Long-term *Listeria monocytogenes* proliferation control in milk by intermittently delivered pulsed electric fields, implications for food security in the low-income countries

Alexander Golberg

Microbial density control is a major challenge for food preservation especially in the low-income countries, where 850 million undernourished people live. In this work, we report on non-thermal, chemical-free microbial cell density control by intermittently delivered pulsed electric fields (IDPEF). We show that IDPEF allows for precise control of *L. monocytogenes* density in contaminated milk, an essential product for small farmers in low-income countries. Using *L. monocytogenes* growth kinetics, 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 hours. In a 12 hours experiment at 32 °C, *L. monocytogenes* density of untreated samples reached (9.1 ± 0.6) · 10⁷ CFU ml⁻¹ and (7.1 ± 0.3) · 10⁸ CFU ml⁻¹ for initial load of (1.4 ± 0.2) · 10³ CFU ml⁻¹ and (3.1 ± 0.3) · 10⁶ CFU ml⁻¹ respectively; the final density in the IDPEF treated samples, however, was 120 ± 44 CFU ml⁻¹ and (1.1 ± 0.3) · 10⁵ CFU ml⁻¹. The energy required for IDPEF storage of milk in the low-income countries could be generated by a small-scale 2kW solar energy system operating 5.5 hours per day in combination with small-scale energy storage system. We believe that IDPEF storage technology can empower millions of small farmers in the low-income countries by providing them a simple and energy efficient technology for milk preservation.

INNOVATION

Milk is a key element for household food security and provides a stable income to farmers including women, who are usually in charge of taking care of the milk-producing animals in the low-income countries. Currently, pathogen growth in milk is managed with refrigeration or with chemicals. Although bacterial growth in milk is managed with refrigeration in the high-income countries, a high cost of infrastructure and a demand for a permanent electricity supply prevent milk refrigeration in the rural areas in the low-income countries. Moreover, certain pathogens, for example *Listeria monocytogenes*, are less sensitive to low temperature; therefore, they can proliferate at the standard refrigeration temperatures used during transportation and storage. For locations where refrigeration is impossible, Codex Alimentarius approved the use of a lactoperoxidase system. However, to activate an endogenous milk lactoperoxidase, an external substrate, thiocyanate, is needed. According to Codex Alimentarius, a critical drawback in using lactoperoxidase system is the difficulty to control its use: lactoperoxidase system may be misused by the farmer to disguise milk produced under poor hygienic conditions. In this work, we report on non-thermal, chemical-free *L. monocytogenes* cell density control in milk by intermittently delivered pulsed electric fields (IDPEF). IDPEF technology does not require a constant electricity supply and can be powered 5.5 hours a day using small, family scale solar panels. We believe that IDPEF can provide a robust, simple and energy-efficient milk preservation system that would decrease the wasted milk thus increasing the income of the small farmers in developing countries.

NARRATIVE

Food security for the growing population is the major challenge for governments, health organizations and food industries. Although the modern rates of food production are high, there are still 850 million people today who are hungry and undernourished¹. Most of the world’s hungry live in the rural areas of South Asia and Sub-Saharan Africa and have insufficient income to adequately feed their families¹. Milk production in the small family farms has a potential to rapidly increase the income of millions of small farmers in the low-income countries (LIC). Milk production can generate a rapid return on investment and involve women, who are usually unemployed². Development of dairies for small farmers in the rural areas in LIC has been under intensive focus of various international agencies and large food companies in the last decades. One of the major obstacles in the successful implementation of the small farmers dairies programs is the problem to preserve milk from the time of milking till the arrival to the large storage and processing facilities³.

Three major methods are currently available for the milk storage. Temperature control is the major method that allows for effective milk preservation. Refrigeration is the major technology used in the world

