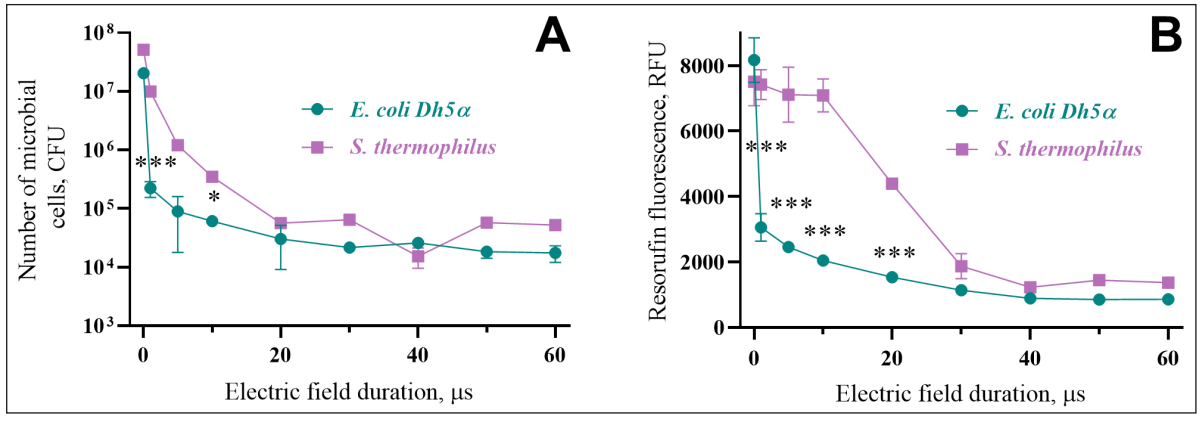

Fig. 3. The dependence of *E. coli* and *S. thermophilus* colony formation (A) and metabolic activity (B) on the strength of the electric field applied. Whiskers represent standard deviation (SD). Significance was measured by using a T-test. *, **, *** represent significance $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively

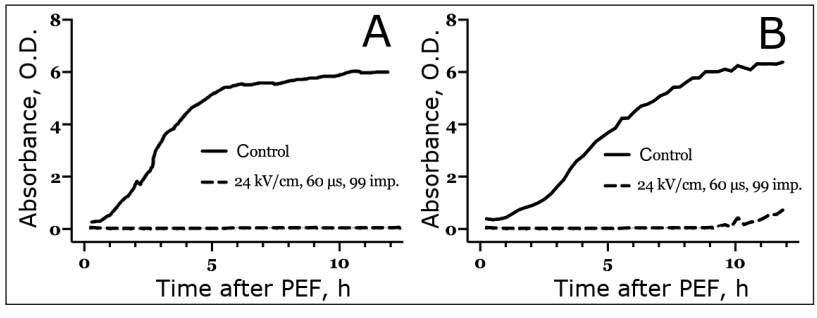

Fig. 4. The change in the cell number in the bioreactor when measuring optical density of bacterial suspension. Straight line marks control and dashed line after application of PEF of *E. coli* (A) and *S. thermophilus* (B)

application of the electric field and compared them to the control. Such an experiment is highly uncommon in PEF treatment, since one usually prevents the bacterial growth but not initiates the growth of what is left after bacteria pasteurisation triggered by PEF: we did not find any publications about performing measurements of bacterial growth in the bioreactor after experiments of PEF application. The most common experiment measurements in the field of PEF application on bacteria is colony-formation and metabolic activity assays.

In Figs 4 A and B, we intentionally used the highest parameters that the electric pulse generators used could apply to investigate the time of the manifestation of the signs of bacterial growth. For that, we chose a 12-hour measurement window. To our understanding, this could be enough to find the signs of bacterial recovery since the PEF-treated bacteria suspensions were grown/incubated in perfect growth conditions for the specific bacterial lines.

As seen in Fig. 4 B, the absorbance, i.e., the quantity of *S. thermophilus* bacteria, started to rise after 9.5 h after PEF treatment. A different observation is obtained in Fig. 4 A, where PEF-treated *E. coli* did not show any change in absorbance, i.e., the number of bacteria did not increase.

DISCUSSION

The results presented here show the possibility of using PEF as a selective nonthermal pasteurisation method (Nowosad et al., 2021). Two bacteria lines were chosen for these experiments: *E. coli* was chosen to represent coliform bacteria, and *S. thermophilus* bacteria was chosen to represent the bacteria line naturally present in the raw material and used for producing milk by-products.

