MICROBIAL LOAD CONTROL BY INTERMITTENTLY DELIVERED PULSED ELECTRIC FIELDS

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Abstract
Cell density control is a major challenge for modern biotechnology and food industries. In this work, we report on non-thermal, chemical-free microbial cell density control by intermittently delivered *pulsed electric fields (IDPEF). This approach facilitates sterile storage without the need for chemical preservatives, additives, radiation or the complex infrastructure demanded, which is not available in many developing countries.* We show that the *IDPEF control Lysteria monocytogenes* density in contaminated milk. Using the microorganism kinetics growth data we designed an IDPEF protocol that consists of *2 sequences of 10 square wave pulses, 50 μs duration, 12.5 kV* cm^{-1} *electric field strength, delivered at 0.5 Hz and 1 min pause between the sequences* applied every 1.5 h. In a 12 hours experiment at 32^0C *the bacteria density of untreated samples* reached $(9.1\pm0.6)\cdot10^7$ *CFU* ml^{-1} and $(7.1\pm0.3)\cdot10^8$ *CFU* ml^{-1} *for* initial load levels of $(1.4\pm0.2)\cdot10^3$ *CFU* ml^{-1} *and* $(3.1\pm0.3)\cdot10^6$ *CFU* ml^{-1}*, respectively; while the final density in the IDPEF treated samples was 120±44 CFU* ml^{-1} *and* $(1.1\pm0.3)\cdot10^5$ *CFU* ml^{-1}*. Furthermore, energy needed for IDPEF storage of milk in developing countries can be generated by a small-scale 2kW solar energy system operating 5.5 hours per day in combination with small-scale energy storage systems.*

Key words: Pulsed electric fields, Electroporation, *Listeria monocytogenes*, Cell density and proliferation control.

1. Introduction

Microorganisms density and proliferation control is a major challenge for food, pharmaceuticals and biotechnology industries. While some applications need intensive biomass accumulation of microorganisms to maximize the production of valuable materials, others demand the elimination of pathogenic microorganisms.

Microsecond to millisecond duration pulsed electric fields (PEF) destroy cells by damaging the cell membrane, a phenomenon known as irreversible electroporation (IRE) [1]. The first work on PEF in the food industry was published by Doevenspeck in the early sixties [2].In the previous study we have we proposed the non-thermal "intermittent delivery of pulsed electric fields" (IDPEF) at specific, planned time intervals for long-term preservation of biological matter exposed to potentially septic conditions during the storage [3]. We showed that IDPEF is comparable with the standard refrigeration during 5 days as a means for microbial load control. Furthermore, energy consumption analyses revealed the advantage of IDPEF over refrigeration [3]. The goal of this work is further explore IDPEF effect on cell density and proliferation control and to demonstrate its efficiency on proliferation control of a food pathogen - *Lysteria monocytogenes.*

L. monocytogenes is a food pathogen, causing foodborne disease. It is found in raw, pasteurized and processed milk products [4]. *L. monocytogenes* can be found in about 5% of raw milk samples [4]. Previous reports show that *L. monocytogenes* may be resistant to traditional milk pasteurization and cold storage methods [4]. Furthermore, case reports show that humans may acquire listeriosis through consumption of pasteurized milk [5, 6].

For instance, the pasteurization method in the Massachusetts 2007 outbreak of listeriosis was efficient and met FDA standards. However, the committee investigating the incident concluded that the contamination took place, most likely, after pasteurization [6]. The recontamination can be caused by cross contamination, potentially through biofilm, [7, 8], or by unsterile transportation and storage [9]. Therefore, PEF among other non-thermal methods is under investigation as an alternative to classial themal methods for *L. monocytogenes* inactivation [4].

In this work we report for the first time the use of IDPEF for long term density control of *L. monocytogenes* in contaminated milk. IDPEF protocols were established through studies of microorganisms growth kinetics at different initial contamination levels. We show that by using IDPEF it is possible to control *L. monocytogenes* load in milk.The IDPEF can be applied in the laboratory and industrial scale to maintain microbial level at the prescribed limits and at the known proliferation phase.