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for milk storage. However, mostly because of unavailability of a constant electricity, refrigeration is hardly possible today in the most of remote rural areas, where the world poor and hungry live. An alternative usage of temperature for milk preservation is boiling. Traditionally, in many remote rural locations, freshly harvested milk is boiled by direct burning of biomass. However, indoor cooking using direct burning of biomass produces dangerous levels of smoke. It is estimated that 1.45 million die every year from household air pollution. The only additional method for milk preservation approved by Codex Alimentarius is a lactoperoxidase system. However, to activate an endogenous milk lactoperoxidase, an external substrate, thiocyanate, is required. Using thiocyanate requires a special training not available in all location where rapid milk preservation is needed. Moreover, according to Codex Alimentarius, a critical drawback in using lactoperoxidase system is the difficulty to control its use. The control of use is needed because lactoperoxidase system may be misused to disguise milk produced under poor hygienic conditions. Given the lack of the progress in the development of technologies for milk storage in the rural areas, where electricity is not available during 24 hours and lactoperoxidase system is not available, there is a need for new technologies for milk preservation. In this work, we report on a new method to preserve milk from bacteria contamination — intermittently delivered pulsed electric fields (IDPEF).

Microsecond to millisecond duration pulsed electric fields (PEF) destroy cells by damaging the cell membrane, a phenomenon known as irreversible electroporation. PEF technology has been previously reported for milk pasteurization with the goal to replace or enhance thermal pasteurization processes. The effects of PEF on milk chemical properties were reported in Ref. 11. In the previous studies we proposed a modification of PEF process that we called IDPEF. At IDPEF, electric fields are applied to the biological matter multiple times at specific time intervals for long-term preservation and cell density control. In the 5 days experiment, we showed that IDPEF is comparable with the standard refrigeration as a means for Escherichia coli load control in milk. Furthermore, energy consumption analyses revealed the advantage of IDPEF over refrigeration for milk preservation. The goal of this work is to further explore IDPEF effect on bacteria cell density and to demonstrate IDPEF efficiency using a milk specific pathogen Listeria monocytogenes. In addition, we performed an energy consumption analyses for the application of IDPEF technologies in developing countries. L. monocytogenes is a food pathogen which causes foodborne disease. A problem of Listeria contamination of milk is global and is reported in both low and high-income countries. L. monocytogenes is found in raw, pasteurized and processed milk products. Case reports show that humans may acquire listeriosis through consumption of pasteurized milk. Previous studies show that L. monocytogenes may be resistant to traditional milk pasteurization methods. Moreover, the committee investigating the Massachusetts 2007 outbreak of listeriosis concluded that the contamination took place, most likely, after milk pasteurization was performed successfully. Indeed, it is a challenge to prevent recontamination after successful pasteurization by thermal or enzymatic methods. The recontamination can be caused by cross contamination, potentially through biofilm on the equipment, or by unsterile transportation and storage. Moreover, previous studies show that L. monocytogenes is less sensitive to low temperature; therefore, it can proliferate at the standard refrigeration temperatures during transportation and storage. It occurred to us that PEF could be used for direct killing of L. monocytogenes in milk during storage. This approach is fundamentally different from the refrigeration that only slows bacteria metabolism.

In this work, we report for the first time the IDPEF for long-term density control of L. monocytogenes in milk. IDPEF protocols were established through studies of microorganisms growth kinetics at low (1.4 ± 0.2) \times 10^3 and high (3.1 ± 0.3) \times 10^6 initial contamination levels. We show that IDPEF allows for controlling L. monocytogenes load in milk in densities lower than the densities that lead to the disease. IDPEF could be applied both at a laboratory and at an industrial scale. Furthermore, we believe that small farmers in the rural areas could apply the proposed IDPEF technology for the control of microbial load in the intermediate storage tanks, where milk can spend up to 24 hours before treatment in the large-scale centralized dairies. If not treated during intermediate storage and transportation, microorganisms in milk can grow and secrete metabolites that can be harmful even after pasteurization.

The IDPEF treatment parameters depend on the initial microbial load type and quantity, the microbial growth kinetics and the nature of the growth media. A general schematic of a possible IDPEF treatment planning protocol is shown on Fig. 1. The various parameters in Fig. 1 are:

- $C_{\text{started}}$ (CFU ml$^{-1}$) — initial microbial concentration before treatment or after recontamination.
- $C_{\text{HL}}$ (CFU ml$^{-1}$) — highest level of microbial concentration that does not lead to disease.
- $C_{\text{LL}}$ (CFU ml$^{-1}$) — lowest level of microbial concentration detectible in the product.
- $C_{\text{BT}}$ (CFU ml$^{-1}$) — microbial concentration before a single PEF treatment.

