**Developing a reversible electroporation model of bacteria based on rate permeabilization measurements of hydrophilic and hydrophobic compounds in a moderate electric field**

**Abstract**

**1. Scientific background**

**1.1 Pulsed electric field**: Applying an external pulsed electric field (PEF) to microbial cells leads to an increase in their membrane permeability, a phenomenon which is termed electroporation (Sale and Hamilton, 1976). Based on theoretical studies and experiments, it was suggested that this phenomenon appears when the external electric field exceeds the capacity of the cell membrane potential. This leads to mechanical changes and the creation of hydrophilic pores where water molecules can enter through the membrane lipid bilayer, thus causing the polar head groups of adjacent phospholipids to face toward the water (Neumann et al., 1999; Gehl 2003; Kotnik et al., 2015). The electric field range created from electroporation depends on the cell size and type along with the medium components such as osmolarity and electrical conductivity [Pucihar et al., 2014; Baldwin et al., 2010]. The electric fields produced by electroporation are divided into four ranges according to the membrane electroporation characterization and are described below [Yarmush el al., 2014]:

1. No detectable electroporation – Below a threshold of a specific electric field strength, regardless of the duration, nodetectable electroporation is produced [Kramar et al., 2007; Pucihar et al., 2014].
2. Reversible electroporation – This electric range is characterized by pore formation which leads to transport of molecules in and out of the cells. In this range the phenomenon of pore resealing, where most of the electroporated cells retain their viability, may occur depending on the electric parameters and environmental conditions.
3. Non-thermal irreversible electroporation – The pores reseal too slowly or not at all, leading to the release of cell contents.
4. Irreversible electroporation with thermal damage – The electric current increases the temperature which leads to a denaturation of the released molecules [Kotnik et al., 2015]. The pore creation occurs in less than a second, while resealing has been reported to occur over a range of minutes or even hours [Kinosita, and Tsong 1977; Lee et al., 1992].

**1.2 Viable but non-culturable (VBNC) cells:** It is common to evaluate bacterial viability based on their ability to replicate [Espina et al., 2016], and the lack of replication is considered as nonexistence of microbial life [Emerson et al., 2017]. However, for many microorganisms such as bacteria the designation of being categorized strictly as a dead or livecellis not clear. In addition, the complete processes involved with cell life to cell death along with how cells repair after injury remain to be elucidated [Davey 2011; Schottroff et al., 2018]. Viable but non-culturable (VBNC) cells, as well as sub-lethally injured microorganisms, are important cell states that may be induced by stressful conditions such as heat treatment, ultraviolet radiation, hydrostatic pressure, cold plasma, pulsed light, and PEF treatment [Schottroff et al., 2018; Colwell 2009]. It is important to be able to identify and differentiate when a cell is injured or when in a state of VBNC. VBNC cells are characterized as being able to maintain membrane integrity while exhibiting low gene expression and reduced metabolic activity. However, in this state the cell’s ability to create colony forming units (CFUs), the golden standard of characterizing cell viability, is inhibited [Ayrapetyan, and Oliver, 2016]. Suitable environmental conditions have been shown to revive cells out of the VBNC state [Ramamurthy et al., 2014]. In contrast to VBNC cells, sub-lethally injured cells can still replicate and form CFUs but they do so very slowly and only when provided with more nutrient rich, non-selective growth media [Li et al., 2014]. After being exposed to more suitable conditions, the cell recovery mechanisms activate and can restore the cells’ ability to function and grow normally again [Espina et al., 2016].

**1.3 PEF factors which influence VBNC bacterial cells:** The ability of PEF to influence cell viability depends on three main parameters and their sub-parameters:

1. The electrical parameters, which include: the electric field strength and treatment duration; pulse number and amplitude as well as pulse width and shape; pulse frequency and unipolar or bipolar mode of pulses. In addition, the electrode configuration, treatment chamber geometry as well as continuous or batch treatment affect cell viability [Raso et al., 2016; Donsì et al., 2005];
2. The targeted cell type,including: eukaryote or prokaryote, genus, species, and if they are in a vegetative or spore state [Pillet et al., 2016], along with cell growth phase, size, shape, orientation in the electric field and cell concentration [Raso et al., 2016];
3. Treatment medium and its composition, pH, temperature, and conductivity values [Raso et al., 2016; Álvarez et al., 2002].

After PEF treatment the combination of these parameters influences cell susceptibility to reversible membrane permeability for a period of time until the membrane returns to its original state: or, certain combinations lead PEF treatment to irreversible permeabilization and cell death [Raso et al., 2016; Mahnič-Kalamiza et al., 2014]**.**

Research indicates that Gram-positive bacteria may be more resistant than Gram-negative bacteria to PEF treatment (REFs). It was suggested that the thick peptidoglycan layerof Gram-positive bacteria protect them from PEF damage [Schottroff et al.,2017; Hülsheger et al., 1983]. However, after testing several types of Gram-negative and Gram-positive bacteria, García et al. (2005) reported that the most PEF resistant bacterial types depended on the medium pH. This research group found the Gram-negative bacteria *Escherichia coli* O157:H7 exhibited higher resistance to PEF treatment at pH 4, while Gram-positive *Listeria monocytogenes* possessed higher resistance to PEF treatment at pH 7 [García et al., 2005]. Due to their larger cell sizes, yeasts are more sensitive to PEF than Gram-positive bacteria. However, yeasts are more resistant to PEF effects than Gram-negative bacteria, probably due to the enrichment of S-S bonds in the yeast walls that seem to stabilize the cells against PEF [Shamtsyan, 2012]. Thus, it is important to understand the impact of the cell wall on the electroporation process and is a major focus of the proposed research.

