Selective Inactivation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* with Pulsed Electric Fields and Antibiotics

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Objective: Increasing numbers of multidrug-resistant bacteria make many antibiotics ineffective; therefore, new approaches to combat microbial infections are needed. In addition, antibiotics are not selective—they kill pathogenic organisms as well as organisms that could positively contribute to wound healing (bio flora).

Approach: Here we report on selective inactivation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, potential pathogens involved in wound infections with pulsed electric fields (PEFs) and antibiotics (mix of penicillin, streptomycin, and nystatin).

Results: Using a Taguchi experimental design *in vitro*, we found that, under similar electric field strengths, the pulse duration is the most important parameter for *P. aeruginosa* inactivation, followed by the number of pulses and pulse frequency. *P. aeruginosa*, a potential severe pathogen, is more sensitive than the less pathogenic *S. epidermidis* to PEF (alone or in combination with antibiotics). Applying 200 pulses with a duration of 60 μs at 2.8 Hz, the minimum electric fields of 308.8 ± 28.3 and 378.4 ± 12.9 V/mm were required to inactive *P. aeruginosa* and *S. epidermidis*, respectively. Addition of antibiotics reduced the threshold for minimum electric fields required to inactivate the bacteria.

Innovation: This study provides essential information, such as critical electric field parameters for bacteria inactivation, required for developing *in vivo* treatment and clinical protocols for using PEF for wound healing.

Conclusion: A combination of PEFs with antibiotics reduces the electric field threshold required for bacteria disinfection. Such an approach simplifies devices required to disinfect large areas of infected wounds.

Keywords: bacterial infection, burn wounds, hurdle technology, pulsed electric fields, electroporation, *Pseudomonas aeruginosa* PAO1, *Staphylococcus epidermidis* RP62A

INTRODUCTION

Wound infection is a stubborn medical and economic problem, which increases hospitalization time and requires more nursing care, additional dressings, and possibly readmission to the hospital and additional surgery.[1,2] As a result, wound infection increases treatment costs, and multiple studies have demonstrated the need to increase investments in wound infection prevention to decrease the overall treatment costs in both high- and low-income countries.[3–5] The most severe

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types of infections are invasive bacterial wound infections, which are associated with extreme toxicity, high fever, a hyperdynamic circulatory state, bacteremia, hypotension, and cardiovascular collapse.[6] In burn patients, infections remain the major cause of patient death.[6,7]

Staphylococcus aureus, *Pseudomonas aeruginosa*, and betahemolytic streptococci are the primary causes of delayed healing and infection in both acute and chronic wounds.[8] In addition, the members of a normal skin flora, such as *Staphylococcus epidermidis*, were shown to slow down the wound-healing process.[9–11] Moreover, it successfully forms biofilms on medical devices and implants, leading to additional infection concern.[12–15] Based on previous exhaustive work on bacteria inactivation in food systems[16–18] and previous work on the low-voltage constant electric fields to facilitate the delivery of antibiotics to otherwise recalcitrant biofilms,[19–24] we proposed to use high-voltage but pulsed electric fields (PEFs) for wound and implant disinfection.[25–27]

PEF is an emerging medical technology[28] currently used for tissue ablation by irreversible electroporation,[29] cancer treatment by electrochemotherapy,[30] and gene electrotransfer.[31] The effect of PEF on cells can be explained by the induced change in biological membrane permeability through a phenomenon known as electroporation.[32] Current consensus describes electroporation as the formation of aqueous pores in the lipid bilayer that enable molecular transport.[32–34] The theory of aqueous pore formation, based on thermodynamics, describes the formation of aqueous pores as started by the penetration of water molecules into the lipid bilayer of the membrane, which leads to the reorientation of adjacent lipids with their polar headgroups toward these water molecules.[33] In wound healing, PEF has been used in *in vivo* experimental models for skin rejuvenation,[35] scar treatment,[36] and genetic engineering to enhance the expression of healing-enhancing factors.[37–39] Our recent *in vivo* study that examined the normal skin response to PEF *in vivo* showed complete scarless regeneration in rats.[40]

Using PEF alone, we showed a reduction of *Acinetobacter baumannii in vivo*,[25,26] and elimination of *P. aeruginosa* ATCC 19660 (strain 180) biofilm on the surgical mesh.[27] However, the application of PEF alone might not be sufficient for clinical applications. Previous studies in food disinfection involving PEF have suggested that a combination of two or more methods simultaneously, known as hurdle technologies,[41] could achieve higher disinfection efficiency than each of the methods alone.[42,43] Similar results describing the combined effects of PEF and oxacillin were recently shown for the inactivation of blood-isolated *S. aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *P. aeruginosa*, and *Candida albicans* in liquid.[44]

The goal of this work is to determine the electric field thresholds required to inactivate *P. aeruginosa*, a common wound pathogen, and *S. epidermidis*, a drug-resistant bacteria that is a part of normal skin flora, but which is considered an "opportunistic pathogen"[10] that can slow down the healing process and demonstrates drug resistance similar to that of *S. aureus*.[45] Using a concentric electrode system that allows single-step determination of the critical electric fields,[46] we determined the thresholds of electric fields when electric fields were applied alone or in combination with an antibiotics mix in different concentrations *in vitro*.

Using the Taguchi robust experimental design approach,[47] we determined the relative importance of each of the PEF parameters on disinfection efficiency. First, the application of PEF *in vivo* induces immune system responses,[48] which are complex and can interfere with the effect of antibiotics. Second, *in vitro* experiments allowed us to test a large number of PEF parameters so that we could investigate their impacts and optimize their values. Using our *in vitro* setup, we followed the 3R principle[49] of reduction and significantly reduced the number of animals that would be required to identify the impact of the each of the experimental parameters on inactivation levels of bacteria in future studies. The demonstration of the hurdle approach for inactivation of potential wound pathogens is expected to overcome the problems associated with the current pharmacologic or only physical means of disinfecting wounds.