2 Materials and methods.

2.1 *Bacteria culture preparation*.

L. monocytogenes 10403S background LLO^- , with natural streptomycin resistance, was kindly provided by Prof. Daniel Portnoy Laboratory (Molecular and Cell Biology Department, UC Berkeley) was used in this study. The starting bacterial culture was prepared by transferring the single colony from Luria-Bertani (LB) plates to 2 mL of the brain heart infusion broth BHI growth medium and incubation at 30°C for 14 hours.

2.2 *Bacterial count.*

The bacterial number was counted by the spread counting method. The samples were diluted tenfold in Dulbecco's phosphate-buffered saline to eliminate the effects of media on cell growth. Samples (100 µL) of each of the tested solutions were plated on Luria-Bertani Miller (LB) agar plates and incubated at 37°C for 24 hours. Three samples were plated for each experimental condition.

2.3 *Pulse Electric Field treatment protocol.*

We used pasteurized milk, purchased at a local store, brought to room temperature (24°C) with added streptomycin (Sigma-Aldrich, USA) to total concentration of 60-µg ml^{-1} to inactive additional bacteria, which could prevent *Listeria* growth. In our previous work we showed that dividing the total amount of pulses into groups leads to a higher PEF inactivation rate [10]. In this work we treated 85 µl of milk immersed in 1mm gap parallel electrodes cuvette (Genesee Scientific, San Diego,CA) by applying 2 sequences of 10 square wave pulses, each 50 µs duration, 12.5 $kVcm^{-1}$ field, delivered at 0.5 Hz with 1 min pause between the sequences using an ESM 830 square pulse generator (BTX, Harvard Apparatus, MA, USA). Twenty pulses were delivered in total in a single treatment. The temperature after the treatment was immediately measured in the cuvette using a Neoptix Reflex® signal conditioner with a 0.7-mm probe covered with polyimide (Neoptix, Québec, Canada). Electric field amplitude and pulse duration were measured by a high impedance Tektronix TDS 210 oscilloscope (Tektronix Inc., OR, US). The bacterial numbers were counted before and after each the treatment by the spread counting method described in section 2.2.

2.2 *The Effect of L. monocytogenes starting concentration.*

The level of possible *L. monocytogenes* concentration in food varies [4]. We tested the effect of a pulse electric field protocol, described in section 2.3 on 5 levels of milk contaminated from 10^6 to 10^2 CFU ml^{-1}. Three repeats were performed for each experimental point.

2.3 *Intermittently delivered pulsed electric field treatment of L. monocytogenes* in milk*.*

We tested IDPEF on $(1.4\pm0.2)\cdot10^3$ CFU ml^{-1} and $(3.1\pm0.3)\cdot10^6$ CFU ml^{-1}, starting concentrations of *L. monocytogenes* in milk. We used fifteen cuvettes (100µl, 1mm gap) for each starting concentration. We treated the samples by pulsed electric field protocol described in section 2.3 at 24°C every 1.5 h and stored them without agitation at 32°C in

the intervals between the treatments. We counted the bacterial load every 3 h as described in section 2.2. At each counting point the total volume of the three cuvettes for each starting concentration were taken for plating. The total experiment length was 12 hours.

2.4 *Statistics*

Three repeats were performed for each experimental point. Statistical analyses were performed using the Microsoft Office Excel 2007 external package, as well as student t-tests with unequal variances.

3 Results and Discussion

The goal of this work is to demonstrate the application of IDPEF to control *L. monocytogenes* density in milk. Since the initial bacterial load in products may vary we tested PEF disinfection efficiency for various initial loads of *L. Monocytogenes.* The applied PEF protocol led to log 0.6 – log 0.8 reduction in the tested range of starting microbial load as shown on Figure 1a. From Figure 1a it is evident that the starting concentration variation does not impact the applied protocol efficiency. Milk temperature increase, as measured immediately after treatment, was 6.4±0.2 °C.