**1.4 Irreversible electroporation** **applications**: The application of irreversible electroporation technology for bacterial disinfection has been widely employed in industries involving water purification and for the disinfection of liquid-based foods. This technology is considered a "clean" method which does not use heat and thereby also does not change food taste, flavor, or color [Evrendilek et al., 2013; Sobrino-López and Martin-Belloso, 2009; Amiali et al., 2006; Puertolas et al., 2009]. In addition, irreversible electroporation has been shown to be useful for the extraction of molecules from cells. For example, in Gateau et al. 2020 the team used PEF to extract 46% of the total protein amount from the microalga *Haematococcus pluvialis.* This was done under a field strength of 1 kV cm-1 and a majority of the extraction occurred within 5 min after PEF treatment (Gateau et al. 2020). In another study, Pankiewicz et al. 2020 enriched the probiotic *Lactobacillus rhamnosus* B 442 bacterial strain with calcium ions by applying a PEF electric field strength of 3.0 kV/cm. The calcium-enriched *L. rhamnosus* B 442 was used to prepare icecream with a high content of protein, carbohydrates, and fat, along with creating the lowest melting rates (Pankiewicz et al. 2020). Reversible electroporation was also used for lipid extraction from the green algae *Chlorella pyrenoidosa* (Han et al. 2019).

**1.5 Modeling of reversible electroporation in bacterial cells**: The molecular mechanisms of PEF which lead to membrane permeabilization have not been entirely elucidated. However, experimental and theoretical studies have demonstrated that PEF forms pores in the microbial lipid bilayer membrane. Relatively simple models are based on dynamic models of diffusion-driven transmembrane transport caused by electropermeabilization (Pucihar et al., 2001; Puc et al., 2003; Henslee et al., 2014; Tylewicz, 2020). Puc et al. (2003) presented a pharmacokinetic model of diffusion-driven transmembrane transport which described how transmembrane transport, caused by electropermeabilization, allows for the study of molecule uptake as a function of elapsed time, voltage and pulse duration small molecules. Specifically, they showed where the permeabilization process was divided into a short permeabilizing phase that takes place during the pulse, and a longer resealing phase that begins after the end of the pulse.

The most influential factor in the basic process is the transmembrane potential difference induced by the electric field (REFs). The basic models used the spherical shape cell approximation to analytically find the induced transmembrane potential difference (REFs). More comprehensive models that provide an extensive view of the pore formation process are the molecular dynamics (MD) simulations and the continuum model (REFs). Although MD models are a powerful tool for studying systems on the molecular level, their use is limited by associated costs involved in high computational requirements. (Rems 2017, 2019). The continuum models describe the electric field, flow field, and temperature distributions in a pulsed electric field (PEF) treatment chamber by solving for the continuity, momentum, energy and electric field equations of different geometries. (Salengke et al., 2012; Knappert et al., 2020).

The proposed study attempts to develop a model based on a continuum model by using numerical simulations to calculate the local treatment conditions (electric field strength, temperature, flow field) inside a treatment chamber, as described in the recent study by Knappert et al. (2020). Such a model will allow for a more fundamental understanding in the design of the experiments and the analysis of their results. In addition, we will apply the dynamic model described in Puc et al. (2003) in order to analyze the experimental results and to find parameters that influence the flow between intracellular and extracellular space, such as the membrane opening size (Puc et al. (2003)).

In the theoretical portion of the proposed study a kinetic model of the electroporated cells will be developed. The model will describe the fraction of the electroporated Gram-negative and Gram-positive bacteria as a function of the electric field strength which will also include experimental examination of rate permeabilization of hydrophilic and hydrophobic molecules with different molecular weights (MW). The model will also describe the pore size until resealing of the bacterial cells. The suggested model is based on the continuum model by using numerical simulation to calculate the local treatment conditions (electric field strength, temperature, flow field) inside a treatment chamber based on the work of Knappert et al. (2020). The model will be implemented in a CFD simulation using the COMSOL® Multiphysics® modeling software, an interactive environment for modeling and simulating scientific and engineering problems. The model will be developed in conjunction with the experimental work to find the most appropriate parameters in the model.

**2. Research Objectives & Expected Significance**

**2.1 Research goal:** The overall research goal of this study is to develop a reversible electroporation model of bacteria based on rate permeabilization measurements of hydrophilic and hydrophobic molecules in a moderate electric field.

**2.2. Research objectives**

1. To examine the permeabilization rate of hydrophilic and hydrophobic molecules after exposure of Gram-negative bacteria to PEF treatment in a moderate electric field (1-4 kV/cm) and variable physical parameters (e.g., pulse number, current density and total specific energy).