CLINICAL RELEVANCE

Currently, local wound infection is addressed by early surgical debridement and skin grafting,[8] topical and prophylactic antibiotics,[8] an enzymatic detachment of biofilms,[50] immunoprophylaxis and immunotherapy,[51] photodynamic therapy,[52] hyperbaric oxygen therapy,[8] or vacuum-assisted wound closure.[8] However, in many cases, especially with the emergence of multidrug-resistant strains,[53,54] these methods are not efficient, and therefore, additional means of disinfecting wounds are clearly needed. Furthermore, *P. aeruginosa* and *S. epidermidis* can cause deep infections in many tissue sites, including joints,[10,55] lung, heart,[56] liver,[57] and implants.[10] To address these problems, we recently

proposed to use non-thermal, high-voltage PEF technology, previously found to be effective for wounds and surgical mesh disinfection.[25–27]

MATERIALS AND METHODS

Bacterial culture

P. aeruginosa PAO1 and *S. epidermidis* RP62A (RP62A kindly provided by Prof. Micha Fridman, School of Chemistry, Faculty of Exact Sciences, Tel Aviv University) were grown first on electroporation low salt (ELS) media-based solid agar. The ELS media composition was as follows: 0.1 mg/mL NaCl (Merck, Darmstadt, Germany), 0.01 g/mL Bactotryptone (Academia, Israel), 0.005 g/mL yeast extract (BD extract of autolyzed yeast, Israel), 0.015 g/mL agar (Bacteriological Agar-Academia, Israel), 0.5 mg/mL glucose-D+ (Sigma-Aldrich, St. Louis, MO), and 0.0239 g/mL HEPES buffer (HEPES 100G-H buffer; Sigma-Aldrich). The reagents were dissolved in the double-distilled water and autoclaved (instrument) for 30 min at 121°C. Each plate was filled with 10 mL ELS media. For starter culture preparation, a single colony was cultured in 2 mL of liquid ELS at conditions of 32°C and 150 rpm for 8 h. One hundred microliters of liquid starter with optical density (OD) 0.22–0.26 (measured using Tecan infinite M200 PRO with 600 nm wave) and pH 7 were spread on solid ELS agar with Dregalski stick and cultivated at 32°C for 8 h before electroporation experiments.

PEF experimental setup for the determination of irreversible electroporation electric field strength threshold with a single step

Concentric ring electroporation as described by Fernand *et al.* was used.[46] The concentric electrode design creates a gradient of disinfection from the center outwards to the periphery. The local electric field strength at each point is described using equation (1) as follows:

$$E(r,V)=\frac{\Delta V}{rln\left(\frac{R2}{R1}\right)} \quad (1)$$

where E (V/mm) is field strength, r is distance from the center of the central electrode, ΔV (V) is potential difference between the central and peripheral electrodes, $R1$ (mm) is the radius of inner electrode, and $R2$ (mm) is the radius of outer electrode. In this study $R1$ was 0.75 mm and $R2$ was 11.95 mm.

Pulses were delivered using a BTX 830 pulse generator (Harvard Apparatus, Inc., Holliston, MA). Currents were measured *in vivo* using a PicoScope 4224 Oscilloscope with a Pico Current Clamp (60 A, AC/DC) and analyzed with Pico Scope 6 software (Pico technologies, Inc., Cambridgeshire, United Kingdom).

Taguchi robust experimental design to determine the individual impact of a number of pulses, pulse length, and frequency of delivery on minimum electric field strength required to inactivate *P. aeruginosa* PAO1

The goal in this series of experiments was to determine the effects of PEF parameters of pulse number, duration, and frequency on the minimum strength of electric field (E_c) required to inactivate *P. aeruginosa* PAO1. The range of PEF parameters and their combinations is large; therefore, to decrease the number of experiments but still allowing to evaluate the impact of each parameter independently, we applied the Taguchi robust design method to the experimental design.[58] The key feature of the Taguchi method is the design of the experiment where process factors are tested with orthogonal arrays. We tested the impact of the following range of PEF settings using L9 Taguchi matrix: pulse length of 40, 50, 60 μs; interval between pulses of 350, 400, 450 ms; and pulse number of 100, 150, 200. Supplementary Table S1 summarizes the experiments conducted for the L9 orthogonal Taguchi array needed to determine the individual effects of each of the tested parameters on E_c. At least 12–16 repeats were performed for each experimental condition. Analysis with "minimum the best target" function, the goal of which is to find the smallest E_c at which the bacteria were inactivated,[36] was done using Minitab 18 (Minitab, Inc., State College, PA).

Determination of the minimum strength of electric field

The digital image of each experiment was captured with Binocular (Leica M420) and analyzed with Image-J (ver 1.6.0; NIH). r_c (mm), the radius from the center where no bacterial growth was observed, was measured at least at four different points. The average of measured radii was taken and used for the calculation of E_c as follows:

$$E_c(N,t_p,T)=\frac{\Delta V}{r_c ln\left(\frac{R2}{R1}\right)} \quad (2)$$

where N is number of pulses, t_p is duration of a single pulse, and T is interval between pulses. Conversion rate was 90 pixels to 0.2 mm, calibrated with a micrometer with $\times 5.6$ magnification (Leica M420; Leica, Wetzlar, Germany).

Determination of invested energy

Energy, W (J), invested in each treatment was calculated with equation (3).

$$W = V \cdot I \cdot t_p \cdot N \tag{3}$$

Hurdle effects of PEF and antibiotics on the inactivation of *P. aeruginosa* and *S. epidermidis*

To test the combined hurdle effect and the impact of PEF and antibiotics, we used the following antibiotic mix (Biological Industries 03-032-1C, Cromwell, CT): penicillin (Penicillin G Sodium Salt; 10,000 units/mL); streptomycin (Streptomycin Sulfate; 10 mg/mL); and nystatin (1,250 units/mL). Previous studies have suggested the control of *P. aeruginosa* with streptomycin[59] and nystatin[60] and *S. aureus* with penicillin.[61] Two microliters of the diluted antibiotic mixture (1/2 to 1/20 dilution factor) was applied at the spot where the central electrode was positioned. Three replicates were done in the same Petri dish with PEF (1,750 V, 200 pulses, 60 μs, chosen from Taguchi experiments), and three replicates were done with PEF but only with antibiotics. When no PEF treatment was applied in the control samples, the equivalent E_c was calculated with equation (2). The total number of replicates per experimental condition was 6–9 for each dilution.

