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Growth in a Biofilm Sensitizes *Cutibacterium acnes* to Nanosecond Pulsed Electric Fields

Asia Poudel
Old Dominion University, apoudel@odu.edu

Adenrele Oludiran
Old Dominion University, aoludira@odu.edu

Esin B. Sözer
Old Dominion University, esozer@odu.edu

Maura Casciola
Old Dominion University, mcasciol@odu.edu

Erin B. Purcell
Old Dominion University, epurcell@odu.edu

See next page for additional authors

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Growth in a biofilm sensitizes *Cutibacterium acnes* to nanosecond pulsed electric fields

Asia Poudel [a], Adenrele Oludiran [a], Esin B. Sözer [b], Maura Casciola [b,c], Erin B. Purcell [a,*], Claudia Muratori [b,*]

[a] Old Dominion University, Department of Chemistry and Biochemistry, USA
[b] Old Dominion University, Frank Reidy Research Center for Bioelectrics, USA
[c] Center for Devices and Radiological Health, US Food and Drug Administration, Silver Spring, MD 20993, USA

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ABSTRACT

The Gram-positive anaerobic bacterium *Cutibacterium acnes* (*C. acnes*) is a commensal of the human skin, but also an opportunistic pathogen that contributes to the pathophysiology of the skin disease acne vulgaris. *C. acnes* can form biofilms; cells in biofilms are more resilient to antimicrobial stresses. Acne therapeutic options such as topical or systemic antimicrobial treatments often show incomplete responses. In this study we measured the efficacy of nanosecond pulsed electric fields (nsPEF), a new promising cell and tissue ablation technology, to inactivate *C. acnes*. Our results show that all tested nsPEF doses (250 to 2000 pulses, 280 ns pulses, 28 kV/cm, 5 Hz; 0.5 to 4 kJ/ml) failed to inactivate planktonic *C. acnes* and that pretreatment with lysozyme, a naturally occurring cell-wall-weakening enzyme, increased *C. acnes* vulnerability to nsPEF. Surprisingly, growth in a biofilm appears to sensitize *C. acnes* to nsPEF-induced stress, as *C. acnes* biofilm-derived cells showed increased cell death after nsPEF treatments that did not affect planktonic cells. Biofilm inactivation by nsPEF was confirmed by treating intact biofilms grown on glass coverslips with an indium oxide conductive layer. Altogether our results show that, contrary to other antimicrobial agents, nsPEF kill more efficiently bacteria in biofilms than planktonic cells.

1. Introduction

Cutibacterium acnes (*C.acnes*), formerly known as *Propionibacterium acnes,* is a Gram-positive, non-spore forming, facultative anaerobic bacterium that colonizes human skin [1–2]. While *C. acnes* is a largely commensal bacterium that coexists in homeostasis with the rest of the skin microbiota, it can act as an opportunistic pathogen whose overgrowth and dominance within the dermal microbiome contributes to the pathophysiology of the skin disease acne vulgaris [3–4]. *C. acnes* is capable of adhering to surfaces, including human skin, in structured microbial communities known as biofilms; cells in biofilms are more resilient to antimicrobial stresses than free-living, or planktonic, cells [5–6]. Acneic strains of *C. acnes* form biofilms inside skin-gland hollows, leading to the formation of follicular plugs and inflammation [7].

Acne vulgaris is an inflammatory disease of the human sebaceous follicle. It involves the interplay of four main factors: pathological overproduction of sebum, abnormal follicular keratinization, colonization of the pilosebaceous duct by *C. acnes* colonies and biofilms, and inflammation [8]. Acne is a very common skin disorder especially in adolescents and young adults [9]. It affects approximately 85% of adolescents but can persist also in adulthood with prevalence increasing especially in adult women 25 years and older [9–10]. The direct cost of acne treatment in the United States is $846 million per year [11].

There are two main classes of topical monotherapies for acne treatment: vitamin A derivatives called retinoids and antimicrobial agents such as antibiotics. Topical retinoids bind to various sets of retinoid acid receptors thereby conferring differences in activity, efficacy and tolerability [12]. Although retinoids are the core of topical acne treatment, their main drawback is that they are associated with a range of cutaneous side-effects in up to 75% of patients such as scaling, erythema, dryness and irritation [13].

Both topical and systemic antibiotics, most commonly clindamycin and erythromycin, are used in combination with benzoyl peroxide or retinoids to treat all grades (mild, moderate to severe) of acne [14]. The use of antibiotics to reduce the bacterial burden often show incomplete responses and may alter the composition of the skin microbiota in unfavorable ways. Following treatment failure, there is a recurrence of inflammation. The failure of antibi-

* Corresponding authors at: Chemistry and Biochemistry, Old Dominion University, (Erin B. Purcell), Frank Reidy Research Center for Bioelectrics, Old Dominion University, (Claudia Muratori), USA.
E-mail addresses: epurcell@odu.edu (E.B. Purcell), cmurator@odu.edu (C. Muratori).

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otic therapy has been associated with the emergence of antibiotic resistance in clinical isolates [15–17]. Studies have shown that up to 94% of acne patients have *C. acnes* strains on their skin that are resistant to at least one antibiotic [18].

From the above discussion it is clear that current acne therapy can be complicated by antibiotic resistance and adverse side effects.