2. To investigate the permeabilization rate of hydrophilic and hydrophobic molecules after exposure of Gram-positive bacteria to PEF treatment in a moderate electric field (1-4 kV/cm) and variable physical parameters (i.e. pulse number and duration, current density and total specific energy).

3.Toanalyzethe bacterial proteome during the reversible electroporation

4. To develop a reversible electroporation model for bacteria by describing: the kinetics of pore formation and size; kinetics of pore resealing; and the transport affinity of hydrophilic or hydrophobic molecules across the pores and their effect of the bacterial cell wall.

5. To elucidate the parameters of the dynamic model which will be found from the experimental results by building a full numerical simulation for the temperature, flow and electric fields distributions within the batch PEF treatment chambers using a Computational Fluid Dynamic (CFD) software. The predicted values and the experimental data will be compared to demonstrate the validity of the proposed model.

**2.3 Expected significance:** The main motivation in developing electroporation models is to evaluate and analyze the experimental results, to predict experimental outcomes, and to use these models for the optimization of experimental protocols. Therefore, these models need to accurately predict the physical quantities that are accessible by experimental measurements. The ability to create reversible electroporation of relatively small and large molecules is an important method involved in many fields including: bioremediation of recalcitrant environmental polluted molecules; molecule extraction from bacterial cells; and genetic engineering processes. In addition, such a model will provide a basic theoretical understanding of processes that are involved in PEF technology. Furthermore, our work attempts to rule out the possibility of a thermal effect on the results.

**3. Detailed description of the proposed research**

**3.1 Working hypothesis:** Pore formation and resealing in electroporated-mammalian, plant, yeast and bacterialcellsare well-known phenomena. The existing models describing electroporation are mostly based on mammalian cells or artificial membranes. Some models attempt to characterize permeabilization using fluorescent dyes like propidium iodide (PI) applied to the electroporated target. However, the transport of PI is influenced by many factors, such as the cell physiology state and the medium composition. The bacterial cell wall, its small size compared to mammalian cells, and the bacterial cytoplasmic membrane composition may influence pore formation and resealing. Exposure of bacterial cells suspended in a phosphate buffered saline (PBS) solution applied to PEF-treatment may lead to pore formation. However, transferring the treated bacteria to a more nutrient-rich medium such as brain heart infusion medium (BHI) may lead to enhanced chances of cell recovery and pore resealing. Pore resealing may occur within a minute to hours and yet the pore size and its affinity to hydrophilic or hydrophobic molecules until resealing is not well understood. The treatment chamber space domain is considered to be a continuum media where the laws of physical conservation can be implemented (REFs). Based on collected data on the permeabilization rate of hydrophilic and hydrophobic molecules to electroporated Gram-positive as well as Gram-negative bacteria, we will develop a dynamic model of diffusion transmembrane transport in a moderate electric field (1 – 4 kV cm-1) with varying physical parameters.

**3.2 Research design & methods**

3.2.1Examination of the pore size resealing time in electroporated Gram-negative bacteria:The experimental conditions are described as follows**.** Abacterial suspension of *Pseudomonas putida* F1 (6899 DSMZ, Germany) will be exposed to a pulsed electric field (PEF) of about 1– 4 kV cm-1 with a frequency of 100 Hz, square pulse shape, with a duration of 10 µs. Pulse variation number between 1,000 to 10,000 in a continuous series of trains which include 500 pulses each will be applied. The duration of a train will be 5 s with a 2 s interval between the trains. The trains will be delivered in a polar mode.

*P. putida* F1 suspended in PBS will be diluted in ultra-pure (UP) water (0 – 24.22 mM PBS) which leads to current densities between 0.02 ± 0.01 to 5.2 ± 0.1 A cm-2.The conductivity before adding the bacteria in the UP water and PBS will be 1 and 155–4058 µS cm-1, respectively. Immediately after the PEF treatment of the bacterial suspension into PBS (four different conductive solutions) with the different current densities, the treated bacterial suspension will be diluted into brain heart infusion (BHI), a rich medium. The BHI medium will contain: a) a fluorescent dye, Lucifer Yellow (457.25 Da), in an attempt to receive crude information on the pores duration until revealing; b) hydrophobic compounds from one aromatic hydrocarbon to 10 rings which include 7 aromatic hydrocarbon and 3 rings of 5 carbons which include benzene, naphthalene, anthracene, pyrene, benzo[e]pyrene and decacyclene with molecular weights of 78.12, 128.174, 178.23,  202.256, 252.3 and 450.5 g/mol, respectively; c) and relatively hydrophilic compoundsincluding phenol, bisphenol A, ellagic acid, epigallocatechin gallate, procyanidin B2 and theaflavin-3-gallate with molecular weights of 94.11, 250.275, 302.197, 458.372, 578.52 and 716.604 g/mol, respectively.

Each of the aforementioned compounds will be examined in PEF-treated bacteria suspended in four different PBS concentrations. It is important to note that most of the chosen hydrophobic and hydrophilic compounds are considered as environmental contaminants and were reported to be biodegradable. Part of these molecules are characterized by slow biodegradation rates (Fernández-Luqueño et al., 2010 to add to references). The control of these experiments will be non-PEF treated suspensions of *P. putida* F1 in corresponding physical and environmental parameters.