Statistical analysis

Statistical analysis was done using Minitab18 (Minitab, Inc.), Matlab (ver. 2013; The MathWorks, Inc., Natick, MA), and Excel (ver. 2013; Microsoft Corporation). For randomization, 103 permutation simulations were done. Results show mean and standard deviation. The minimum number of repeats per experimental condition was 15. For the linear regression model developed in this study, we calculated the total relative error (TRE) using equation (4):

$$\mathrm{TRE} = \frac{100}{m}\sqrt{\sum_{i=1}^{m}\left(\frac{S_i - PV_i}{PV_i}\right)^2} \tag{4}$$

where m is number of measurements, S_i is measured value, and PV_i is predicted value.

RESULTS AND DISCUSSION

The experimental setup with concentric electrodes for one-step determination of the minimum electric field required for irreversible electroporation of bacteria with PEF is shown in Fig. 1a. The dynamic current of the individual pulse of 60 μs duration is shown in Fig. 1b. Figure 1c shows the PEF effect on the culture of *P. aeruginosa*. A clean area with dead cells closer to the center is apparent, and unaffected cells remain on the edges, where the strength of the electric field was insufficient to kill the bacteria.

In the tested ranges, increasing pulse duration (t_p), increasing number of pulses, and energy invested in the whole treatment decreased E_c (Figs. 2a, c, d and 3 and Table 1). Increasing the pulse interval had almost no effect on E_c (Figs. 2b and 3). In studies using Taguchi orthogonal arrays and the individual parameters of a pulse, pulse duration had the strongest effect on E_c, followed by pulse number (Fig. 3). The interval between pulses had the lowest impact on E_c (Fig. 3). The lowest E_c was observed when 200 pulses of 60 μs duration were delivered with 350 ms interval.

Using a multivariable regression approach, we constructed a linear regression model to describe the dependence of E_c (V/mm) of *P. aeruginosa* on the tested parameters of the electric pulse ($n = 150$) as in equation (5),

$$E_c = \alpha_0 + \alpha_1 t_p + \alpha_2 N + \alpha_3 I + \epsilon \tag{5}$$

where α_0 is intercept, and α_1, α_2, α_3 are linear coefficients of pulse duration (t_p, μs), N is number of pulses, I is interval between pulses (ms), and ε is model error.

The determined coefficients were: 962.15 (α_0), –9.55 (α_1), –1.89 (α_2), 0.77 (α_3), indicating that t_p is the strongest predictor among measured parameters in the tested ranges of E_c. The model p-value was $<2.2\ 10^{-16}$, the adjusted R^2 was 0.868, and the corresponding TRE was 0.78%.

Next, using the PEF protocol with the smallest E_c ($t_p = 60$ μs, $I = 350$ ms, $N = 200$), we investigated the combined effect of PEF and antibiotics on *P. aeruginosa* and *S. epidermidis*. For *P. aeruginosa*, the addition of antibiotics reduced E_c in comparison to PEF alone (Figs. 4 and 5 and Table 2). The hurdle effect of PEF and antibiotics was also stronger than the effect of the same dose of antibiotics alone [equivalent E_c was calculated from the inhibition radius when no PEF was applied using the same Eq. (1)] (Fig. 5 and Table 2). For example, E_c was reduced from 308.8 V/mm at PEF-alone treatments to 155.6 V/mm at PEF+Pen G 5,000 units/mL, Strep 5 mg/mL, and Nys 625 units/mL ($p = 0.000$); at antibiotic mix alone, the equivalent E_c was 189.6 V/mm. Increasing the concentration of antibiotics significantly reduced E_c in the tested range of concentrations (Fig. 5 and Table 2).

In comparison, in our preliminary work, we showed that for $t_p = 50$ μs, $I = 500$ ms, and $N = 150$,

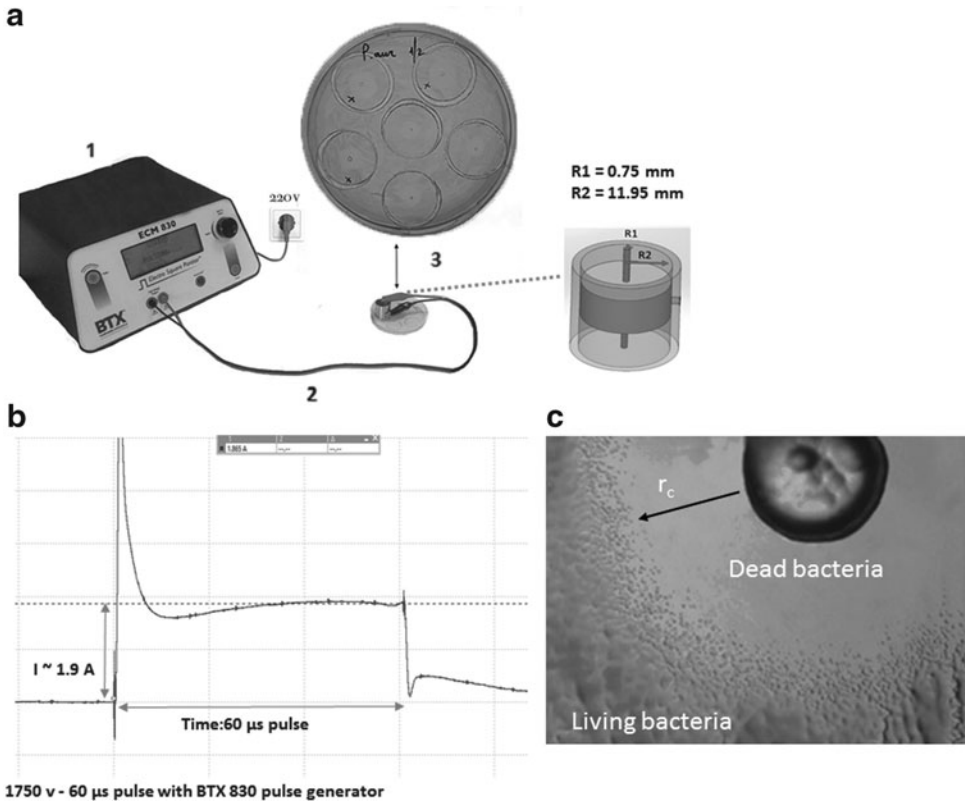


Figure 1. **(a)** Experiment setup with concentric electrode electroporation for the determination of critical electric field (E_c) for bacteria inactivation in a single step. **(b)** The shape of an electric pulse applied to the Petri dish with bacteria. **(c)** Digital image of the observed disinfected area of *Pseudomonas aeruginosa* after the application of PEF. PEF, pulsed electric field.