In the present study we measured the efficacy of nanosecond pulsed electric fields (nsPEF), a new promising cell and tissue ablation technology, to inactivate *C. acnes*. The ability of pulsed electric fields (PEF) to inactivate microorganisms has been known for over 60 years [19]. Indeed, PEF are among the most promising microbial inactivation methods for liquid food [20] and wastewater [21–22]. Conventional PEF treatments use pulses of millisecond or microsecond duration to compromise the integrity of the cell plasma membrane, a process referred to as electroporation. Depending on the pulse parameters, electroporation can be reversible or irreversible. Reversible electroporation denotes the formation of pores in the cell membrane which can reseal after a specific time, while irreversible electroporation indicates a permanent damage which leads to cell death. Compared to conventional PEF, nsPEF utilizes much shorter pulses (down to 10 ns) to target not only the plasma membrane but also intracellular structure such as the endoplasmic reticulum (ER) and mitochondria [23–27]. Killing of eukaryotic cells with nsPEF has been extensively explored *in vitro* [28–35], followed by successful tumor ablation trials in animals [34–38] and in humans [39–40], without recurrence and with minimal side effects.

Recent literature has shown that nsPEF also affects bacterial cell viability [41–47]. Most studies used 10 ns pulses to treat *E. coli*. For instance, Guionet *et al*. observed a 1.5 log10 decrease in *E. coli* viability with 500, 10 ns pulses at 100 kV/cm, 1 Hz while Perni *et al*. reported a 2 log10 reduction with 9000 pulses at 100 kV/cm, 30 Hz [43–44]. These studies used different pulse parameters, exposure solutions and methods, highlighting the importance of these factors for bacterial inactivation by PEF. As with mammalian cells, the current dogma is that bacterial inactivation by PEF occurs through irreversible electroporation of the plasma membrane. However, Pillet *et al*. have recently shown that PEF exposure causes a structural disorganization of the cell wall and partial destruction of the spore coat architecture of *Bacillus pumilis* [48]. Furthermore, Chalise *et al.* have recently reported that *E. coli* inactivation by 32 ns pulses may occur as a result of damage to intracellular components [49].

Our results show that *C. acnes* in biofilm is much more sensitive to nsPEF than planktonic cells in exponential growth phase as measured by increased cell death at consistent nsPEF treatments (up to 2000 pulses, 280 ns pulses, 28 kV/cm, 5 Hz; 4 kJ/ml). Moreover, we found that pretreatment with lysozyme, a naturally occurring cell-wall-weakening enzyme found in bodily secretions (tears, saliva, and milk), increased planktonic *C. acnes* vulnerability to nsPEF.

2. Materials and Methods:

2.1. Bacterial strains and growth conditions

The bacterial strain used in this study is *C. acnes* ATCC 29,399 (American Type Culture Collection, Manassass VA). All anaerobic bacterial culture took place at 37 °C in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, 5% H_2. Cell growth was monitored by measuring the optical density at 600 nm using an Ultraspec 10 Biochrom cell density meter (Biochrom, Cambridge, United Kingdom) for up to 3 d. All plastic consumables were allowed to equilibrate in the anaerobic chamber for a minimum of 72 h prior to use. Unless otherwise noted, all reagents and supplies were purchases from Fisher Scientific (Fisher Scientific, Waltham MA).

Bacterial cultures were grown in TY medium containing 3% peptone, 2% yeast extract and 0.1% sodium thioglycolate and colonies were maintained on TY plates with 3% agar. To avoid contamination, liquid TY media was supplemented with 20 μg/ml of metronidazole (TY-Met) (Beantown Chemical, Hudson NH). Other culture media utilized in this study are 2.5% Brain-Heart Infusion supplemented with 0.5% Bacto-BD yeast extract (BHIS) and 3% agar, chocolate agar plates (Thomas Scientific, Swedesboro NJ), and anaerobic blood agar plates (Thomas Scientific, Swedesboro NJ).

C. acnes biofilm were grown for 72 h in plastic 24 well plates, either directly on the plate wells bottoms or on glass coverslips. Growth surfaces were coated with 100 μl of 0.1% fibronection (Thomas Scientific, Swedesboro NJ), poly-D-lysine (Millipore Sigma, Burlington MA), or poly-L-lysine (Thomas Scientific, Swedesboro NJ) and dried for 4 h before equilibrating for another 72 h in anaerobic chamber prior to usage. Exponentially growing *C. acnes* cultures at OD_{600} between 0.5 and 0.8 were diluted at 1:3 into fresh TY-Met media and 2 ml of the diluted culture were directly applied on to the plates which were further incubated for 72 h for the biofilm formation. To visualize biofilm formation, liquid cultures were removed by pipetting and surfaces were washed with phosphate buffered saline (PBS) at pH 7.0. 0.1% crystal violet was applied for 30 min and washed twice with PBS.

2.2. Pulsed electric field exposure methods.

C. acnes samples were prepared by inoculating single colonies into 3 ml of TY-Met medium and growing for 48 h at 37 °C. Starter cultures were diluted 1:10 into TY-Met medium to reach OD_{600} = 0.5–0.8. One hundred μl samples of this suspension were loaded into 1 mm gap electroporation cuvettes (BioSmith, San Diego, CA), which were closed, sealed with parafilm and brought outside of the anaerobic chamber for nsPEF treatments. In each experiment, samples were kept outside of the chamber for a maximum of 1 h. Samples were exposed to either nsPEF or sham exposure in TY medium with a conductivity of 0.73 S/m at room temperature (22 ± 2 °C). Trapezoidal pulses of 280 ns duration (100 to 2000, 5 Hz, 28 kV/cm; 0.2–4 kJ/ml) were produced by a custom pulse generation system (Fig. 1A) with an adjustable pulse amplitude (up to 15 kV), duration (200 to 1000 ns) and frequency (1–100 Hz; Pulse Biosciences, Inc., Hayward, CA). The waveform of a 280 ns pulse, 28 kV/cm is reported in Fig. 1B.