3.3.2 Methods and Calculations: After the suspension of the PEF-treated bacteria in BHI, the permeabilization rate of each molecule will be examined using High Performance Liquid Chromatography (HPLC) at differing time intervals (for example every 15 min) until the permeabilization rate will reduce to zero. The measurements will be conducted on the supernatant. To understand the level of the bacterial enzyme activity during the pore formation and resealing, the degradation rate for two selected compounds (hydrophobic and hydrophilic) will be examined. For this, the bacterial sediment will be extracted using a sonicator (name, version, manufacturer, location) and the intermediate metabolites will be measured using HPLC. CFUs will be examined immediately after the suspension into BHI and at the same intervals as described in paragraph 1.

The total specific energy (WT) will be calculated as described in the research performed by Raso et al. (2016). Equation 1 calculates the specific energy input per pulse (*W*). The *W* is the integral over time of the recorded pulse shape of voltage and current that was measured on the treatment chamber during the pulse (τ).

**Equation 1:**

where *m* is the sample mass, *U(t)* is the voltage, and *I(t)* is the current measured on the PEF chamber during load pulse (τ). The total specific energy (*WT*) for each treatment will be determined in Eq. 2 by multiplying the pulse number (*n*) with specific energy per pulse (*W*).

**Equation 2:**

The same experiments as performed with *P. putida* F1 will be done with *Staphylococcus* *aureus*, a Gram-positive bacterium (25923 ATCC, USA).To understand the influence of the bacterial cell wall on the electroporation kinetics, the permeabilization of two select compounds, one hydrophilic and one hydrophobic, will be examined to protoplast bacterial cells (*P. putida* F1 and *S.* *aureus*). The peptidoglycan will be digested using murein hydrolases where the commonly used enzyme is the hen egg white lysozyme. Since the outer membrane of Gram-negative bacteria mostly prevents entry of enzymes towered the peptidoglycan, these types of cells require pre-treatment with a chelating agent (e.g. EDTA) or detergent (e.g. Triton X-100) for removing the outer membrane [Salazar and Asenjo 2007]. The protocol for preparing protoplast is described by Figueroa et al., 2018.

To understand if and how bacterial pore resealing occurs following the electroporation process, analysis of the proteome will be conducted. At selected times, electroporated bacteria and the control, non-treated bacteria will be collected and centrifuged. The proteins from the washed sediment will be sonicated and treated with urea, ammonium bicarbonate, and DTT as described in our previous study (Emanuel et al, 2019). Mass spectrometry analysis will be performed at the Smoler Proteomics Center at the Technion, Israel. Details of this processes are described in Emanuel et al., 2019. The data will be quantified by label-free analysis using the same software, based on extracted ion currents (XICs) of peptides, thus enabling quantification from each LC/MS run for each peptide identified in the experiments.

3.3.3. Development the bacterial electroporation model

The presented theoretical work aims to build a numerical simulation of cell permeabilization by PEFs. The governing equations are based on the conservation equations for mass, momentum, energy and charge (Knappert et al., 2020). The model is composed ofthe conservation equation for mass, momentum, energy, electric potential electric and the transport equation for the activity of passive biological tracers as detailed in Eqs. 3 through 7, below:

**Equations 3-7:**

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where *t* is the time, **u** is the fluid velocity vector,  is the fluid density, *p* is the pressure, is the fluid dynamic viscosity and **g** the vector of gravitational acceleration. *c*p is the heat capacity of the fluid, *T* the total temperature and *k* is the fluid thermal conductivity, and *F*p the permeabilized cells population fraction (activity of passive biological tracers). The term e represents a source term for the internal energy.

**Equation 8:**

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where  is the electrical conductivity of the fluid and *E* represents the local strength of the electric field. The electric field can be computed from Eq. 9:

**Equation 9:**

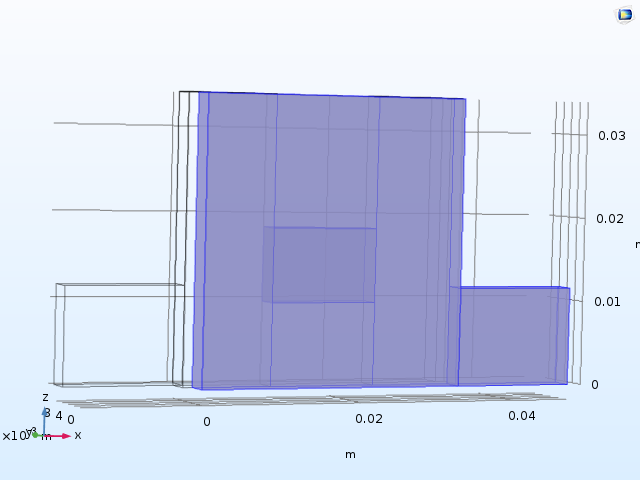
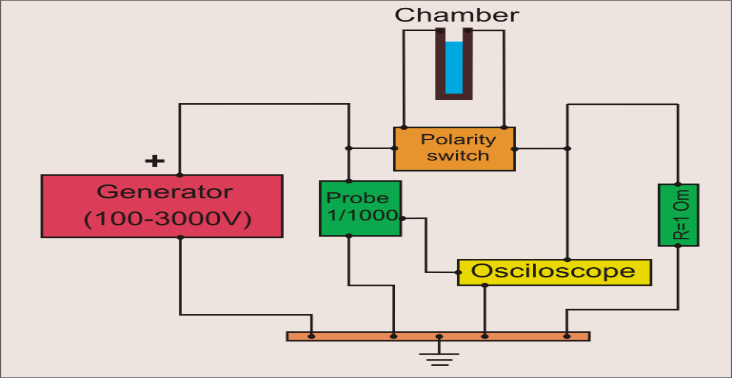
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The source term for the fraction of perforated cells isFp. It is a function of the electric field strength and the treatment temperature and will be derived from experimental data. The numerical tool which will be employed isa commercially available CFD software package (COMSOL Multiphysics®). It is used to solve numerical 3D transient models by calculating the temperature response in place and time.