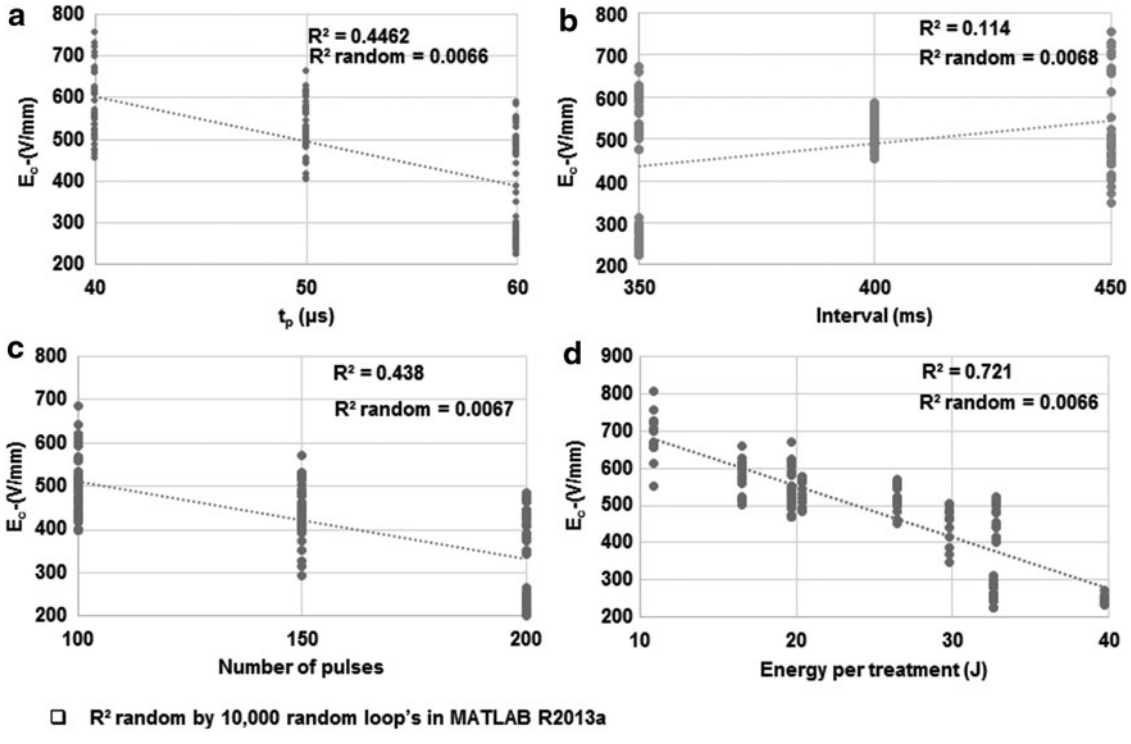
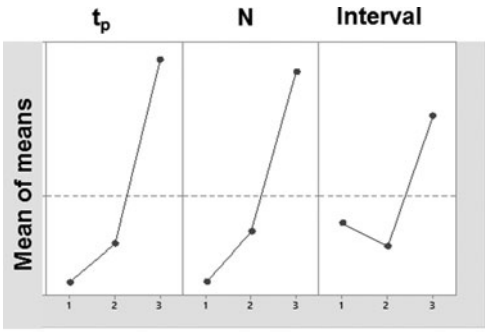


Figure 2. Individual impacts of PEF parameters on the threshold of electric field required to inactivate *P. aeruginosa* as done using Taguchi orthogonal array described in Table 2. The impacts of **(a)** pulse duration, **(b)** interval between pulses, **(c)** total number of pulses, and **(d)** total invested energy in the treatment are shown.



Level	t_p	N	Interval
1	1.836	1.844	3.119
2	2.676	2.939	2.621
3	6.692	6.422	5.465
Delta	4.856	4.578	2.844
Rank	1	2	3

Figure 3. Analysis of process factor impacts and their ranking of importance on the threshold of electric field required to inactivate *P. aeruginosa* as done using Taguchi methodology. The *top* plot shows the impact of change of each of the process factors on E_c. The *bottom* shows the ranking of each of the parameters.

the E_c for *P. aeruginosa* biofilms on surgical mesh was 235 ± 6.1 V/mm; and for $N = 300$ it was 121 ± 14 V/mm.[27] Interestingly, low-voltage (5 V), high-frequency (200 Hz) electric fields were shown to prevent biofilm formation of *P. aeruginosa.*[62] Future combinations of high- and short-voltage fields could provide an effective protection from *P. aeruginosa* biofilms by simultaneous killing and developmental prevention.[63] It is important to mention that an 8-h culture of *P. aeruginosa* was treated in this work. This may be a limitation, since the treatment of old, stable cultures may be required to truly simulate clinical infections. Additional work on the impact of culture age on PEF resistance is warranted.

For *S. epidermidis*, the addition of antibiotics reduced E_c in comparison with PEF alone (Figs. 3 and 6 and Table 3). The hurdle effect of PEF and antibiotics was also stronger than the effect of the same dose of antibiotics alone [equivalent E_c was calculated from the inhibition radius when no PEF was applied using the same Eq. (2)]. For example, E_c was reduced from 378.41 V/mm at PEF-alone treatments to 348.51 V/mm at PEF+Pen G 5,000 units/mL, Strep 5 mg/mL, and Nys 625 units/mL ($p = 0.020$). Using antibiotic mix alone, the equivalent E_c was ∞ V/mm (Table 3). However, unlike *P. aeruginosa* cultures, increasing the concentration of antibiotics significantly did not significantly reduce E_c in most of the tested concentration ranges for *S. epidermidis* (Table 3). This can be potentially explained by the fact that we may have reached drug saturation and membrane damage, and hence effectiveness on this bacteria. Stronger field strengths should be tested in future studies to determine if additional synergistic effects are possible. To the best of our knowledge, there are no previous reports describing the inactivation of *S. epidermidis* with PEF and electroporation technology. Eradicating *S. epidermidis* with PEF alone or in combination with antibiotics, as shown in this study, could provide a new direction for treating wounds[64,65] and disinfection of medical equipment,[14] where biofilms are problematic and lead to infections.