**Figure 1** (a) The concept of solar powered pulsed electric field system for milk preservation for the small farmers in the low-income countries. (b) Schematic diagram of microbial growth under intermittently delivered pulsed electric fields (IDPEF) mediated storage protocol. The scheme describes the behavior of the simplest first order kinetic model described in Equation (1). Here we assume that exponential growth of bacteria occurs between the PEF treatments ($C_{\text{AT}}$ to $C_{\text{BT}}$).

**Table 1** The parameters in Fig. 1 are:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
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<tbody>
<tr>
<td>$C_{\text{started}}$</td>
<td>Initial microbial concentration before treatment or after recontamination.</td>
</tr>
<tr>
<td>$C_{\text{HL}}$</td>
<td>Highest level of microbial concentration that does not lead to disease.</td>
</tr>
<tr>
<td>$C_{\text{LL}}$</td>
<td>Lowest level of microbial concentration detectible in the product.</td>
</tr>
<tr>
<td>$C_{\text{BT}}$</td>
<td>Microbial concentration before a single PEF treatment.</td>
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C_{AT} (CFU ml\textsuperscript{−1}) — microbial concentration after a single PEF treatment.

The objective function of an IDPEF treatment is to maintain a microbial concentration below a particular level between C_{LT} and C_{UL}. 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}. These 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\textsuperscript{−1}), and that the time interval between treatments is T_{period} (h), the following equation is relevant to treatment design:

\[ C_{BT} = C_{AT} \times \exp(kT) \]
\[ T_{period} = \left( \frac{\ln(C_{BT}/C_{AT})}{k} \right) \]  

(1)

IDPEF treatment should be correlated with microbial growth rate at specific medium and specific environment conditions. To determine the growth kinetics parameters, we constructed the growth curves for (1.4 ± 0.2) \times 10\textsuperscript{7} CFU ml\textsuperscript{−1} and (3.1 ± 0.3) \times 10\textsuperscript{6} CFU ml\textsuperscript{−1} starting concentrations of L. monocytogenes in milk stored without agitation at 32 °C. We calculated the treatment T_{period} from the average value of growth rate derived from these curves.

To determine the effect of the initial microbial load on the PEF efficiency, we applied a single PEF treatment, consisting of 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm\textsuperscript{−1} field, delivered at 0.5 Hz with 1 min pause between the sequences, on milk contaminated with various initial loads of L. monocytogenes. Five initial concentrations were tested: (1) 2.2 \times 10\textsuperscript{7}; (2) 2.4 \times 10\textsuperscript{7}; (3) 2.2 \times 10\textsuperscript{8}; (4) 3.4 \times 10\textsuperscript{8}; (5) 2.8 \times 10\textsuperscript{9} CFU ml\textsuperscript{−1}. The applied PEF protocol led to the following Log reduction (Fig. 2). ANOVA analyses showed there was no significant difference in the log reduction between groups (p > 0.05); therefore, we concluded that for the tested initial concentrations level and the applied PEF protocol the initial load of bacteria did not affect the PEF disinfection efficiency. Milk temperature increase, as measured immediately after treatment, was 6.4 ± 0.2 °C.

During IDPEF storage, contaminated milk samples were subjected to 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm\textsuperscript{−1} field, delivered at 0.5 Hz with 1 min pause between the sequences every 1.5 hours, and stored without agitation at 32 °C in the intervals between treatments. The total treatment time was 12 h.

For the IDPEF storage experiment with the high initial load of bacteria, 5 ml of a disinfected milk sample were contaminated with bacteria at an initial concentration of (3.1 ± 0.3) \times 10\textsuperscript{6} CFU ml\textsuperscript{−1}. In the untreated samples of milk, L. monocytogenes proliferated without interruption and reached the “plateau” stage (7.4 ± 0.3) \times 10\textsuperscript{5} CFU ml\textsuperscript{−1} already 9 hours after contamination. In contrast, 12 hours after the contamination, the concentration of L. monocytogenes in the IDPEF samples was (1.1 ± 0.3) \times 10\textsuperscript{5} CFU ml\textsuperscript{−1} (Table 1, Fig. 3).