The IDPEF treatment parameters depend on the initial microbial load type and quantity, the microbial growth kinetics and the nature of the growth media [3]. A general schematic of a possible IDPEF treatment planning protocol is shown in Figure 1b. The design goal of the IDPEF treatment protocol is to maintain a microbial load between a particular level between C_{LL} and C_{HL}. To this end we are seeking to find an optimal way of applying the PEF to reduce the cell density from C_{BT} to C_{AT} at prescribed time intervals T_{period}. The IDPEF treatment parameters will obviously depend on the growth kinetics of PEF treated microorganisms. Assuming that after a PEF treatment, the microorganisms experience exponential growth phase with a constant rate constant k (h^{-1}), and that the time interval between treatments is T (h), the following equations are relevant to IDPEF delivery design:

$$C_{BT} = C_{AT} \cdot \exp(kT) \tag{1}$$

$$T_{period} = \frac{\ln C_{BT} / C_{AT}}{k}$$

To test the effect of IDPEF on *L. Monocytogenes* in milk we used two different initial bacterial loads. In the first, high initial load, 5 ml of a milk sample were contaminated with bacteria at an initial concentration of $(3.1\pm0.3)\cdot10^6$ CFU ml^{-1}. In the non-treated samples *L. monocytogenes* proliferated without interruption and reached the "plateau" stage $(7.4\pm0.3)\cdot10^8$ CFU ml^{-1} already 9 hours after contamination. In the IDPEF treated samples the concentration was maintained in the range of 10^5-10^6 CFU ml^{-1} as shown on Figure 1c. 12 Hours after the contamination the concentration of *L. monocytogenes* in the IDPEF samples was $(1.1\pm0.3)\cdot10^5$ CFU ml^{-1}. In the second, low initial load, 5 ml of a milk sample were contaminated with bacteria at an initial concentration of $(1.4\pm0.2)\cdot10^3$ CFU ml^{-1}. In the non-treated samples of milk *L. monocytogenes* proliferated without interruption and reached $(9.1\pm0.6)\cdot10^7$ CFU ml^{-1} in 12 hours. In the IDPEF treated samples the concentration was maintained in the range of 120-160 CFU ml^{-1} as shown on Figure 1d during the whole experiment. Twelve hours after the contamination the concentration of *L. monocytogenes* in the IDPEF samples was 120±44 CFU ml^{-1}.

The data from the linear region of the semi-logarithmic plot of cell number vs. time of the untreated cultures in Figure 1c and Figure 1d was used to evaluate the basic microorganism growth rate constant (k, h^{-1}) for $(1.4\pm0.2)\cdot10^3$ CFU ml^{-1} and $(3.1\pm0.3)\cdot10^6$ CFU ml^{-1} initial concentrations. The calculation was done using the following equation:

$$k = \frac{\ln[C(T2)/C(T1)]}{T2 - T1} \tag{2}$$

Where *C(T1)* and *C(T2)* are initial and final concentration of bacteria at times *T1* and *T2* respectively. We found that for $(3.1\pm0.3)\cdot10^6$ CFU ml^{-1} starting concentration the growth rate of *L. monocytogenes* in milk at 32^0C was 0.3 h^{-1} while for $(1.4\pm0.2)\cdot10^3$ CFU ml^{-1} starting concentration it was 2 h^{-1}. The generation half time (g,h) is directly related to growth rate constant, and is given by Equation 3. The calculated average generation time for *L. monocytogenes* in milk at 32^0C was approximately 1.5h. This, therefore, was the time interval used between the PEF treatments in the applied IDPEF protocol.

$$g = \frac{\ln 2}{k} \qquad (3)$$

In this work we demonstrated IDPEF applicability to milk preservation. Although no studies on the initial concentration of *L. monocytogenes* in milk were found in the FDA/FSIS study [11], the report suggests that in the affected products the concentration of bacteria varies between 0.04 to 250 CFU ml^{-1} [12].