For nsPEF exposure of biofilms-derived cells, samples were harvested by scraping the biofilm from the growth surface. Cells were suspended in 1 ml TY medium and vortexed to disrupt clumps [50] before being aliquoted into electroporation cuvettes for the treatment described above. Samples from each experiment were diluted to the same optical density (OD_{600} = 0.5–0.8). Exposure of intact biofilms was accomplished by growing *C. acnes* biofilms on glass coverslips with an indium tin oxide (ITO) conductive layer and placing these coverslips into 1 mm gap electroporation cuvettes filled with 100 μl of TY medium. The ITO layer was deposited on one side of the glass coverslips by Diamond Coatings (Halesowen, UK). During nsPEF exposure, the glass surface of the coverslip was resting on the anode and the ITO surface with cells was facing the cathode. The electric field (E) distribution was calculated with Sim4Life light (ZMT ZurichMedTech AG, Zurich, Switzerland) in ohmic quasi-static conditions similarly to what was previously described [51–52]. Two parallel electrodes (20.55 × 12.00 × 1.58 mm^3, perfect electric conductors) mimicking the electroporation cuvette were filled with medium (0.73 S/m). The coverslip was modeled as a dielectric cylinder of 8.2 mm diameter, 125 μm thickness with conductivity 0.0043 S/m and coated with a 25 μm think layer of ITO (1.3 MS/m). The coverslip was placed in contact with

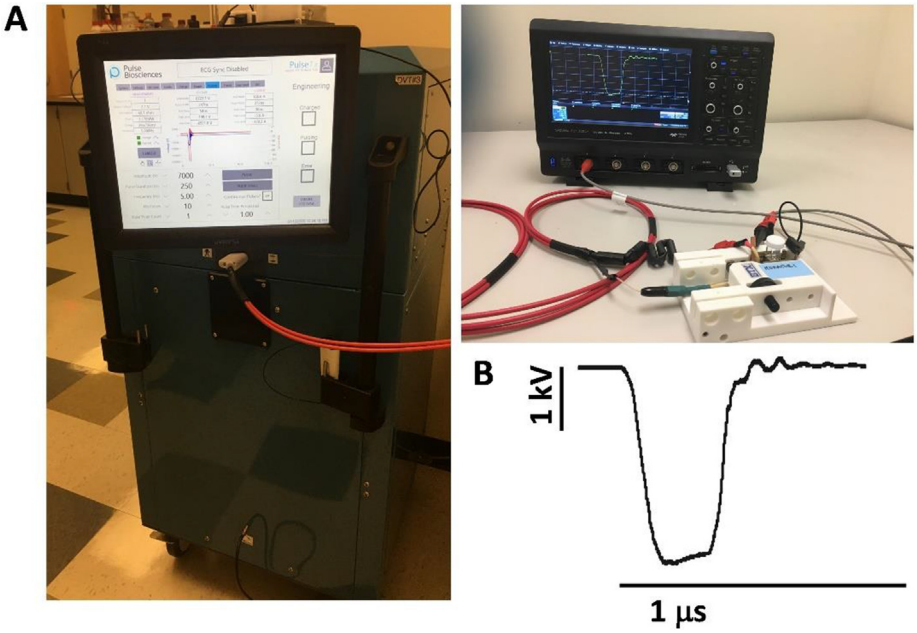

Fig. 1. Pulsed electric field exposure system. A. Trapezoidal pulses of 280 ns duration were produced by a custom pulse generation system and delivered to 1 mm electroporation cuvettes. A digital oscilloscope was used to monitor the pulse amplitude and shape at the cuvette. B. The shape of a 280 ns, 28 kV/cm electric pulse.

the anodic electrode with the ITO layer facing the cathode. A 50 × 50 × 50 mm^3 cube of air surrounded the electroporation cuvette.

The mesh chosen to discretize the domain of simulation resulted in 6. 6.762 MCells elements. For every 100 V applied across a 1 mm gap cuvette, the simulated E field at the ITO surface was 0.37 kV/cm. Considering variation in the cuvette gap and in the coverslip thickness the E field was rounded to 0.4 kV/cm. Since in this study 900 V were applied during the experimental procedure, the E field applied to the bacteria was 3.6 kV/cm.

Treatment energies are reported based on estimation of the total energy (W) delivered to the electroporation cuvettes using the equation $W = \frac{V^2}{Z} \times t_p \times n_p$, where V is the voltage across the cuvette (2.8 and 0.9 kV for suspension and ITO coverslips, respectively), Z is the impedance of the cuvette containing the samples (~10 Ω),t_p is the pulse duration (280 ns) and n_p is the number of pulses (100 to 2000).

In selected experiments, both planktonic cells and biofilm-derived cells were incubated anaerobically with lysozyme (1 to 10 mg/ml; MP Biomedicals, Santa Ana, CA) for 1 h at 37 °C prior to nsPEF treatment.

Sample heating was measured using a thermocouple thermometer (Physitemp, Clifton, NJ).