Using a combination of PEF with antibiotics, we showed that a much lower dose of antibiotics is needed to inactivate both organisms when PEF is used. This suggests that a combined therapy where antibiotics are assisted by PEF, as adjuvant, could dramatically reduce the volumes of used antibiotics, contributing to the minimization of antibiotic resistance.[66,67] Previous studies in food preservation also showed the hurdle effects of PEF with various antibiotic compounds.[68,69] We found that *P. aeruginosa* is more sensitive to PEF alone or in combination with antibiotics than *S. epidermidis.* These findings are important as *P. aeruginosa* is a much more infectious agent (25% patients with surgical wound infections had *P. aeruginosa* vs. 7% who had *S. epidermidis*[70]), suggesting that milder protocols would be used more often in clinical applications.

The observed higher sensitivity of *P. aeruginosa* to PEF than *S. epidermidis* could partially be explained by the difference in cell size and shape that affects induced transmembrane potential.[71] Previous work has shown that rod cells experience 15% higher induced transmembrane potential than elliptical cells.[72] *P. aeruginosa* cells are rods of 0.3–0.5×3 μm,[73] and the *S. epidermidis* shape is closer to spheroidal with 1–2 μm radius.[74] Previous theoretical analysis of the induced transmembrane voltage (equations 6–8 in Ref.[71]) suggests that *P. aeruginosa* cells will experience unequal induced transmembrane potential depending on the angle between surface vector of the membrane and exter-

Table 1. *L9 Taguchi matrix of pulsed electric field inactivation of* Pseudomonas aeruginosa

Experiment Number	*Voltage at* R_1	*Pulse Length* (μs)	*Number of Pulses*	*Pulse Interval (ms)*	E_c*(V/mm)*
1	1,700	40	100	450	705.7 ± 65.4
2	1,700	50	150	400	552.7 ± 35.6
3	1,700	60	200	350	295.3 ± 35.3
4	1,725	40	150	350	593.1 ± 52.5
5	1,725	50	200	450	478.2 ± 38.5
6	1,725	60	100	400	527.4 ± 40.4
7	1,750	40	200	400	520.9 ± 34.1
8	1,750	50	100	350	581.8 ± 43.8
9	1,750	60	150	450	452.9 ± 49.1

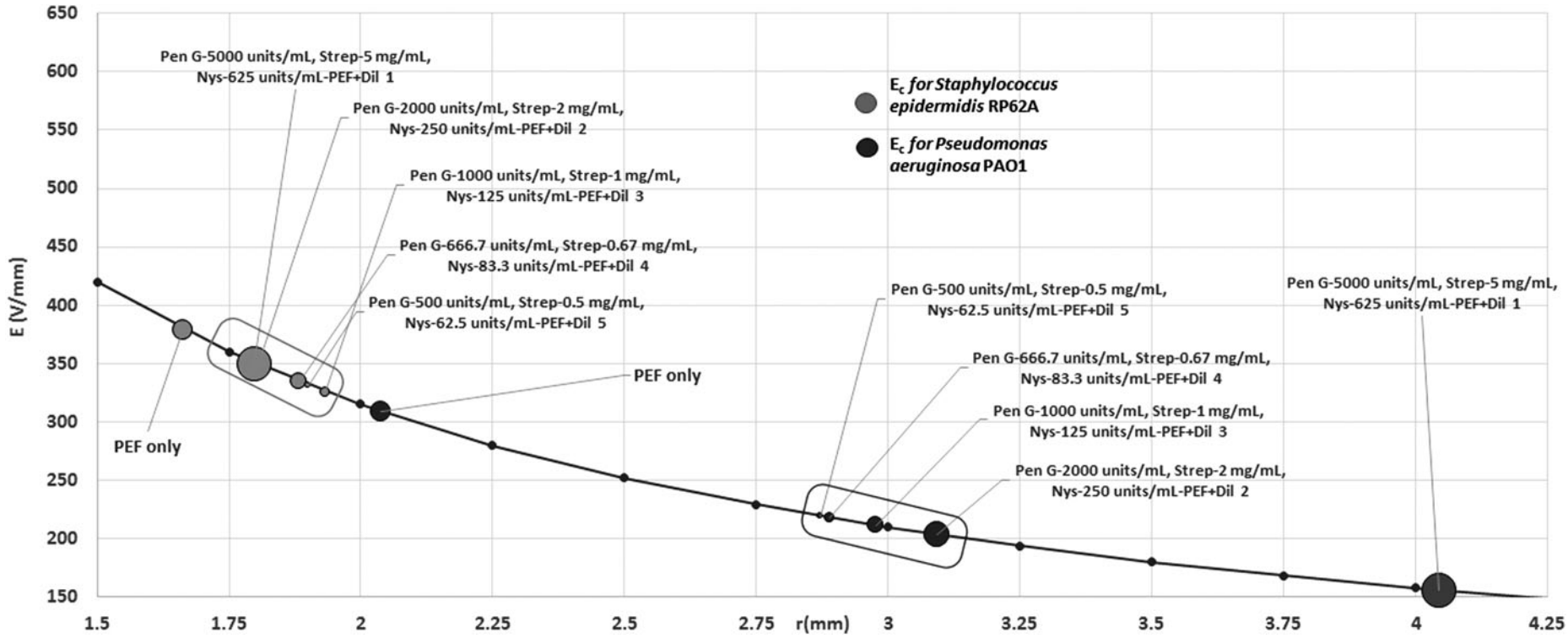


Figure 4. The threshold of electric field strength for the inactivation of *P. aeruginosa* and *Staphylococcus epidermidis* by PEF and antibiotics mix. The values of E_c for a specific dose of the antibiotic mix are shown in *dots* on the electric field curve that starts from the center of the concentric system. *r* is distance from the central point. PEF parameters: pulse duration = 60 μs; interval between pulses = 350 ms; number of pulses = 200. For each point, $N = 48$ for *P. aeruginosa* and 36 for *S. epidermidis*.

