

Combination of Microsecond and Nanosecond Pulsed Electric Field Treatments for Inactivation of *Escherichia coli* in Water Samples

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Abstract Inactivation of microorganisms with pulsed electric fields is one of the nonthermal methods most commonly used in biotechnological applications such as liquid food pasteurization and water treatment. In this study, the effects of microsecond and nanosecond pulses on inactivation of *Escherichia coli* in distilled water were investigated. Bacterial colonies were counted on agar plates, and the count was expressed as colony-forming units per milliliter of bacterial suspension. Inactivation of bacterial cells was shown as the reduction of colony-forming units per milliliter of treated samples compared to untreated control. According to our results, when using microsecond pulses the level of inactivation increases with application of more intense electric field strengths and with number of pulses delivered. Almost 2-log reductions in bacterial counts were achieved at a field strength of 30 kV/cm with eight pulses and a 4.5-log reduction was observed at the same field strength using 48 pulses. Extending the duration of microsecond pulses from 100 to 250 μ s showed no improvement in inactivation. Nanosecond pulses alone did not have any detectable effect on inactivation of *E. coli* regardless of the treatment time, but a significant 3-log reduction was achieved in combination with microsecond pulses.

Keywords Inactivation · Bacteria · Nanosecond · Microsecond · Electric pulse · Pulsed electric field treatment · Electroporation · *E. coli* · Water

Introduction

Electroporation is a method for cell membrane permeabilization. Its effect is a significant increase in electrical conductivity and permeability of the membrane, caused by externally applied electrical pulses. When cells are exposed to an external electric field of sufficient amplitude and duration, the cell membrane is electroporated. Aqueous pores are assumed to be induced in the cell membrane, and they increase in size and number with pulse duration (Neumann and Rosenheck 1972; Chang and Reese 1990; Weaver 2003; Saulis 2010). This is just one of various theoretical models that have been proposed to explain electroporation, with structural reorganization and creation of hydrophilic pores remaining directly unobserved (Rols 2006). Depending on the parameters of the electric pulses, the membrane can become either transiently or permanently permeable, thus making electroporation either reversible or irreversible. When electric pulse parameters are below the threshold of electroporation, the pores in the cell membrane can reseal and the cell survives. With electric pulse parameters exceeding critical threshold, the size and number of the induced pores achieve a critical value. Through these pores, the cell loses internal components, which leads to its death (Gusbeth et al. 2009; Saulis 2010).

Reversible electroporation requires the cell to survive exposure to an external electric field and return to its natural state afterward. It is most widely used in biotechnology and medicine for electrofusion and electrotransfer of drugs, genes and other large molecules to cells, both as a research tool and as a clinical technique. One such clinical technique is electrochemotherapy, where cancer cells are treated by permeabilizing the cell membrane and allowing chemotherapeutic agents to enter at greater concentrations

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and kill the targeted cells (Marty et al. 2006; Bertacchini et al. 2007; Sersa et al. 2008). A new and developing field of research is the application of drugs and genes to brain tissue in humans (Agerholm-Larsen et al. 2011), where the blood–brain barrier could theoretically be overcome with the use of reversible electroporation.

In addition to reversible electroporation, electric pulses can be used for inactivation of microorganisms, where all biological activities in the cell are terminated. This has been extensively studied since the beginning of the electric power industry, with the first studies dating as far back as the nineteenth century (Fuller 1898). Irreversible electroporation uses an electric field to directly destroy cells. The pores created by the applied electric field are unable to reseal, which prevents maintenance of homeostasis (Rubinsky 2007). In medicine, the effects of irreversible electroporation are being clinically used as a method for tissue ablation (Davalos et al. 2005). This has the potential to become an alternative method of ablation for solid tumors (Lee et al. 2010). However, irreversible electroporation reaches far beyond medical purposes. It has found its way into the food industry, where it is used as a non-thermal method for inactivating microorganisms in liquid food products (pasteurization). Pulsed electric field (PEF) technology utilizes short electric pulses to preserve the food. The main advantage over other methods is that food products are treated at lower temperatures, so they retain nutritional and organoleptic characteristics. Irreversible electroporation is also more cost-effective than conventional systems (Barbosa-Cánovas et al. 1999). In industry it is also used to treat different water samples, for example, hospital wastewaters (Rieder et al. 2008; Gusbeth et al. 2009) that are usually contaminated with pathogenic bacteria (Kümmerer 2001). The main advantages of PEF technology in water treatment are less unwanted by-products compared to other inactivation techniques such as chlorination, ozonation and UV irradiation (Rook 1977; Paraskeva and Graham 2002; Schwartz et al. 2003) and no developed adaptation to electric field by the descendants of treated bacteria (Gusbeth et al. 2009).