2.3. Determination of inactivation rates

Immediately after treatment, both planktonic and biofilm bacteria samples were returned to the anaerobic chamber. Nine hundred µL of TY medium was added to each cuvette mixed by pipetting. The resulting 1 ml samples were serially diluted to 10^{-5}, 100 µl of each sample was plated on triplicate TY-met plates and colonies were enumerated after 72 h. Only counts between 0 and 300 CFU per plate were considered. Inactivation rates are expressed as $\log_{10}$ CFU/ml differences between sham and nsPEF exposure samples. All experiments were performed in triplicates and repeated at least three times unless otherwise stated.

2.4. Statistical analyses

Data are presented as mean ± SE for n independent experiments. Statistical analyses were performed using a two-tailed t-test where p less than 0.05 was considered statistically significant. Statistical calculations, including data fits, and data plotting were accomplished using Grapher 11 (Golden Software, Golden, CO).

3. Results

3.1. Optimization of C. Acnes growth in vitro

C. acnes is a slowly growing bacterium that exists in nature largely as biofilms; as such, it has been difficult to cultivate clinical samples *in vitro*, which has led to underdiagnosis in implant devices or bone or joint infections [53–54]. Reported cultivation times range from 7 to 14 d for its isolation and identification [55–56]. Reported doubling times *in vitro* are 5–6 h, with liquid cultures reaching stationary phase in 3–4 d [2]. Such prolonged cultivation increases the risk of laboratory contamination [57–58]. Our initial experiments sought to establish optimal laboratory growth conditions for the planned experiments. Therefore, we tested different growth media commonly used to culture anaerobes and incubation times. Cells were cultured on supplemented brain heart infusion (BHIS) agar, tryptone yeast (TY) agar, chocolate agar or anaerobic blood agar [59–61]. All media yielded round, opaque, and mucoid colonies after 72 h of growth (Fig. 2A). The chocolate agar plates showed both small and large colonies which could have been due to *C. acnes* heterogeneity or contamination (Fig. 2A). Because TY medium showed high consistent colony counts and is cheaper than the other options tested, growth in liquid culture was assessed in TY. TY medium contains sodium thioglycolate, previously shown to facilitate *C. acnes* growth by regulating medium redox potential [56–57]. To avoid contamination, liquid medium was supplemented with 20 µg/ml metronidazole, to which *C. acnes* is resistant [62–63]. We found that *C. acnes* grows at a moderate speed in this medium and reaches a stationary optical density of 1.2–1.4 within 72 h (Fig. 2B). 72 h was also