**4. Preliminary Results**

**4.1 Design and construction of the electroporator****:** A high voltage generator was set to administer an electric field between 100 V and 3000 V on the bacterial suspension. The produced voltage pulses were under the control of a synthesized function generator. To obtain the current ICH value, the voltage UR at a resistance R=1Ω was connected in series to the current circuit (ICH=UR/R) (Figure 1A). Current density was calculated in accordance with cross section S (JCH=ICH/S).

**4.2 Construction of the electroporator chamber:** The electroporator chamber was made from two stainless-steel plates, each with a thickness of 3 mm. Dimensions are shown in Figure 1B.



Crocodile hook area

A

B

Liquid sample

Figure 1. Schematic drawing of (A) the experimental arrangement (electronic circuit) and (B) the electroporator chamber.

**4.3 Total specific energy and the heat transfer model**: To investigate the possible thermal effects on the results, energy balance and heat transfer analyses were performed to find the temperature distribution during the PEF treatment. Measurements were taken for the highest conductive sample (1050 µS cm-1). The current and electrode potential were measured during the PEF treatment. The current and potential measurements are shown in Figure 2A. The total specific energy (WT) was calculated using Eqs. 10 and 11. The *W* is the integral over time of the recorded pulse shape of voltage and current that was measured in the treatment chamber during the pulse (τ).

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| **Equation 10:** |  | **Equation 11:** |  |

where *m* is the sample mass, *U(t)* is the voltage, and *I(t)* is the current measured in the PEF chamber during load pulse (τ). The total specific energy (*WT*) for each treatment was determined in Eq. 2 by multiplying the pulse number (*n*) with the specific energy per pulse (*W*). The total specific energy for the highest conductive sample (1050 µS cm-1) was found to be 224 kJ kg-1. This value was used as the heat source for the heat transfer modeling. The temperature response in the system was calculated using COMSOL Multiphysics® numerical software (COMSOL Multiphysics®, Release 5.4, manufacturer, location) . The calculation was performed using Eqs. 12 and 13. The 3D transient heat transfer model was based on the electrodes’ domain, the heat convection at the electrodes’ boundaries and on the conduction heat transfer in the bacterial suspension. The heat generation source term was taken from the total specific energy calculations. The initial temperature of the bacterial suspension and electrodes was 22°C.

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| **Equation 12:** |  | **Equation 13:** |  |

where *T* is the temperature in the space and time T(x,y,z,t). *q'''* is the heat source (W m-3), is the heat diffusivity, and is the heat conductivity ( = for the electrodes, or s for the sample). As a boundary condition, convection heat transfer at the electrode walls was applied, where is the convection heat coefficient and is the ambient temperature. The temperature map after 70 s is presented in Figure 2B.

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| **A** | **B** |

Figure 2. Potential and current time response during a pulse for two cases of opposite polarities. (A) Voltage input ( ); Voltage output ( - - - ); current output ( ). (B) Electrode sample system’s temperature map after 70 s operation time.

It was found that after an operation time of 70 s, the average temperature in the sample was approximately 35 °C. The temperature predictions using the COMSOL Multiphysics numerical software were consistent with the experimental measurements using a temperature probe (Thermocouple).

**4.4 Effect of the solution conductivity on bacterial viability:** *P. putida* suspensions in DI water as well as in different PBS concentrations were exposed to electric fields of 4.0, 2.8, 2.0 and 1 kV cm-1. The PEF-treated suspensions were incubated at 37 ºC for 1.5 h, followed by viable count analysis. The electric parameters applied to the bacterial suspensions that were treated by 1 kV cm-1 were as follows: square pulse shape with duration of 10 µs and frequency of 100 Hz. and 5000 pulses were performed by a continuous polar series of trains, each train consisting of 500 pulses. Each train duration was 5 s with a 2 s interval between each train (Figure 3A). The first and second columns represent the CFU mL-1 of the bacteria that were suspended in DI water without PEF-treatment (control) and with PEF-treatment, respectively. The rest of the columns represent samples of bacterial cells suspended in different PBS concentrations (correlated to current densities of 0.5, 1.2, 3.1 and 5.2 A cm-2)that were exposed to PEF-treatment. As seen in Figure 3A, a linear correlation was found between the bacterial viabiliy and the current density; and with the bacterial viability with the total specific energy. This phenomenon was also observed when the cells were exposed to 4.0, 2.8 and 2.0 kV cm-1 (data not shown).