Several studies have investigated the inactivation of bacteria predominantly in relation to microbial inactivation in liquid food (Calderon-Miranda et al. 1999; Heinz et al. 2002; Wu et al. 2005) and wastewater (Rieder et al. 2008). Results of those studies showed significant inactivation but with variable results depending on the microorganisms, the medium in which they were treated and the different electrical parameters that were used. Electrical field strengths ranged from 10 to 40 kV/cm, with pulse duration from 1 μ s to 100 ms (Hamilton and Sale 1967; Teissié et al. 2002; Mosqueda-Melgar et al. 2007). Besides microsecond and millisecond electric pulses used for electroporation of cell membranes, Schoenbach et al.

(2000) reported that nanosecond pulse durations can be used to efficiently eradicate bacteria from liquid samples. However, reduction of pulse duration from micro- to nanoseconds must be compensated by an increase in the electric field intensity (Schoenbach et al. 2000; Kotnik and Miklavcic 2006; Saulis 2010). When exposing bacterial cells to nanosecond pulses, electric field strength is around 100 kV/cm and pulse duration is in the range of tens of nanoseconds (Schoenbach et al. 2000; Perni et al. 2007). The mechanism of inactivation with nanosecond pulses is different from that caused by reversible and irreversible electroporation with micro- and millisecond pulses (Schoenbach et al. 2000; Weaver 2003). Micro- and millisecond pulse durations induce a voltage on the cell plasma membrane which reduces the energy necessary for membrane lipid rearrangements, leading to cell membrane permeabilization. Exposure of the cell to an external electric field also induces voltage on cell organelles that is several orders of magnitude smaller than the voltage on the cell membrane. Such voltage is too low to induce organelle membrane permeabilization. In nanosecond pulse application, the pulse duration is shorter than the charging time of the outer membrane, for bacteria typically less than 10 ns; besides the effects on cell membrane integrity, additional disruption of internal cell organization is observed (Schoenbach et al. 2000; Perni et al. 2007). Theoretical evaluation of such effects of nanosecond electric pulses was presented for mammalian cells containing organelles by Kotnik and Miklavcic (2006); however, it cannot be directly applied to bacteria due to different internal organization of eukaryotic and prokaryotic cells.

Our study concentrated on inactivation of bacteria in water samples. The aim was to investigate how different electrical pulse parameters affect the inactivation efficiency. We used different protocols, applying microsecond and nanosecond pulses and systematically changing only one parameter at a time, to ascertain the parameters that crucially contribute to the best inactivation results. Microsecond and nanosecond pulses were also used in combination for a potential synergistic effect between the two different pulse protocols.

Materials and Methods

Preparation of Water Samples with Bacterial Culture

The *Escherichia coli* K12 ER1821 strain (New England BioLabs, Frankfurt am Main, Germany) was used as a test microorganism. Luria broth (Sigma-Aldrich, Munich, Germany) was inoculated with *E. coli* cells in an Erlenmeyer flask and incubated for 16–18 h with continuous shaking at 37 °C. In order to prepare samples for the

experiments, a given volume of broth was centrifuged at 4,200 RCF for 30 min at 4 °C. Supernatant was carefully removed, and the cell pellet was resuspended in distilled water, to obtain a final concentration of about 2×10^9 CFU/ml, from which serial dilutions ranging from 10^{-1} to 10^{-7} were prepared. The negative control was the untreated suspension of bacteria. Samples for treatment were diluted suspensions (10^{-1}) of bacterial culture.