The results show a clear distinction between the chosen bacterial lines after the application of PEF. Compared to *S. thermophilus* bacteria, *E. coli* loses viability significantly faster. That can be explained through the parameters of both bacteria lines. *E. coli* is a bacillus-shaped bacteria of approximately 2–6 μm in length and 1.1–1.5 μm wide, with round ends. In comparison, *S. thermophilus* bacteria is coccus-shaped with a diameter of 0.7 to 0.9 μm. These sizes will impact PEF efficiency since it is a key factor for the induction of transmembrane potential due to the external electric field (Weaver, Chizmadzhev, 1996). A simplified model of transmembrane potential evaluation is presented in equation 2:

$$U = 1.5 \cdot r \cdot E \tag{2}$$

where U indicates the transmembrane potential triggered by an external electric field, r is the radius of the bacterium perpendicular to the electrodes, and E is the triggered external electric field. By this equation, *S. thermophilus* at 10 kV/cm (the biggest difference between bacterial lines) induced transmembrane potential between 1.05 V and 1.28 V, and when 26 kV/cm (highest voltage used) was used, the induced transmembrane potential was between 2.73 V and 3.33 V. At the same electric field parameters, *E. coli* gains either 3–6 V transmembrane potential if the length of bacillus is perpendicular to the electrodes, or 1.82–2.31 V if the width of the bacillus is perpendicular to the electrodes when the applied electric field is 10 kV/cm. Even higher values are obtained when 26 kV/cm is used: 7.8 V to 15.6 V if the length of the bacillus is perpendicular to the electrodes and 4.73 V to 6 V if the width of the bacillus is parallel to the electrodes. Such huge differences are crucial because transmembrane potential is the primary parameter that triggers electroporation and thus induces PEF. Also, the calculated numbers make sense, since at 10 kV/cm, the transmembrane potential is triggering similar values in *E. coli* as compared to *S. thermophilus* with 26 kV/cm voltage applied. On those two experimental points, bacterial viability was profoundly decreased in both bacterial lines.

Differences are observed when comparing the metabolic activity and the colonies formed. To our understanding, that can be because of the triggered mitotic arrest. This assumption is supported by our observations that the colonies

formed had a much smaller area (data not presented). The mitotic arrest could have decreased the metabolic activity at the chosen measuring time. Even though there are some differences, the trend of the values is the same. To our understanding, this was sufficient to prove selective pasteurisation by measuring metabolic activity of the affected bacteria.

Finally, we present the growth of the PEF-treated bacteria in the bioreactor. As stated before, such an experiment is highly uncommon in PEF treatment since one usually prevents bacterial growth but not initiates the growth of what is left after bacteria pasteurisation triggered by PEF. We did not come across any publications that report bacterial growth measurements in the bioreactor after experiments of PEF application as the most usual experiments are colony formation and metabolic activity assays. The results show that the quantity of PEF-treated *S. thermophilus* bacteria rises 9.5 h after application of electric pulse. The control of *S. thermophilus* bacteria was growing at a high expected rate.

A different observation is obtained with PEF-treated *E. coli*, which does not show a change in the quantity of bacteria. In such good conditions, healthy bacteria were growing regularly (control of *E. coli*). In this way we have received a double confirmation that *E. coli* cells did not recover to show growth signs up to 12 h after the treatment.

CONCLUSIONS

By measuring the metabolic activity, the ability to form colonies, and the change in bacteria growth rate in the bioreactor, we showed that a PEF-induced selective nonthermal pasteurisation method could be applied in the industry where coliform bacteria can be eliminated while leaving other needed bacteria intact.

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SELEKTYVI KOLIFORMINIŲ BAKTERIJŲ INAKTYVACIJA IMPULSINIAIS ELEKTROS LAUKAIS (PEF)

Santrauka

Koliforminės bakterijos dažniausiai atsiranda žarnyne, kai maisto pramonėje neužtikrinamas sterilumas. PEF sukelta selektyvi neterminė pasterizacija gali pasterizuoti koliformines bakterijas, nepažeisdama naudingųjų bakterijų. Norėdami pagrįsti šią hipotezę, koliforminių bakterijų reprezentacijai pasirinkome *Escherichia coli dh5α* (*E. coli*) padermę; taip pat naudojome *Streptococcus thermophilus* bakterijas kaip pieno produktuose naudojamų naudingųjų laktobakterijų analogą. Gauti rezultatai rodo, kad taikant PEF (impulsinius elektros laukus) galima sukelti selektyvią bakterijų žūtį. Bakterijų gyvybingumas buvo matuojamas pasitelkus kolonijų formavimo testą, metabolinis aktyvumas - naudojant „Alamar Blue“ rinkinį. Bakterijų augimas įvertintas bioreaktoriuje. Tyrimas atskleidė, kad PEF sukelta selektyvi neterminė pasterizacija gali būti taikoma pramonėje. Proceso metu galima pašalinti nepageidaujamas koliformines bakterijas, o reikalingos bakterijos lieka nepaveiktos.

Raktažodžiai: elektroporacija, selektyvus bakterijų inaktyvavimas, impulsiniai elektros laukai, koliforminės bakterijos