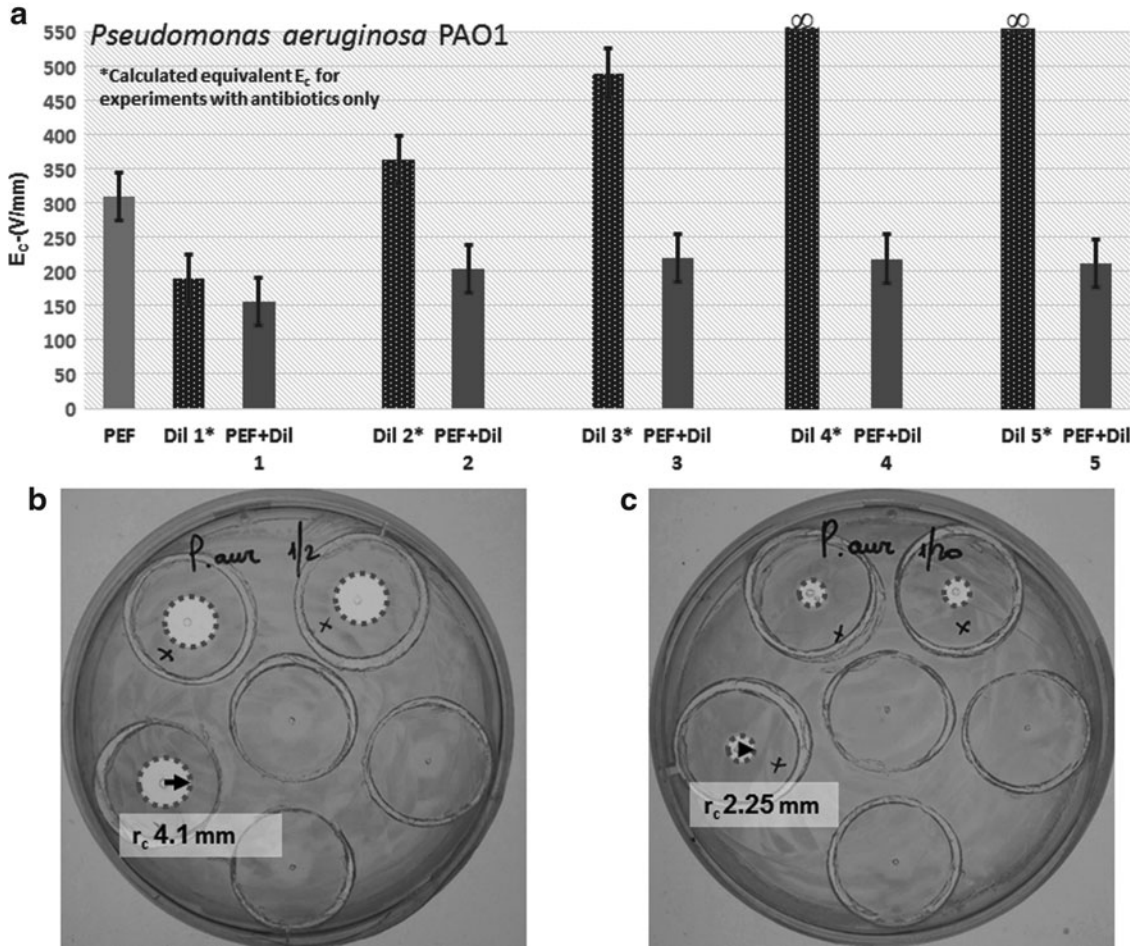


Figure 5. **(a)** The threshold of electric field, E_c, required to inactivate *P. aeruginosa* by PEF and antibiotics. For antibiotic mix treatment only, we calculated the equivalent E_c from the observed radius of inactivation. Dil 1–5 are as described in Table 3. $n=63$. **(b)** Digital image of a plate with PEF: 1,750 V, 200 pulses, 60 μs pulse length, 350 ms pulse interval combined with Pen G 5,000 units/mL, Strep 5 mg/mL, Nys 625 units/mL (Dil 1). **(c)** Digital image of a plate with PEF: 1,750 V, 200 pulses, 60 μs pulse length, 350 ms pulse interval combined with Pen G 500 units/mL, Strep 0.5 mg/mL, Nys 62.5 units/mL (Dil 5). Dil, dilution; Nys, Nystatin; Pen, Penicillin; Strep, Streptomycin.

Table 2. *Hurdle effects of pulsed electric field and antibiotics on* P. aeruginosa *inactivation*

E_c/p-*Value*	*PEF Alone*	*Dil 1*	*Dil 2*	*Dil 3*	*Dil 4*	*Dil 5*	*PEF+Dil 1*	*PEF+Dil 2*	*PEF+Dil 3*	*PEF+Dil 4*	*PEF+Dil 5*
PEF alone	308.86 V/mm/$p=0.000$										
Dil 1	$p=0.000$	189.65 V/mm*									
Dil 2	$p=0.004$	$p=0.000$	362.99 V/mm*								
Dil 3	$p=0.025$	$p=0.003$	$p=0.015$	488.93 V/mm*							
Dil 4	∞**/$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	∞**						
Dil 5	∞**/$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	—	∞**					
PEF+Dil 1	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	155.67 V/mm				
PEF+Dil 2	$p=0.000$	$p=0.022$	$p=0.000$	$p=0.002$	$p=0.000$	$p=0.000$	$p=0.000$	203.53 V/mm			
PEF+Dil 3	$p=0.000$	$p=0.022$	$p=0.000$	$p=0.002$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.013$	219.07 V/mm		
PEF+Dil 4	$p=0.001$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.002$	$p=0.112$	$p=0.282$	217.78 V/mm	
PEF+Dil 5	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.022$	$p=0.062$	$p=0.118$	211.34 V/mm

PEF parameters: pulse duration = 60 μs; interval between pulses = 350 ms; number of pulses = 200; $n=63$. Dil 1: Pen G 5,000 units/mL, Strep 5 mg/mL, Nys 625 units/mL; Dil 2: Pen G 2,000 units/mL, Strep 2 mg/mL, Nys 250 units/mL; Dil 3: Pen G 1,000 units/mL, Strep 1 mg/mL, Nys 125 units/mL; Dil 4: Pen G 666.7 units/mL, Strep 0.67 mg/mL, Nys 83.3 units/mL; Dil 5: Pen G 500 units/mL, Strep 0.5 mg/mL, Nys 62.5 units/mL.