Electroporation Protocols

Suspension of bacteria in water was electroporated in cuvettes with integrated aluminum electrodes (Eppendorf, Hamburg, Germany) with a 1- or 2-mm gap. For our experiment, we used different pulsing protocols, which consisted of microsecond, nanosecond and a combination of these pulses. To generate different sequences of microsecond pulses, we used the electric pulse generator HVP-VG (Igea, Carpi, Italy). Nanosecond pulses were generated by a custom-designed high-voltage nanosecond generator built in the Laboratory of Biocybernetics at the Faculty of Electrical Engineering of the University of Ljubljana. The nanosecond generator was built as a diode opening switch generator (Reberšek and Miklavčič 2011) with air-core inductors (Sanders et al. 2009). The electrical field strength (E) was evaluated as

$$E = \frac{U}{d} \quad (1)$$

where U is the applied voltage of the pulses (1,500 and 3,000 V) and d is the gap between electrodes in the cuvettes (1 and 2 mm).

For electroporation with microsecond pulses at different electrical field strengths (7.5, 15, 30 kV/cm) we used eight rectangular unipolar pulses with pulse duration of 100 μ s and repetition frequency 1 Hz (Fig. 1a). The correlation between the number of pulses and inactivation was determined by using 8-, 24- and 48- μ s pulses at 15 and 30 kV/cm, with pulse duration of 100 μ s. The effect of two different pulse durations was determined by comparison of 100 and 250 μ s at 30 kV/cm.

Nanosecond pulses were delivered in trains ranging from 10 to 1,000 pulses at 10-Hz frequency, with pulse duration of 10 ns and electrical field strength of 80 kV/cm (Fig. 1b).

The combination of microsecond and nanosecond pulses started with application of 8×100 - μ s pulses ($E = 30$ kV/cm, 1-Hz repetition frequency) followed by $1,000 \times 10$ -ns pulses at 10-Hz repetition frequency, with pulse duration of 10 ns and electrical field strength of 80 kV/cm (Fig. 1c). We also studied the possible effect of the time gap between applying microsecond and nanosecond pulses. In the first set of experiments, there was a 5-s time gap between sets of

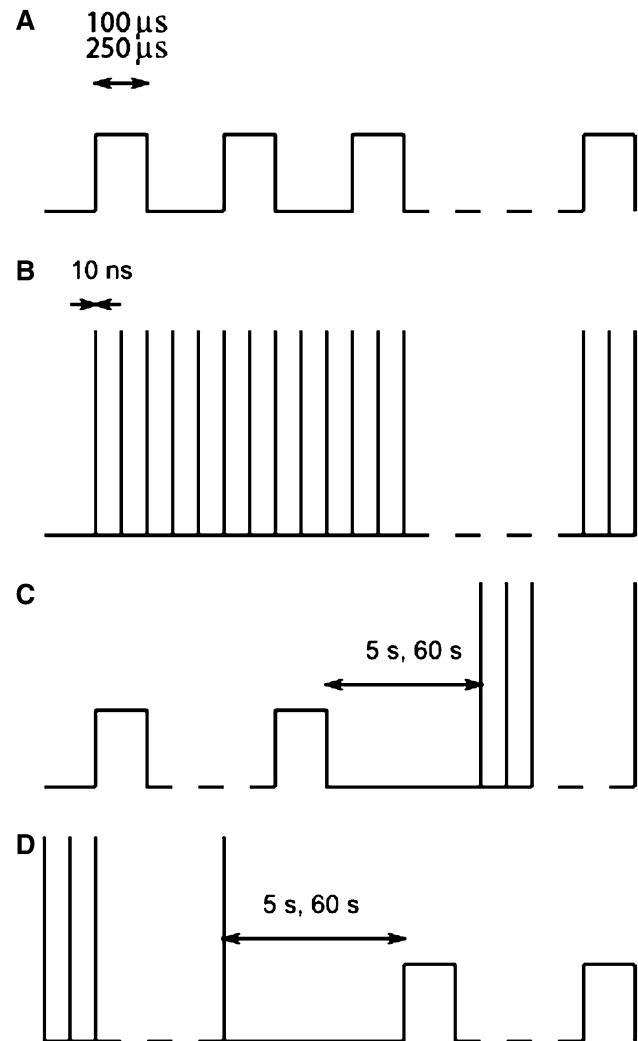


Fig. 1 Schematic representation of different pulsing protocols used in our experiments. Microsecond pulses (a), nanosecond pulses (b), combination of microsecond and nanosecond pulses (c) and combination of nanosecond and microsecond pulses (d)