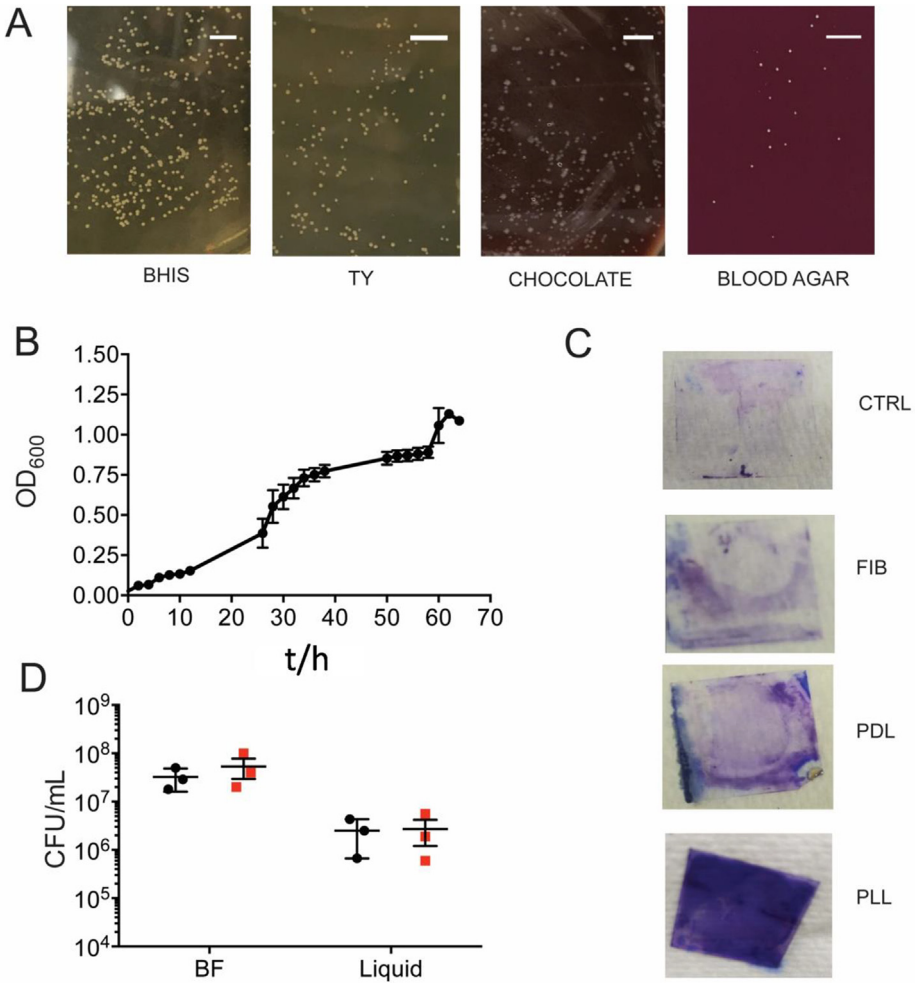

Fig. 2. *C. acnes* growth condition optimization: planktonic vs. biofilms. A. *C. acnes* cells from a frozen glycerol stock were aliquoted into TY medium, let it grow overnight, and plated on the indicated agar plates. Cells on BHIS, TY, and chocolate agar plates were plated at a 10^5 dilution while cells on blood agar plates were seeded at a 10^6 dilution. Scale bar:5 mm. B. Growth of planktonic *C. acnes* over 72 h. C. Crystal violet staning of *C. acnes* biofilms grown for 72 h on glass coverslips with no coating (CTRL) and coated with 0.1% fibronection (FIB), 0.1% poly-D-lysine (PDL), or 0.1% poly-L-lysine (PLL). D. Biofilm (BF) and liquid culture viability after 60 min incubation in an electroporation cuvette in anaerobic (black) or aerobic (red) conditions. Mean +/- s.e. for both B and D.

sufficient time for the growth of robust *C. acnes* biofilms on glass or plastic (not shown) surfaces. Cellular adhesion to abiotic surfaces was greatly enhanced by coating with poly-L-lysine while coating with fibronection or poly-D-lysine had more modest effects (Fig. 2C). Because *C. acnes* cultures in electroporation cuvettes needed to be taken out from the anaerobic chamber for a maximum of 1 h for the pulse treatment, the effect of oxygen exposure from seal leakage on cell viability was assessed. Planktonically growing cells and cells scraped out of biofilms were diluted and plated for viability after 1 h of incubation at 22 °C in aerobic or anaerobic conditions. After 1 h, all samples were returned to the anaerobic chamber, diluted, and plated to assess viability. We found that 1 h of passive exposure to environmental oxygen had no meaningful effect on *C. acnes* viability, consistent with previous reports of its aerotolerance (Fig. 2D) [56,64].

3.2. Planktonic C. Acnes in exponential growth phase are resistant to nsPEF

PEF treatments are intended to be a non-thermal method to inactivate microorganisms. However, it is well known that an increase in temperature due to Joule heating can be associated with high pulse doses. To exclude any potential heat inactivation effect, in preliminary experiments we measured the temperature increase associated with the pulse treatment. The highest pulse dose we could test with our exposure system, namely 2000 pulses, 280 ns, 28 kV/cm at 5 Hz, increased the sample temperature from 20.4 ± 0.2 to 30.9 ± 0.8 °C. Because *C. acnes* exhibits maximum growth at human body temperatures (30 °C and 37 °C) [65], nsPEF-induced heating was discounted as an inactivating variable. We then tested the effect of increasing numbers of 280 ns pulses on the viability of planktonic *C. acnes* in exponential growth phase. The viability of the planktonic cells was not meaningfully affected by any of the pulse doses tested (Fig. 3). Specifically, 2000 pulses, 280 ns pulses, 28 kV/cm at 5 Hz (4 kJ/ml), led to only 0.2 ± 0.02 log10 reduction in *C. acnes* viability.

3.3. Lysozyme increases planktonic C. Acnes sensitivity to nsPEF

Different types of bacteria have different sensitivity to PEF. For instance, it is well established that Gram-positive bacteria are less susceptible to electrotransformation than Gram-negative bacteria due to the structure and density of their cell walls [66]. In many cases increasing the fragility of the cell wall increases the transformation efficiency significantly [67]. Among these cell-wall-weakening agents, lysozyme (LY) is a naturally occurring enzyme found in bodily secretions such as tears, saliva, and milk, and is considered a part of the innate immune system in most mammals

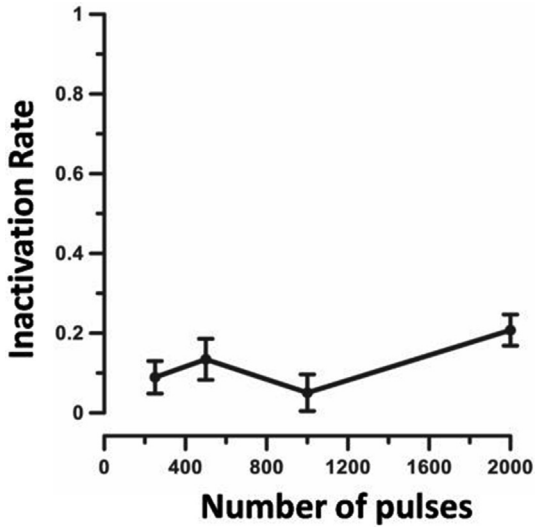

Fig. 3. Effect of nsPEF on planktonic *C. acnes* viability. Cells in exponential phase were treated with increasing numbers of 280 ns pulses (250 to 2000 pulses, 28 kV/cm, 5 Hz; 0.5 to 4 kJ/ml) and viability was measured at 72 h post treatment. Inactivation rates are expressed as $\log(CFU/ml)_{sham} - \log(CFU/ml)_{nsPEF}$. Mean +/- s.e., n = 3–5.