**4.5 Bacterial membrane permeability and relative cell size as a function of current density****:** *P. putida* F1 bacteria were treated with an electric field intensity of 1 kV cm-1  as described in section 7.1.3.3.3**)**, followed by incubation of the bacterial suspension at 37ºC for 1.5 h and the addition of PI for 5 min at 37ºC. The samples were examined via flow cytometry (FCM) and the membrane permeability of about 50,000 cells was examined. The histograms of cell number as a function of PI fluorecence intensity showed that increasing the current density led to an increase in cell populations that were PI positive (Figure 3B). The percentage of PI-positive cells in the untreated samples (control) was 10 ± 0.9%. PI permeability at the lower current density of 0.02 ± 0 .01 A cm-2 was found to be 14 ± 0.9%. These results indicated that there is no significant change in cell permeability at the low (0.02 ± 0 .01 A cm-2) current density compared to the control. At a current density of 1.2 ± 0.1 A cm-2, PI permeability was 53 ± 5.1% and at the maximum tested current density (5.2 ± 0.5 A cm-2) permeability was 65 ± 0.3% (Figure 3C). In conclusion, a linear correlation was found between increasing current density and bacterial cell permeability. This phenomenon was also observed in the electric field ranges of 2.0, 2.8 and 4.0 kV cm-1 (data not shown).

Bacterial relative cell size as a function of current density is shown in Figure 3D. The PEF-treated and untreated cells were stained with PI. Each examined sample included about 50,000 cells, so that the area under each curve was equal (except the upper curve that represent only the PI stained cells). The overlay offset graph shown in Figure 3D presents the different scattering of *P. putida* F1 cell size under PEF treatments relative to untreated cells. As shown, the peak of the curve of the untreated sample (control) was shown to be approximately 2 × 104 FSC (blue line). The peak of the majority of the PEF- treated cells shifted to the right, indicating an increase in cell size (red, green and purple line) relative to the control (blue line).

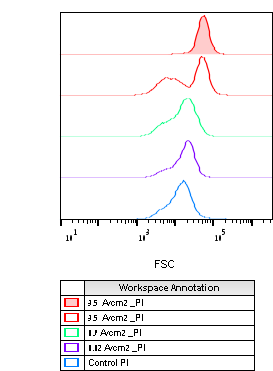


**A**

**B**

**A**

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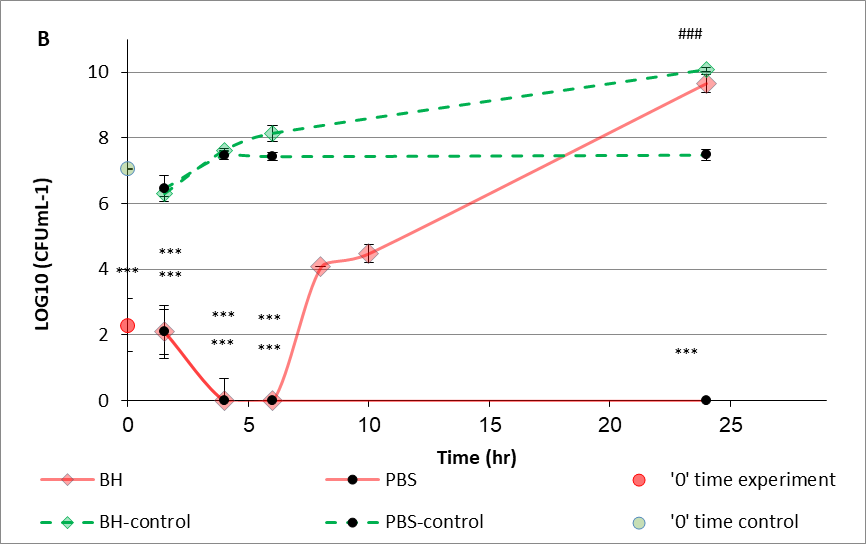
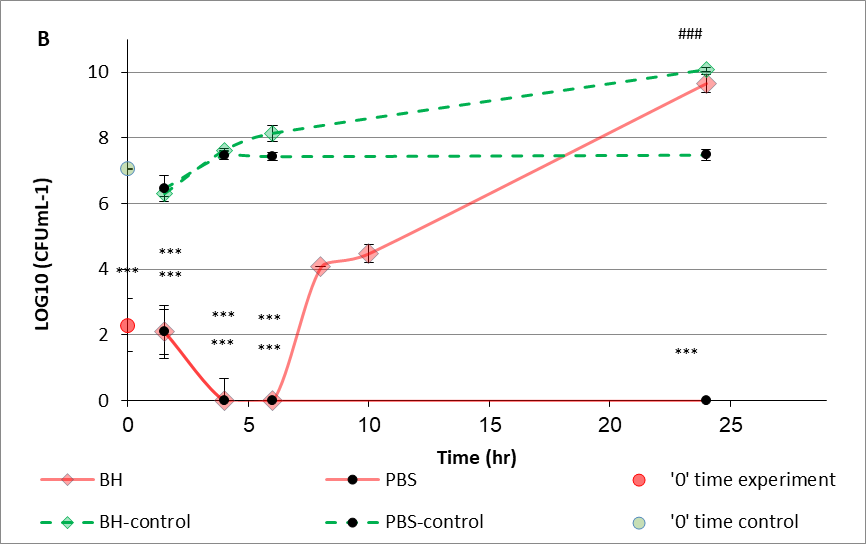
**D**