pulses. A second set of experiments had a time gap of 1 min. All experiments were repeated with pulses delivered in reverse order, applying first nanosecond and then microsecond pulses (Fig. 1d).

Viable Cell Count

The plate count method (Reasoner 2004) was used to determine cell viability in negative controls (untreated samples) and treated samples. Serial dilutions of each sample were evenly spread using a spread plate technique on Luria agar (Sigma-Aldrich). Plates were incubated for 24 h at 37 °C. Bacterial colonies were counted manually, and the count was expressed as colony-forming units per milliliter of sample. To test the possible toxic effect of

treatment medium on bacteria at chosen electrical parameters, we electroporated the distilled water in cuvettes without bacterial cells and added it to untreated bacterial suspension at a ratio of 1:1, while we did a parallel experiment with untreated distilled water.

The inactivation level of bacterial cells was determined by calculating the \log_{10} of the survival fraction ($S = N/N_0$). N is the number of colony-forming units per milliliter of treated sample, and N_0 is the number of colony-forming units per milliliter of untreated sample. The results are mean values from at least three experiments, with standard deviations shown by error bars. The number of experiments was higher for the most crucial pulse parameters: $8 \times 100 \mu\text{s}$, 30 kV/cm, where ten experiments were performed; $1,000 \times 10\text{-ns}$ pulses, where five experiments were performed; and combination of $8 \times 100 \mu\text{s}$, 30 kV/cm and $1,000 \times 10\text{ ns}$ pulses, where five experiments were performed.

Statistics

Statistical tests were performed on all results (SigmaPlot 11.0; Systat Software, Richmond, CA). A paired t -test was performed on results obtained from the same sample and on normalized results if they were obtained from different samples. Respectively, if the normality test (Shapiro-Wilk) failed, the Wilcoxon signed rank test and Mann-Whitney rank sum test were used instead. Results were considered statistically different at $P > 0.05$.

Results

Microsecond Pulses

The effect of different electrical field strengths on the inactivation curve of *E. coli* is shown in Fig. 2a. Electrical field strengths were 7.5, 15 and 30 kV/cm. The inactivation level increased with applied electrical field strengths, for 7.5 ($P = 0.039$), 15 ($P = 0.041$) and 30 ($P < 0.001$) kV/cm compared to control samples not exposed to electric pulses.

Increasing the pulse number from eight to 48 at electrical field strengths of 15 kV/cm, we achieved an almost 2.5-log reduction, which was not statistically significant ($P > 0.05$). For electric field amplitude 30 kV/cm, increasing the pulse number from eight to 24 resulted in significant inactivation ($P = 0.014$), and a similar effect was obtained when comparing eight and 48 pulses ($P = 0.014$). With 48 pulses and 30 kV/cm, a more than 4.5-log reduction was observed.

With eight microsecond pulses of $100 \mu\text{s}$ we were able to achieve an almost 2-log reduction of *E. coli* ($P < 0.001$).