[68]. LY degrades peptidoglycan in the bacterial cell wall and is it used in skin care and as a safe adjunct to antifungals. Incubation of *C. acnes* supernatant with LY at various concentrations reduced the *C. acnes* activity [69], and LY-triclosan complexes were found to significantly enhance bactericidal activity against several strains of Gram-positive and Gram-negative bacteria [70]. We therefore asked whether destabilizing the cells wall of *C. acnes* with LY increased cells sensitivity to nsPEF. Planktonic *C. acnes* cells were treated with 0, 1 or 10 mg/ml LY at 37 ^{0}C. After 1 h, samples were either exposed to 280 ns pulses (1000 and 2000 pulses, 28 kV/cm, 5 Hz; 2 and 4 kJ/ml) or left untreated as parallel sham controls, and colonies were counted at 72 h post treatment. Our results show that treatment with either nsPEF alone or lysozyme alone did not affect *C. acnes* viability (Fig. 4). Indeed, lysozyme increased C. acnes growth suggesting that perturbation of the cell wall may accelerate cell division. However, combining a pretreatment of *C. acnes* with 10 mg/ml LY with nsPEF significantly increased *C. acnes* inactivation. Our results reveal for the first time the synergistic effect between LY and nsPEF at killing Gram-positive bacteria.

3.4. nsPEF inactivate C. Acnes in biofilms

Recent studies indicate that *C. acnes* biofilm formation plays a significant role in the chronic course of acne vulgaris [1]. To measure the effect of nsPEF on *C. acnes* biofilms viability, cells were grown on poly-L-lysine coated plastic well for 72 h. In contrast to our results with planktonic cells (Fig. 3), Fig. 5 A shows that biofilms were significantly inactivated by nsPEF (100 to 2000 pulses, 280 ns pulses, 28 kV/cm, 5 Hz; 0.2 to 4 kJ/ml). Because planktonic cells were collected in exponential growth phase while biofilm were harvested in stationary phase, we asked whether nsPEF sensitivity correlated with the bacteria growth phase. In Fig. 5 B, *C. acnes* cells were grown as biofilm for 3 d and both free-floating bacteria in the biofilm supernatant and plastic attached biofilms were treated with nsPEF. Our results show that even when planktonic cells and biofilms were kept under the exact same culture conditions, nsPEF killed more efficiently bacteria in biofilms (Fig. 5 B).

Our results show that while all tested nsPEF doses failed to inactivate planktonic *C. acnes*, they significantly impaired bacteria in biolfilms. We therefore tested whether a pretreatment with LY could further increase biofilm sensitivity to nsPEF. *C. acnes* biofilms were pretreated with 10 mg/ml LY for 1 h before nsPEF (500 and 1000 pulses, 280 ns pulses, 28 kV/cm, 5 Hz; 1 and 2 kJ/ml) and viability was measured at 72 h post treatment. Fig. 5 C shows that LY failed to boost *C. acnes* biofilm sensitivity to nsPEF. Because biofilms produce a thick extracellular matrix which is highly resistant to drug penetration, one can speculate that LY in our experiments did not reach a sufficiently high concentration to affect the bacteria cell wall.

In Fig. 5 biofilms were disrupted with a scraper in order to transfer the sample into the electroporation cuvettes. In order to test intact biofilms on their original substrate, we next grew biofilms on glass coverslips with an indium oxide (ITO) conductive layer. When using these coverslips, nsPEF exposures are accomplished simply by aseptically placing a coverslip with the adherent

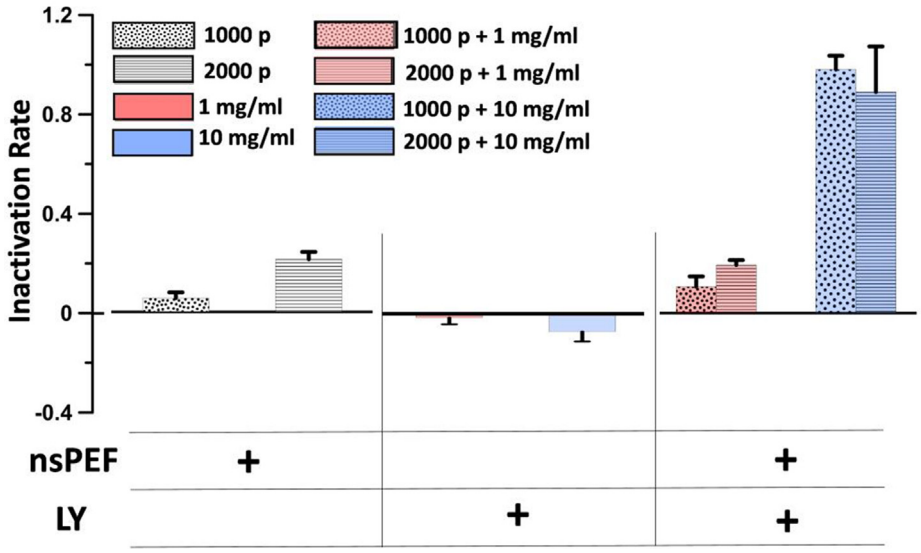

Fig. 4. Synergistic cytotoxicity from combination of LY treatment and nsPEF. Planktonic *C. acnes* cells in exponential phase were treated with either nsPEF (1000 and 2000 pulses, 280 ns, 28 kV/cm, 5 Hz; 2 and 4 kJ/ml) or LY (1 or 10 mg/ml) for 1 h or both and viability was measured at 72 h post treatment. Inactivation rates are expressed as $\log(CFU/ml)_{sham} - \log(CFU/ml)_{nsPEF}$. Mean +/- s.e., n = 3–5.