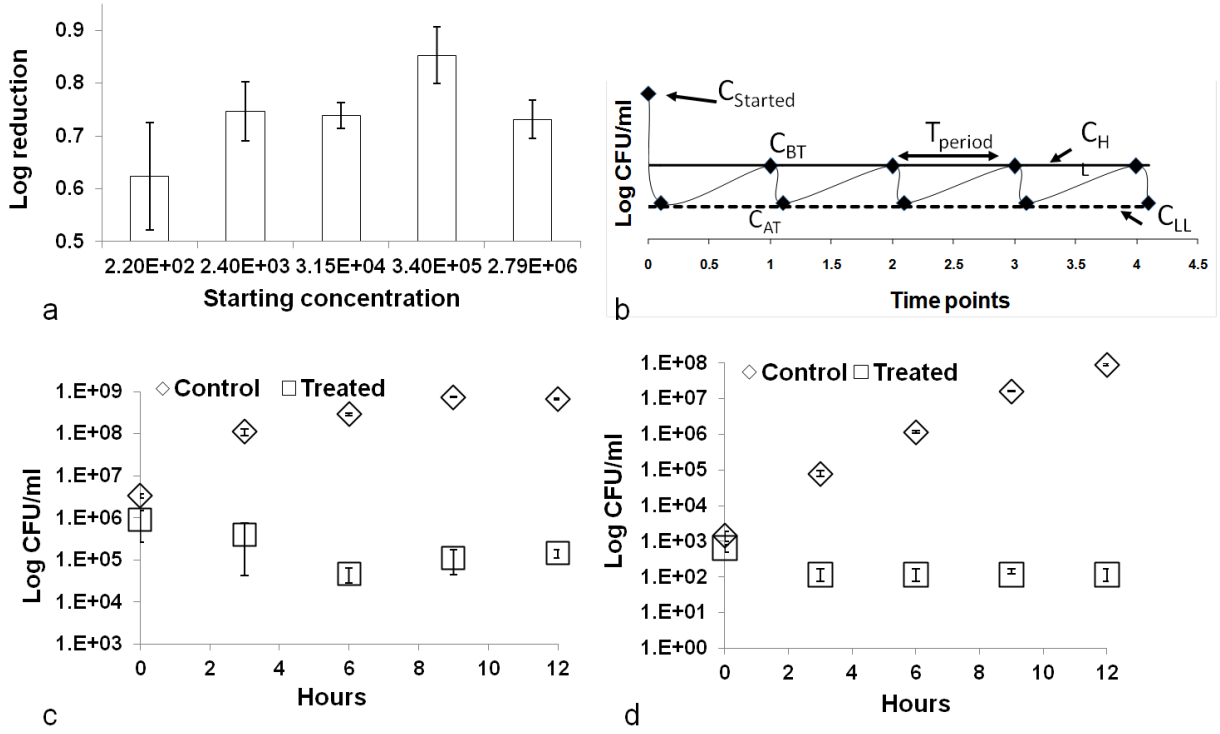

***Figure 1 a**. The effect of contamination load on* L. monocytogenes *PEF treatments. **b.** Schematic diagram of microbial growth under IDPEF mediated storage. The scheme describes the behavior of the simplest first order kinetic model described in Equation 1. Here $C_{started}$ (CFU ml^{-1}) is an initial microbial concentration before treatment. C_{HL} (CFU ml^{-1}) is the highest level of microbial concentration allowable in the product .C_{LL} (CFU ml^{-1}) – highest level of microbial load found in the product. C_{BT} (CFU ml^{-1}) is the microbial concentration before an PEF treatment.C_{AT} (CFU ml^{-1}) is the microbial concentration after an PEF treatment.T_{period} (h) is the time interval between intermittent PEF treatments. **c.** The effect of IDPEF on milk with initial concentration of $(3.1\pm0.3)\cdot10^6$ CFU ml^{-1} of* L. Monocytogenes. ***d.** The effect of IDPEF on milk with the initial concentration of $(1.4\pm0.2)\cdot10^3$ CFU ml^{-1} of L. monocytogenes Error bars represent ± 1 standard deviation.*