**C**

**Figure 3.** **Viability, pemeability and relative cell size of PEF- treated *P. putida* F1 as a function of current density.** Viability of bacterial cells were calculated in CFU mL-1. *P. putida* F1 membrane permeability was examined using flow cytometry. **(A)** A histogram of cell number as a function of propidum iodide (PI) fluorecence intensity. **(B)** PI-unstained cells (control) are in gray; PI-stained cells (control) are in blue; PEF-treated cells (PI stained) after application with a current density of 0.02 A cm-2 are in purple; current density of 1.2 A cm-2 in green; and 5.2 A cm-2 in red. **(C)** The percentage of PI positive cells. In subfigures Aand C, the first column (control) and the second column represent bacterial cells suspended in DI water without PEF treatment and PEF-treated cells after application with a PEF current density of 0.02 A cm-2, respectively. The columns 0.5 – 5.2 A cm-2 represent bacterial suspension in solutions with ionic strengths of 2.42 – 24.22 mM. P value (T-test): p < 0.05 = \*; p < 0.001 = \*\*\*); **(D)** *P. putida* F1 relative cell size was measured using flow cytometry. Untreated cells (control) are indicated in blue; PEF-treated cells treated with a current density of 0.02, 1.2 and 5.2 A cm-2 are as displayed in purple, green, and red lines, respectively; only PI-positive stained cells are in solid red.

**4.6 Viability of PEF-treated *P. putida*** **as a function of suspension in a rich medium, BHI medium and PBS:** In this experiment, the *P. putida* and *S. aureus* bacteria in PBS were…. Data are shown for *P. putida* where similar results were found with slight differences that were observed for *S. aureus* when exposed to PEF treatment of 2.9 kV cm-1 and at a current density of 3.4 ± 0.1 A cm-2. Physical parameters for these experiments were described in section **7.1.3.3.3**.

A PEF-treated bacterial suspension (0.02 OD 600 nm) was divided into three 100 µL portions each. The first portion was transferred to XXX (time '0') immediately after the PEF treatment to examine bacterial viability (in CFU mL-1). The second portion was suspended into 900 µL of 0.54 mM PBS and designated as PEF-treated bacteria in PBS, while the third portion was suspended into 900 µL BHI and designated as PEF-treated bacteria in BHI. The same procedure was performed on bacterial suspensions that were not exposed to PEF treatment and designated as non-treated bacteria in BHI and non-treated bacteria in PBS (control). The PEF-treated and non-treated bacteria were incubated for 24 h at 37ºC, and at indicated times during 24 h,a viable count assay was performed (Figure 4A).

**Figure 4. Viability assays of PEF-treated and non-treated *P. putida* F1 (CFU mL-1).** All samples were collected at various time-points between 1.5 h and 24 h after treatment unless specified otherwise. **(A)** PEF-treated bacteria at time '0' (); non-treated bacteria at time '0' (); PEF-treated bacteria in BHI (); PEF-treated bacteria in PBS (); non-treated bacteria in BHI (); non-treated bacteria in PBS().Significance was assessed using a Student’s t-test and examined CFU counts at each time collection point in relation to its controlP < 0.001\*\*\***.** Significance tests of treated bacterial quantities were assessed in PBS related to BHI, at each time-point P < 0.001 ### **(B)** Proportions of over-expressed proteins in PEF-treated *P. putida* F1 in BHI, compared to the non-treated sample.

At time '0', the PEF treatment led to a reduction of 4.8 log10. The non-treated bacteria in PBS remained at the same concentration during the entire experiment. The non-treated bacteria in BHI continued to replicate, reaching 1.20E+10 CFU mL-1 after 24 h. However, no CFUs of the PEF-treated bacteria in PBS or in BHI were observed from the fourth to the sixth hours after exposure. The PEF-treated bacteria in BHI began to replicate after the sixth hour and by the eighth hour they reached 1.18E+04 CFU mL-1. At the end of the experiment (24 h), the CFU countswere similar to those in the BHI control (the non-treated bacteria).

The same experiment but with the Gram-positive bacteria *S. aureus* showed that these bacteria were more resistant to PEF treatment, compared to the Gram-negative bacteria *P. putida*. The PEF treatment of *S. aureus* led to a reduction of approximately 3.2 log10 CFU mL-1. In addition, the PEF-treated *S. aureus* suspended in BHI maintained a count of 1.84E+04 CFU mL-1 for about 1.5 h, and then began to multiply (data not shown). This is an interesting contrast compared to the PEF-treated *P. putida* which decreased to zero CFU mL-1 at the fourth hour and remained there for about 2 h, and then began to multiply. It was previously reported that the thick peptidoglycan layer and structural properties of a Gram-positive bacterial membrane protect them from PEF damage. We suggest that the results from the PEF-treated *S. aureus* in BHI are due to multiple studies that found Gram-positive bacteria to be more resistant than Gram-negative bacteria to PEF treatment [7,54].