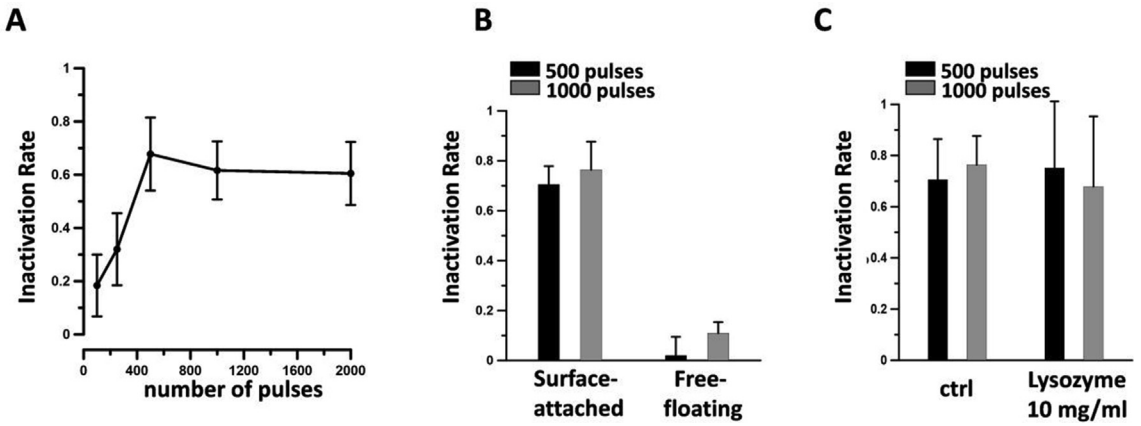

Fig. 5. Effect of nsPEF on *C. acnes* biofilms. In A *C. acnes* cells were grown as biofilms for 72 h and, before nsPEF, rinsed with growth medium to remove planktonic bacteria, scraped from the plastic surface, and aliquoted in electroporation cuvettes. Samples were treated with increasing numbers of 280 ns pulses (100 to 2000 pulses, 5 Hz, 28 kV/cm; 0.2 to 4 kJ/ml) and viability was measured at 72 h post treatment. In B *C. acnes* biofilms were grown for 3 d and both surface attached biofilms and free-floating cells in the biofilm suspension were treated with nsPEF (500 and 1000 pulses, 300 ns duration, 28 kV/cm, 5 Hz; 1 and 2 kJ/ml). Viability was measured at 72 h post treatment. In C *C. acnes* biofilms were treated with 10 mg/ml LY or left untreated (ctrl). After 1 h cells were treated with nsPEF (500 and 1000 pulses, 280 ns duration, 28 kV/cm, 5 Hz; 1 and 2 kJ/ml) and viability was measured at 72 h post treatment. Inactivation rates are expressed as $\log(\text{CFU/ml})_{\text{sham}} - \log(\text{CFU/ml})_{\text{nsPEF}}$. Mean +/- s.e. n = 5–8, n = 3–8 and n = 3–5 for A, B and C, respectively.

biofilm into 1 mm gap electroporation cuvette and delivering nsPEF, thus eliminating the steps of biofilm detachment (Fig. 6 A). Cells were exposed to 500 or 1000 pulses (280 ns, 5 Hz; 0.1 and 0.2 kJ/ml) at 900 V, which generated a practically uniform electric field of 3.6 kV/cm at the coverslips surface. Even under these conditions, which eliminated stressful cell handling and possible confounding impact of detachment of cells, *C. acnes* biofilms were efficiently killed by nsPEF (Fig. 6 B). Notably, our results are in agreement with previous research showing that nsPEF treatments of cells on ITO coverslips are highly efficient, requiring about 10-fold lower electric fields than the one used for cells in suspension [71].

Altogether our results show that, contrary to other antimicrobial agents, nsPEF kills more efficiently bacteria in biofilms than planktonic cells.

4. Discussion

Dysbiosis in the growth of *C. acnes* turns a commensal skin bacterium into an opportunistic pathogen whose effects can range from a decrease in quality of life due to acne vulgaris to life-threatening deep-tissue infections due to *C. acnes* biofilm growth on surgical implants [11,62,72]. Treatment of acne vulgaris with antibiotics can contribute to the spread of antimicrobial resistance (AMR) and is frequently unsuccessful due in part to the increased resilience of bacterial cells growing in biofilms [5,73–74]. The need for new treatment options that are effective against biofilms and do not contribute to AMR is evident.

The effect of electrical currents and electrical fields on bacterial biofilm viability has been subject of a limited number of prior investigations. For instance, long, 50 μs duration electric pulses were used to measure critical electric fields and number of pulses needed to kill *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilms and were proposed as a treatment option in combinatory protocols with systemic antibacterial therapy [75]. Another approach utilizes continuous DC low electric fields to treat biofilms derived from multiple bacteria species including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *P. aeruginosa*, either alone or in combination with standard biocides [76–78]. While these studies show promising results, none has directly compared the sensitivity to electrical stimuli of planktonic cells to that of the same bacteria strain grown as a biofilm.

Our results show that *C. acnes* cells growing planktonically in liquid culture are minimally affected by nsPEF even though the estimated induced membrane potential based on Schwan equation [79] exceeds the commonly accepted irreversible electroporation threshold of 1 V for most of the cell surface except the equatorial regions (cell radius is assumed to be 1 μm). This estimation, however, does not account for the effect of the cell wall and non-spherical shape of the bacterial cells, which can be significant.