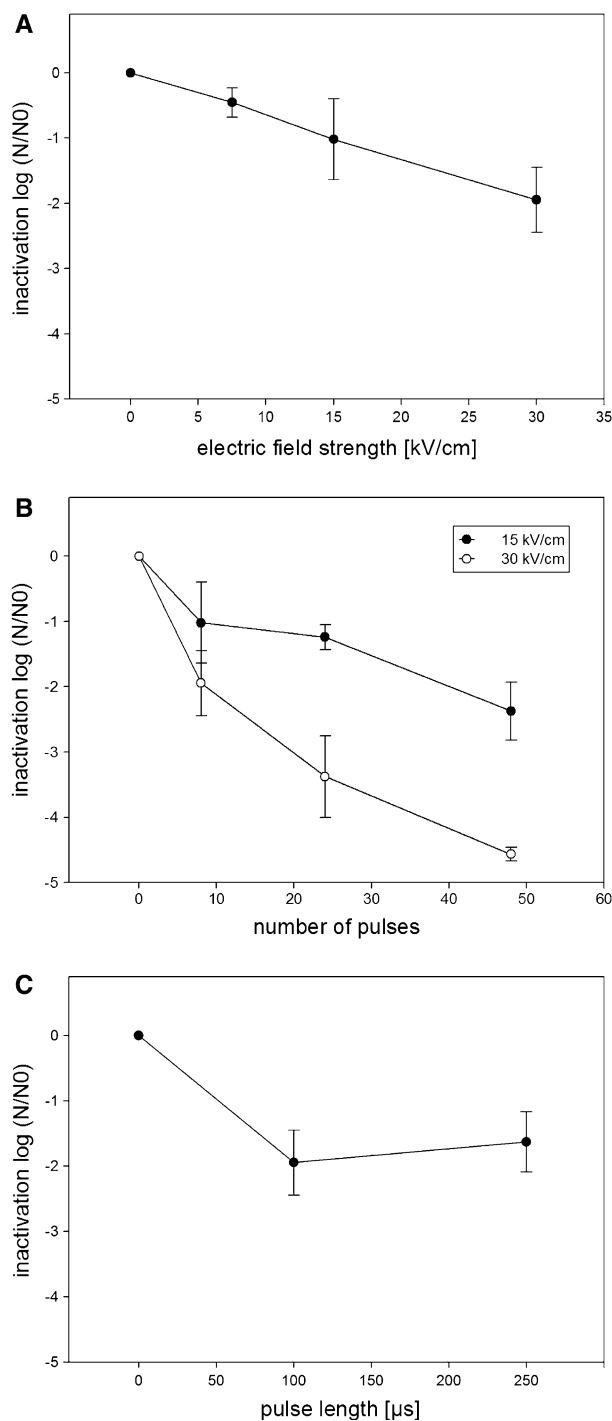


Fig. 2 Inactivation curve of *E. coli* cells by microsecond electric pulses at different electrical field strengths (7.5, 15, 30 kV/cm) with eight pulses of $100 \mu\text{s}$ (a), with different numbers of $100\text{-}\mu\text{s}$ pulses at 15 kV/cm (filled circle) and 30 kV/cm (open circle) (b) and with two different pulse durations, 100 and $250 \mu\text{s}$. A train of eight pulses with a repetition frequency of 1 Hz and electrical field strength of 30 kV/cm was applied (c). Inactivation is presented as a $\log N/N_0$, where N is the number of colony-forming units per milliliter of treated sample and N_0 is the number of colony-forming units per milliliter of untreated sample. Results are means from at least three experiments, with standard deviations shown by error bars

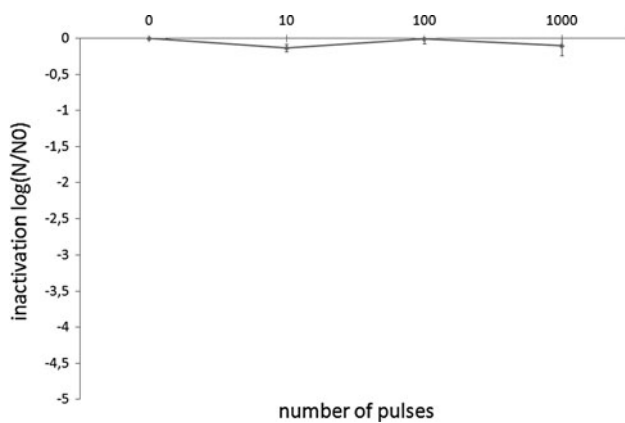


Fig. 3 Inactivation curve of *E. coli* with nanosecond pulses for different numbers of pulses (10, 100, 1,000). Pulse duration was 10 ns and electrical field strength was 80 kV/cm at 10-Hz frequency. Inactivation is presented as a $\log N/N_0$, where N is the number of colony-forming units per milliliter of treated sample and N_0 is the number of colony-forming units per milliliter of untreated sample. Results are means from at least three experiments with standard deviations shown by error bars

Increasing pulse duration to 250 μ s achieved the same inactivation as 100- μ s pulses ($P = 0.353$), as shown in Fig. 2c.