**4.7 MS analysis of the proteins from PEF-treated *P. putida* F1 suspension, compared to non-treated bacteria:** Bacterial (*P. putida*) suspension in 0.54 mM PBS were PEF-treated (2.9 kV cm-1) followed by suspension in BHI. For MS analysis, two samples were taken (three replicates of each) and the following conditions were applied: the PEF-treated bacteria were immediately diluted to 1:10 in BHI; and the non-treated bacteria, which were also diluted in BHI. The samples were taken six hours after the PEF treatment. It is important to note that the growth rate of the PEF-treated bacteria in BHI from the sixth to the eight h was 4.68 h-1. Meanwhile, in the untreated culture the growth rate at the beginning of the log phase was 1.08 h-1. We assume that the PEF-treated culture in BHI by the sixth hour was not composed of dead cells, but rather a large population of them were in a stressed state which may be considered VBNC or sublethally injured cells. Thus, the appropriate control for MS analysis of the PEF-treated bacteria in BHI was untreated culture grown for the same time (6 h), and not a control culture at the end of the lag phase. All samples were washed in PBS (x 3). The proteins in the sediment were extracted and digested. The MS analysis was performed at the Smoler Proteomics Center at the Technion, Israel. As shown in Figure 4B, the results of the proteins consisted of three main groups: 55% were found to be related to stress conditions; 36% to various proteins; and 9% to uncharacterized proteins (Figure 4B).

**5. Available equipment, personnel, and collaborations**

The microbiology experiments will be performed in the labs and under the supervision of Prof. Rivka Cahan. The lab is equipped with a high-voltage generator for applying an electric field on bacterialsuspensions with a signal generator (Stanford Research System DS45, 30 MHz) to control voltage pulses. The lab also contains a homemade electroporator chamber made of two stainless-steel plates, incubators for bacterial growth, UV-Vis spectrophotometer, analytical scale, orbital shakers, laminar safe cabinet, chemical hood, centrifuge with fix-angle and swinging bucket rotors, CG-FID, HPLC, autoclaves, refrigerators, freezers and ‐80ºC freezers. We will have access to the shared facilities of Ariel University. Major equipment includes flow cytometer (FCM), GC/MS, SEM, AFM, confocal microscopy, and fluorescence microscope. Prof. Cahan's lab group consists of one post-doctoral fellow, four PhD students, one MSc and four undergraduate students. The microbiology part of the project will be conducted by PhD and postdoc students.

The reversible electroporation model on bacteria will be conducted by Dr. Gad Pinhasi. His lab group focusses on theoretical, experimental and numerical studies on transport phenomena, *i.e.* fluid dynamics, heat and mass transfer. His lab group will perform the numerical analyses and computational fluid dynamics portion of this project. His group will investigate the mechanisms of two-phase flashing flow and combustion. Dr Pinhasi's lab group consists of two PhD students and five undergraduate students. The modeling part of the mentioned study will be conducted by a PhD student.

**Collaborations**

**6. Expected results and pitfalls**

Duration of the PEF-induced pores will be found by applying a fluorescent dye (Lucifer Yellow) to Gram-negative, Gram-positive and mammalian cells. One anticipated pitfall of the proposed research is thatthe MW of theLucifer Yellow is 457.25 Da: The data on when the beginning of the pores open and the exact time of resealing will not be accurate for the low and high MW of the differing selected compounds. In addition, the time duration of pore openings may change due to the selected compound properties. These limitations may be adressed by expanding (correlated to the data of Lucifer Yellow) the time for examining the permeability rate of the different compounds. The permeability rate of the hydrophilic and hydrophobic compounds will be determined. Another anticipated pitfall of this project includes the fact thatsome of the selected compounds, when used in high concentration, may cause a damage to the cells.This may be solved by reducing the concentration of the selected compound.

**7. Timeline**

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Example of significantly increased **stress proteins** (**total 22**) are Alkyl hydroperoxide reductase and TonB-dependent siderophore receptors. Alkyl hydroperoxide reductase (A5W5H2) is an enzyme related to a large family of thiol-specific antioxidant proteins which reportedly protect bacteria from abiotic stresses [62]. The alkyl hydroperoxide reductase is a crucial enzyme for gut [bifidobacteria](https://www-sciencedirect-com.mgs.ariel.ac.il/topics/immunology-and-microbiology/bifidobacterium), helping to manage reactive oxygen species (ROS) effectively under conditions of [oxidative stress](https://www-sciencedirect-com.mgs.ariel.ac.il/topics/immunology-and-microbiology/oxidative-stress) [63]. Three types of TonB-dependent siderophore receptors were identified (A5VXD9, A5W124 and A5W341). These proteins are located in the bacteria’s outer membrane; they are known for binding and transporting ferro-chelating siderophores, vitamin B12, carbohydrates and nickel complexes. A positive correlation was reported between the expression of iron-uptake systems in *P. aeruginosa* and the response to oxidative stress [65].