*Equivalent to E_c.

**∞ no delay effect.

—, no value; Dil, dilution; Nys, Nystatin; PEF, pulsed electric field; Pen, Penicillin; Strep, Streptomycin.

Gray shading shows no significant difference.

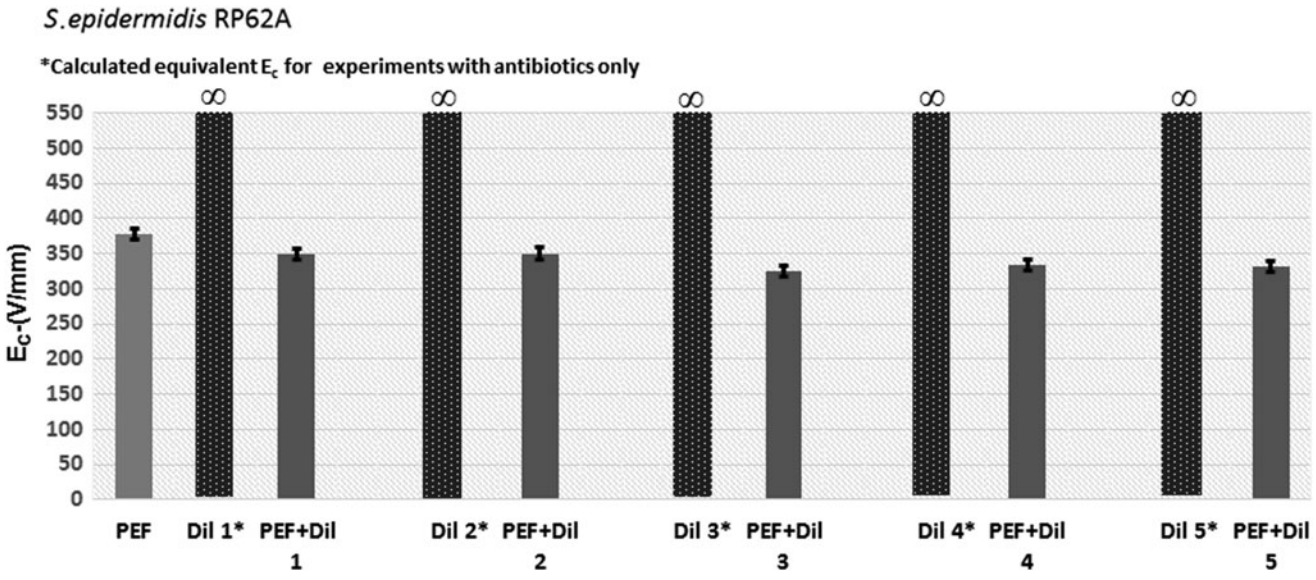


Figure 6. The threshold of electric field, E_c, required to inactivate *S. epidermidis* by PEF and antibiotics. For antibiotic mix treatment only, we calculated the equivalent E_c from the observed radius of inactivation. $n=36$. Dil 1–5 are as described in Table 4.

nal electric field lines. A higher induced transmembrane voltage develops on the cell membrane when the long side of the rod is orthogonal to the lines of external electric fields.[71] At the same time, spherical cells of *S. epidermidis* will experience equal induced transmembrane potential in all parts of the membrane. These differences imply that larger areas of the *P. aeruginosa* cell surface are exposed to larger induced critical transmembrane potential than surface areas of *S. epidermidis*.[71] We previously showed, in the example of *Listeria monosetogenes*, that large electroporated fractions of the cell membranes are correlated with cell death.[75]

As in this study, we used a concentric electrode setup, where the disinfected area around the central electrode (Fig. 1a) shows the potential disinfected areas for actual disinfection applications around a single needle (Table 4). Increasing the drug concentration increased the treated areas for *P. aeruginosa*, but had no significant effect on *S. epidermidis* disinfected areas. This could probably be explained by differences in mechanisms of bacterial resistance to drugs[76] and differences in membrane structure,[77] which impacts the PEF. Similar differences were shown for resistance to cold plasma.[78] Such an approach could address the issue of large infected surfaces if a multi-needle device is developed. Previous studies have shown that electrode shapes with a single needle could create a point of singularity that create high elec-

Table 3. *Hurdle effects of pulsed electric field and antibiotics on* S. epidermidis *inactivation*

E_c/p-*Value*	*PEF Alone*	*Dil 1*	*Dil 2*	*Dil 3*	*Dil 4*	*Dil 5*	*PEF+Dil 1*	*PEF+Dil 2*	*PEF+Dil 3*	*PEF+Dil 4*	*PEF+Dil 5*
PEF alone	378.41 V/mm/$p=0.000$										
Dil 1	$p=0.000$	∞*									
Dil 2	$p=0.000$	—	∞*								
Dil 3	$p=0.000$	—	—	∞*							
Dil 4	$p=0.000$	—	—	—	∞*						
Dil 5	$p=0.000$	—	—	—	—	∞*					
PEF+Dil 1	$p=0.020$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	348.51 V/mm				
PEF+Dil 2	$p=0.010$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.438$	350.12 V/mm			
PEF+Dil 3	$p=0.001$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.025$	$p=0.033$	325.67 V/mm		
PEF+Dil 4	$p=0.003$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.013$	$p=0.039$	$p=0.226$	334.04 V/mm	
PEF+Dil 5	$p=0.006$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.000$	$p=0.039$	$p=0.024$	$p=0.355$	$p=0.281$	331.10 V/mm

PEF parameters: pulse duration = 60 μs; interval between pulses = 350 ms; number of pulses = 200; $n=36$. Dil 1: Pen G 5,000 units/mL, Strep 5 mg/mL, Nys 625 units/mL; Dil 2: Pen G 2,000 units/mL, Strep 2 mg/mL, Nys 250 units/mL; Dil 3: Pen G 1,000 units/mL, Strep 1 mg/mL, Nys 125 units/mL; Dil 4: Pen G 666.7 units/mL, Strep 0.67 mg/mL, Nys 83.3 units/mL; Dil 5: Pen G 500 units/mL, Strep 0.5 mg/mL, Nys 62.5 units/mL.