In the reported outbreaks investigation up to 10^9 CFU ml^{-1} were found in products, most probably due to severe post contamination and storage regime abuse [12]. The number of microorganisms consumed at one meal that cause illness to a healthy individual

was estimated to be $7.7 \cdot 10^4$ CFU ml^{-1} [4]; however, this concentration is much lower for immunosuppressed immune-system individuals, children or elder people . Here we report that IDPEF controls *L. monocytogenes* proliferation in milk, when applied during the entire storage period. Specifically, we show that for the initial concentration level of $(1.4 \pm 0.2) \cdot 10^3$, IDPEF storage prevented bacterial concentration to reach the critical value of $7.7 \cdot 10^4$ CFU ml^{-1} that may cause a disease [4]. Indeed, after 12 hours of incubation at 32^0C the concentration of *L. monocytogenes* in the untreated sample was $(9.1 \pm 0.6) \cdot 10^7$ CFU ml^{-1}, while in the IDPEF enabled storage the concentration was only 120±44 CFU ml^{-1} (Figure 1d). In addition, we show that in the high initial concentration load, the applied IDPEF protocol is able to control microorganism proliferation (Figure 1c).

Currently, the biotechnology and food industries use thermal and chemical methods for microbial density control. Although chemical preservatives are widely used in the food industry, there is a concern of constant adaptation of the microorganisms to these agents. Indeed, antimicrobial resistance was reported in *Listeria* species, including *L. Monocytogenes* [13]. Furthermore, chemical additives themselves can become health hazards [14]. Hydrogen peroxide, forbidden to usage in milk in many developed countries, was proposed to use in the developing countries where other storage methods are not available [15]; however, the supply and distribution chain for the recommended usage is complex due to the high level of control needed. Continuous refrigeration is a standard method for control of microbial growth rate, through reduction of cell metabolism. Although this method effectively slows the majority of known pathogens it still allows the growth of psychrophiles such as *L. monocytogenes* [4]. Furthermore, refrigeration demands a continuous supply of electricity, which is unavailable in many parts of the world.

While refrigeration slows the growth of microorganisms, PEF directly kills them. An energy consumption of a PEF system can in the range of 44-244 J ml^{-1} [16]. An majority of families in India have 2 cows, which produce 800- 3676 kg of milk per animal annually or 2.1-10 kg milk per day [17]. The family uses 50% of the produce, while another 50% is local milkman [17]. These data imply that to treat the produced milk by IDPEF protocol introduced in this study sold to the (16 treatments per 24 hours) there is a need to supply 1.5-39 MJ daily. This energy can be generated, for example, by a small scale 2kW solar energy system operating 5.5 hours per day in combination with small scale energy storage already available. Therefore, IDPEF presents a reasonable solution to the problem of post process recontamination in a "chemical free" way in locations where refrigeration is not available.

In summary, we have shown that IDPEF can control microbial proliferation at high and low initial concentration. We demonstrated the application of IDPEF on milk storage and showed that IDPEF controls *L. monocytogenes* proliferation. The IDPEF method advantages for food preservation are as follows. First, PEF actually kill the bacteria, in comparison with thermal methods that slow the bacteria metabolism. Second, PEF treatment does not require a continuously operating electrical infrastructure as refrigeration. Finally, PEF protocol is insensitive to temperature or chemical resistance of bacteria. However, research remains to be done to understand the kinetics of microorganism growth during IDPEF and to optimize the choice of the optimal PEF parameters and the energy consumption and PEF device use. Furthermore, the proposed process can applied in the dairy industry for reduction of microbial load in the storage tanks, where milk can be stored several up to 24 h before treatment. During this period microorganism can growth and produce metabolites that can be harmful. For the biotechnology IDPEF presents an opportunity to keep microbial culture in the constant exponential growth phase, maximizing the total product produced in the given volume.

5. Conclusions

IDPEF is an emerging method for cell proliferation and growth control with application in both basic research and industry. The advantage of the method is in its non-thermal, chemical free nature. In this work we demonstrated the applicability of IDPEF for the control

of *L. monocytogenes* proliferation in milk. Further work is needed to characterize the fundamental impact of IDPEF on microbial cell culture and products to develop precisely controlled protocols.

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