Nanosecond Pulses

The results obtained by treating cell suspensions of *E. coli* with a train ranging from 10 to 1,000 pulses at 10-Hz repetition frequency (10 ns, $E = 80$ kV/cm) are shown in

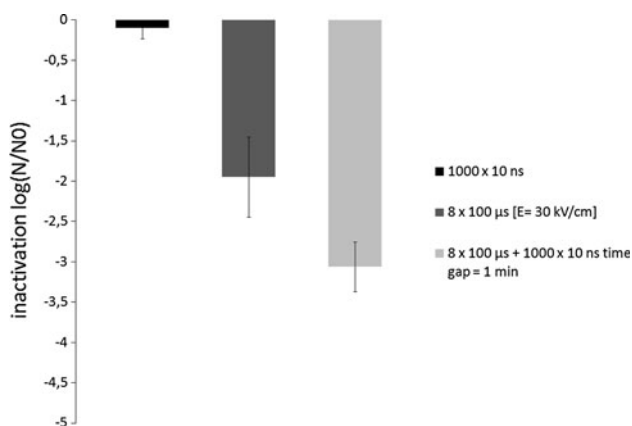


Fig. 4 Comparison of inactivation of *E. coli* using a combination of nano- and microsecond pulses. The pulsing protocol consisted of 1,000 pulses of 10 ns ($E = 80$ kV/cm), eight pulses of 100 μ s ($E = 30$ kV/cm) and a combination of eight pulses of 100 μ s ($E = 30$ kV/cm) followed by 1,000 pulses of 10 ns ($E = 80$ kV/cm). Inactivation is presented as a $\log N/N_0$, where N is the number of colony-forming units per milliliter of treated sample and N_0 is the number of colony-forming units per milliliter of untreated sample. Results are means from at least three experiments, with standard deviations shown by error bars

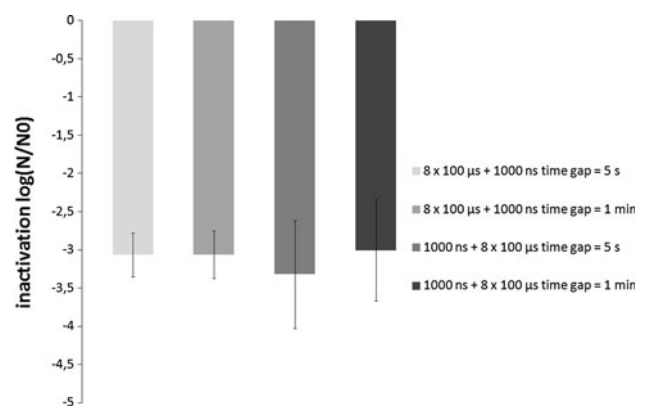


Fig. 5 Effect of the time gap and order of application of nanosecond and microsecond pulses on the inactivation of *E. coli*. Nanosecond pulses consisted of 1,000 pulses of 10 ns ($E = 80$ kV/cm), while microsecond pulses were applied as a train of eight pulses of 100 μ s ($E = 30$ kV/cm). The time gap between nanosecond and microsecond pulses was 5 s and 1 min, respectively. Nanosecond pulses were delivered in two different sequences: microsecond pulses, followed by nanosecond pulses or in the reverse order. Inactivation is presented as a $\log N/N_0$, where N is the number of colony-forming units per milliliter of treated sample and N_0 is the number of colony-forming units per milliliter of untreated sample. Results are means from at least three experiments, with standard deviations shown by error bars

Fig. 3. Nanosecond pulses with these parameters did not have any effect ($P > 0.05$) on inactivation of *E. coli* regardless of the number of pulses delivered.