*Equivalent to E_c.

** ∞ no delay effect.

—, no value.

Gray shading shows no significant difference.

Table 4. *Disinfected areas with a single-needle electrode area*

	P. aeruginosa ($mm^2 \pm SD$)	S. epidermidis ($mm^2 \pm SD$)
PEF	13.30 ± 2.24	8.71 ± 0.61
PEF+Dil 1	53.87 ± 15.47	10.28 ± 0.81
PEF+Dil 2	30.24 ± 3.16	10.20 ± 0.96
PEF+Dil 3	26.32 ± 4.24	11.81 ± 1.31
PEF+Dil 4	26.55 ± 3.72	11.18 ± 0.82
PEF+Dil 5	27.98 ± 2.34	11.47 ± 1.52

PEF parameters: pulse duration = 60 μs; interval between pulses = 350 ms; number of pulses = 200; n = 48 for *P. aeruginosa* and n = 36 for *S. epidermidis*. Dil 1: Pen G 5,000 units/mL, Strep 5 mg/mL, Nys 625 units/mL. Dil 2: Pen G 2,000 units/mL, Strep 2 mg/mL, Nys 250 units/mL. Dil 3: Pen G 1,000 units/mL, Strep 1 mg/mL, Nys 125 units/mL. Dil 4: Pen G 666.7 units/mL, Strep 0.67 mg/mL, Nys 83.3 units/mL. Dil 5: Pen G 500 units/mL, Strep 0.5 mg/mL, Nys 62.5 units/mL.

tric fields without electrolysis around the electrodes.[79–81] Multi-needle electrode configurations, previously developed for precise tissue volume ablation and electrochemotherapy, could be used for large surface disinfection with the parameters found in this study.[82–85]

This *in vitro* study allowed us to determine the effective protocol for bacterial inactivation by PEF alone or in combination with antibiotics. Our previous *in vivo* work on burn disinfection[25,26] showed the feasibility for the use of PEF alone in small animal models. Further translation of the PEF to wound healing clinics will require detailed safety studies as PEF will affect both bacteria and host cells. Studies on irreversible electroporation safety in humans have demonstrated that the procedure is safe,[86,87] especially if the delivery of pulses is electrocardiographically synchronized.[88] Pain studies on patients with deep-tissue tumors showed no difference in comparison with other ablation methods.[89,90] In addition, skin DNA vaccination with PEF in a pain study reported that the procedure is well tolerated.[91] However, it is important to note that the parameters used in our studies have not been tested for pain in patients. Although we have recently demonstrated a full regeneration of normal rat skin ablated by PEF,[40] human skin is different, and further studies on normal or wounded skin responses to PEF in humans are needed. Moreover, the effect of the rapid release of bacterial content in tissues after PEF on procedure safety is still to be investigated.

Furthermore, we and others have shown that PEF cell inactivation is not a deterministic, but rather a statistical event.[92,93] Numerous previous works on bacteria inactivation in the food industry led to the development of a function, which describes bacteria inactivation levels as a function of process parameters.[93,94] This implies that

KEY FINDINGS

- PEFs inactivate *P. aeruginosa* PAO1 and *S. epidermidis* RP62A.
- Combination of PEFs with antibiotics reduces the threshold of the electric field required for inactivation.
- Combination of PEFs with antibiotics increases the disinfection radius for a single-point electrode.

complete, 100%, kill of bacteria by PEF alone is not expected, and additional effects either from activated immune system responses or antibiotics are needed.

The important still open question for future studies is the role of the survived bacteria in the wound-healing process and if these bacteria could develop resistance to PEF. A previous study that used PEF to eliminate *Pseudomonas putida* in the wastewater has shown that the inactivation rate (percentage of survived bacteria) remained constant over 30 generations when each generation was grown from the survival fraction of the PEF-treated culture.[95] The fraction of bacteria could survive because of the natural variance in the membrane structure. We showed previously that a variation in membrane surface charge leads to a variance in the bacteria survival ratio.[75] One of the approaches to keep bacteria concentration lower than the level that might lead to an abnormal healing could be the intermittent delivery of PEF treatment, shown by us to be effective in water and food systems.[63,96] Although, in these previous studies, we did not find increased bacteria resistance to PEF with treatment cycles,[63,96] additional tests with a much larger number of generations are needed to investigate the long-term impact of PEF on bacteria resistance.

INNOVATION

Although the burden of wound infection is a major clinical and economical problem, no single approach to date has been found to be effective in preventing deep infection and biofilm formation in infected patients.[97] We found that the combination of PEF with antibiotics decreases the minimum threshold required to inactivate bacteria. In addition, we determined the parameters needed to disinfect specific areas with a single electrode by PEF alone or in combination with antibiotics. Larger disinfection areas were achieved using a combined approach than by antibiotics alone, suggesting that this approach could reduce the overuse of antibiotics that might lead to the emergence of antibiotic-resistant strains.

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Abbreviations and Acronyms

ΔV = potential difference between the central and peripheral electrodes (V)
E = electric field strength (V/mm)
E_c = *critical electric field required for bacteria inactivation (V/mm)*
ELS = electroporation low salt
N = *number of pulses*
Nys = nystatin
PEF = pulsed electric field
Pen = penicillin
$R1$ = radius of the inner electrode (mm)
$R2$ = radius of the outer electrode (mm)
r_c = *critical radius with no bacteria growth (mm), radius of inactivation*
Strep = streptomycin
t_p = *single pulse duration (μs)*
TRE = total relative error