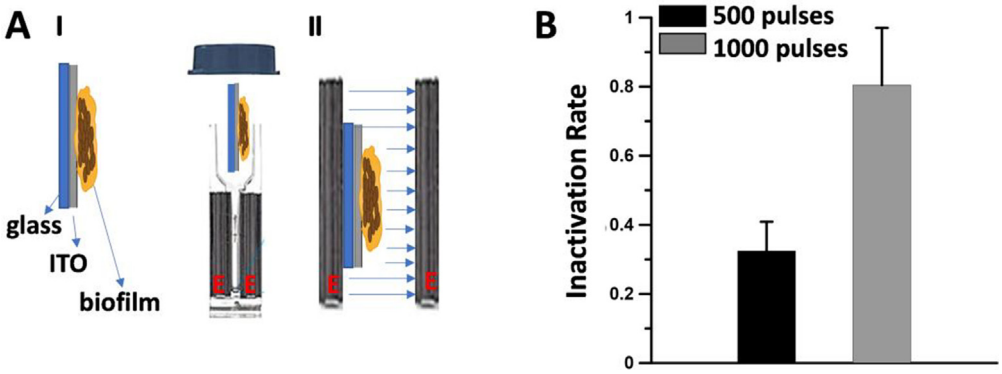

Fig. 6. Effect of nsPEF on undisrupted biofilms. A. Schematic explaining nsPEF delivery to biofilms on ITO glass coverslips in an electroporation cuvette (I). Panel II shows the enlarged view of the gap between the two electrodes (E) with the electric field lines. In B biofilms were exposed to either 500 or 1000 pulses (280 ns, 5 Hz, 3.6 kV/cm; 0.1 and 0.2 kJ/ml) and viability was measured at 72 h post treatment. Inactivation rates are expressed as $\log(\text{CFU/ml})_{\text{sham}} - \log(\text{CFU/ml})_{\text{nsPEF}}$. Mean +/- s.e., n = 9.

Notably, our results are consistent with prior studies using Gram-positive bacteria [47,80–81]. For instance, using nsPEF doses similar to the one utilized in this study, namely 1000, 300 ns pulses at 20 kV/cm, Vadlamani and colleagues reported a 0.2 log10 reduction of *Staphylococcus aureus* viability [81]. Moreover, Martens and colleagues measured less than 0.4 log10 reduction in *Lactobacillus acidophilus* viability when using 1000, 600 ns pulses at 13.5 kV/cm [80]. Altogether these results are consistent with previous literature showing that bacteria are far more resistant to PEF treatments than mammalian cells with Gram-positive bacteria being the most resilient.

Surprisingly, and contrary to the effect of any other antimicrobial agent, nsPEF appear to affect the viability of *C. acnes* in biofilms more effectively than that of cells growing in suspension. A biofilm is defined as a microbial aggregate embedded in an extracellular matrix (ECM) which protects cells from harmful environmental challenges, such as UV exposure, metal toxicity, acid exposure, dehydration, phagocytosis and several antibiotics and antimicrobial agents [82]. The ECM is composed of polysaccharides, proteins, nucleic acids, lipids and other biomolecules [83–84]. These components serve as key structural elements but also support other functions such as serve as signals, promote migration and genetic exchange, and serve as ion reservoirs. It is therefore reasonable to assume that the damage caused by intense electric fields to the structure and biological processes associated with the ECM can explain our results. For instance, damage to the dielectric components of the ECM may contribute to nsPEF cytotoxic effects. Electric fields are well known to cause structural defects in lipid bilayers [85]. Therefore, in addition to the cell membrane, nsPEF exposure may cause disruptions to extracellular lipidic structures in the ECM such as outer membrane vesicles (MVs). Both Gram-negative and Gram-positive bacteria including *C. acnes* produce extracellular MVs. These vesicles have been shown to contribute to diverse biological processes, including biofilm development, electron transfer, virulence, quorum sensing, phage decoy and horizontal gene transfer [86–89]. MVs contain peptidoglycan, virulence factors, cytoplasmic proteins, as well as DNA and RNA [86–87]. In biofilms MVs interact with eDNA in the ECM to enhance structural integrity and to serve as decoys to protect biofilm cells from antibiotics [90–91]. Moreover, intense electric fields could potentially affect the conformation and function of the ECM associated proteins [92–93] as well as the highly negatively charged phosphate backbone of DNA in extracellular DNA (eDNA).

Compared to planktonic cells, bacteria in biofilms are well-organized communities capable of coordinated behavior. In planktonic populations, danger and chemical signals produced by the cells are simply not concentrated enough when passed through the medium to be sensed by nearby cells. However, in biofilms, the ECM holds cells close together allowing concentrations of cell-produced chemical signal molecules to build up in sufficient quantity to cause changes in cellular behavior. Therefore, one can speculate that the damage caused by nsPEF to both the bacteria plasma membrane and ECM components can be sensed and communicated via cell-to-cell signaling in bacteria in biofilms much more effectively than in planktonic cells thus resulting into an enhanced collective cytotoxic response.

Considering that 60 to 80% of bacterial infections in humans are believed to be caused by bacteria growing in biofilms, our findings warrant further research into the effects of PEF treatments on microbial communities.

5. Conclusion

Our results show for the first time that bacteria growing as biofilms are more sensitive to PEF treatments than their planktonic counterpart. Future work will focus on extending our findings to other species of bacteria biofilms, assessing the relevance of pulse parameters such as pulse width and amplitude as well as measuring potential synergistic effects between PEF treatments and standard biocides.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution

C.M. and E.B.P. conceived and designed the study. A.P., A.O., M.C. and C.M. conducted the experiments and data analysis. All authors discussed and interpreted the data. C.M., E.B.P., A.P. and E.B.S. wrote and edited the manuscript.

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