Combination of Microsecond and Nanosecond Pulses

Using a combination of microsecond and nanosecond pulses resulted in a pronounced reduction (three log) in the bacterial count compared to using only microsecond ($P = 0.003$) or nanosecond ($P = 0.008$) pulses (Fig. 4). Extending the time gap between electroporation from 5 s to 1 min did not seem to have any effect on inactivation of cells. Changing the order in which the microsecond and nanosecond pulses were delivered also had no effect on inactivation ($P > 0.05$) (Fig. 5).

Discussion

Based on experimental work, this study presents inactivation of bacteria in water samples with microsecond pulses, nanosecond pulses and a combination of both protocols. We investigated how various field amplitudes, increasing number of pulses and different pulse durations affected the inactivation of *E. coli*.

Inactivation of bacteria with microsecond pulses increases with higher electric field amplitudes (Fig. 2a). These results are in agreement with the findings of other authors (Hülshager et al. 1981; Schoenbach et al. 2000; Álvarez et al.

2003; Pataro et al. 2011). A theoretical explanation is available for the effects of microsecond pulses on bacterial inactivation. Transmembrane potential induced on the cell membrane by external electric pulses leads to creation of small, metastable, hydrophilic pores and pore evolution in terms of pore size enlargement and increase in the number of pores. The rate of pore formation depends strongly on transmembrane potential and electric field amplitude. It is important to note that for bacterial inactivation postpulse events in the cell are also important. Namely, cell viability is strongly related to the pore resealing process and to leaking of material out of the cell as the majority of transport occurs after electric pulse application (Saulis 2010). Even though electric pulse duration is one of the factors affecting cell survival, in our study extending the duration of microsecond pulses from 100 to 250 μ s did not affect the inactivation of *E. coli*, which is consistent with the theory of electroporation that indicates that increasing electric pulse amplitude is more effective than lengthening treatment time (Saulis 2010). However, results show that microbial inactivation rate increased with increasing number of pulses (Fig. 2b). Authors working with different microorganisms report similar results (Hülshager et al. 1981; Jayaram et al. 1992; Martín-Belloso et al. 1997; Calderon-Miranda et al. 1999). Regarding pulse parameters affecting bacterial inactivation caused by micro- and millisecond pulse duration, lipid rearrangements leading to cell membrane permeabilization (Kotnik and Miklavcic 2006) and postpulse resealing (Saulis 2010) are the main factors involved in the process. Besides, when a large number of or long pulses are applied, PEF treatment can produce toxic side products due to electrochemical reactions such as electrolysis of the media, release of the ions from electrodes and generation of free radicals. Bacterial inactivation could be caused by those toxic products, which was not the case in our study as additional experiments were performed. Only the electroporation medium was exposed to PEF treatment and added to intact bacteria, which then grew just as well as bacteria exposed to untreated electroporation medium. No effect on bacterial viability was detected, indicating that in our case no toxic products which would affect bacterial inactivation were formed.

In case of nanosecond pulse application the microbial inactivation observed could not be due only to the effects caused on the cell membrane level (Schoenbach et al. 2000; Perni et al. 2007). In our study, when nanosecond pulses were used to inactivate *E. coli*, no effect was observed regardless of the number of pulses (ranging 10–1,000) to which the samples were exposed (Fig. 3). However, Perni et al. (2007) reported an almost 2-log reduction for *E. coli*. The electric pulse parameters used for this effect were 32-ns pulses at field strength of 100 kV/cm and 30-Hz frequency. Samples were treated for 300 s,

receiving 9,000 pulses in total (Perni et al. 2007). The differences between our results and those reported by Perni and coworkers can be attributed to the different electric pulse protocols used. In our study lower field strength (80 kV/cm), shorter pulse duration (10 ns) applied at lower frequencies (10 Hz) and lower number of pulses (maximal 1,000) were used. It is therefore possible that the overall treatment with our nanosecond pulse parameters was not severe enough to reduce the viability of treated bacteria (Fig. 3). Perni et al. (2007) observed that at the end of the treatment only approximately 1 % of the surviving populations remained uninjured. They assumed that the mechanism of bacterial inactivation with such short pulses may have affected the internal structures of bacterial cells (Perni et al. 2007), as proposed earlier by Schoenbach et al. (2000). Theoretical evaluation of the effect of microsecond and nanosecond electroporation of mammalian cells containing organelles showed that the cell membrane is affected by nanosecond electric pulses and that organelles are permeabilized if the electric properties of the cytosol and the organelle interior are different (Kotnik and Miklavcic 2006). However, the theoretical findings on mammalian cells cannot be directly applied to bacteria due to the different internal organization of eukaryotic and prokaryotic cells, and currently only experimental evidence on the effect of nanosecond pulses on bacteria is available. A large majority of theoretical studies are devoted to explaining the mechanism involved in the electroporation of eukaryotic cells, while the pathways leading to microbial inactivation remain obscure (Saulis 2010). Nevertheless, some experimental evidence is available. Effects of PEF treatment on internal cell structures were observed when bacteria were exposed to microsecond pulses. Electron microscopy revealed that approximately 25 % of the *E. coli* cells exposed to PEF treatment at 30 kV/cm, 20 pulses and 4- μ s duration had an altered internal organization, while rupture of cell membranes could not be confirmed by use of transmission electronic microscopy (Aronsson et al. 2001). The critical pulse duration that results in intracellular effects is for bacteria in the range of tens of nanoseconds (Schoenbach et al. 2000). From this point of view it is interesting that Aronsson et al. (2001) observed alterations in intracellular structures with longer microsecond pulses that should have had a main effect at the cell membrane level. From the data available in the literature (Schoenbach et al. 2000; Weaver 2003; Kotnik and Miklavcic 2006; Perni et al. 2007; Saulis 2010) we can expect that microsecond pulses affect cell membrane integrity, while nanosecond pulses additionally affect internal organization. Since a wide variety of microbial inactivation processes are known to cause death through injury accumulation (Perni et al. 2007), we decided to combine microsecond and nanosecond pulses. Our results

suggest a great synergistic effect between microsecond and nanosecond pulses, with a more than 3-log reduction for *E. coli*. According to our results (Figs. 2b, 4), using only microsecond pulses, $20 \times 100\text{-}\mu\text{s}$ pulses would be needed to achieve the same level of inactivation as eight microsecond pulses in combination with 1,000 nanosecond pulses. The combination of nanosecond and microsecond pulses is a unique method of inactivation and has two main advantages, energy efficiency and less joule heating, compared to microsecond pulses. We roughly estimated the electrical energy needed for each type of electric pulse. Supposing that the electrical pulses are rectangular and that the electric field in cuvettes is homogeneous allows us to use the following equation to estimate the pulse energy,

$$W_p = E^2 \times \sigma \times V \times t_p \quad (2)$$

where W_p is the pulse energy, E is the electric field intensity between the electrodes, σ is the electrical conductivity of cell suspension, V is the volume of suspension and t_p is the pulse duration. The ratio between the energy of one $100\text{-}\mu\text{s}$ pulse (W_μ) and the energy of one 10-ns pulse (W_n) is 1,400. One $100\text{-}\mu\text{s}$ pulse equals $1,400 \times 10\text{-ns}$ pulses from the pulse energy point of view.

Although nanosecond pulses alone have no effect on inactivation, the results of combining them with microsecond pulses suggest that there clearly is some mechanism involved in which nanosecond pulses contribute to inactivation. The effect of the time gap from 5 s up to 1 min between applying microsecond and nanosecond pulses as well as the order in which the pulses were delivered showed no difference in inactivation.

In conclusion, our results confirm that bacterial inactivation is affected by electric pulse parameters such as pulse amplitude and number of pulses when we applied only microsecond pulses. A synergistic effect was observed when nanosecond and microsecond pulses were combined even though nanosecond pulses alone did not affect bacterial inactivation. Further studies are needed to determine the exact mechanisms of action of such a pulse combination on bacteria.

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