For the IDPEF storage experiment with the low initial load of bacteria, 5 ml of a milk sample were contaminated with bacteria at an initial concentration of (1.4 ± 0.2) \times 10\textsuperscript{5} CFU ml\textsuperscript{−1}. In the untreated samples of milk, L. monocytogenes proliferated without interruption and reached (9.1 ± 0.6) \times 10\textsuperscript{4} CFU ml\textsuperscript{−1} in 12 hours. In contrast, in the IDPEF treated samples, 12 hours after the treatment, the concentration of L. monocytogenes in the IDPEF samples was 120 ± 44 CFU ml\textsuperscript{−1} (Table 2, Fig. 4).

The data from the untreated cultures in Fig. 3 and 4 were used to evaluate the basic microorganism growth kinetic parameter. The values of the growth rate constant (k, h\textsuperscript{−1}) for (1.4 ± 0.2) \times 10\textsuperscript{5} CFU ml\textsuperscript{−1} and (3.1 ± 0.3) \times 10\textsuperscript{6} CFU ml\textsuperscript{−1} were 6.4 ± 0.3 and 12.0 ± 0.2 °C, respectively.
The calculated average generation time for *L. monocytogenes* in milk at 32 °C was approximately 1.5 hours:

\[
g = \frac{\ln 2/k_1 + \ln 2/k_2}{2}
\]  

(4)

This, therefore, was the time interval used between the PEF treatments in the described IDPEF experiment.

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*. 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. Energy consumption of a PEF system is in the range of 44–244 J ml⁻¹·h⁻¹. A 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. The family uses 50% of the produce, while another 50% is sold to the local milkman. These data imply that to treat the produced milk by IDPEF protocol introduced in this study (16 treatments per 24 hours) there is a need to supply 1.5–39 MJ daily. This energy could be generated, for example, by a small-scale 2 kW solar energy system operating 5.5 hours per day in combination with small-scale energy storage already available. Therefore, IDPEF presents a reasonable solution in a "chemical free" way in remote rural locations where refrigeration is not available. The challenge to the future work, on which we plan to focus in the coming months, is to develop a robust PEF generator and milk storage chamber that is easy to operate in the rural areas. Furthermore, we believe that the use of this new storage technology will empower the small farmers to produce more milk, which could generate more income for the family, thus reducing the poverty and the hunger.

**Table 2** IDPEF effect on *L. monocytogenes* concentration in milk at an initial concentration of (1.4 ± 0.2) · 10⁴ CFU ml⁻¹.

<table>
<thead>
<tr>
<th>Time Points</th>
<th>Untreated milk (CFU ml⁻¹)</th>
<th>IDPEF milk (after PEF) (CFU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time 0</td>
<td>(1.4 ± 0.2) · 10⁴</td>
<td>649 ± 142</td>
</tr>
<tr>
<td>3 hours</td>
<td>(8.1 ± 0.2) · 10⁴</td>
<td>120 ± 44</td>
</tr>
<tr>
<td>6 hours</td>
<td>(1.2 ± 0.1) · 10⁶</td>
<td>120 ± 44</td>
</tr>
<tr>
<td>9 hours</td>
<td>(1.6 ± 0.4) · 10⁴</td>
<td>120 ± 34</td>
</tr>
<tr>
<td>12 hours</td>
<td>(9.1 ± 0.6) · 10⁷</td>
<td>120 ± 45</td>
</tr>
</tbody>
</table>

(3.1 ± 0.3) · 10⁶ CFU ml⁻¹ initial concentrations were calculated from the linear region of the semi-logarithmic plot of log cell number vs. time (Fig. 3 and 4). In order to calculate the kinetics parameters we used the regions of growth where the exponential growth took place. The calculation was done using the following equation:

\[
k = \left(\ln[C(T2)/C(T1)]\right)/(T2 – T1)
\]  

(2)

where C(T1) and C(T2) are initial and final concentration of bacteria at times T1 and T2 respectively. For the high dose group we used T3 h and T7 h data points. For the lower dose group we used T3 h and T12 h data points.

We found that for (3.1 ± 0.3) · 10⁶ CFU ml⁻¹ starting concentration, the growth rate of *L. monocytogenes* in milk at 32 °C was 0.3 h⁻¹ (k1), while for (1.4 ± 0.2) · 10⁶ CFU ml⁻¹ starting concentration it was 2 h⁻¹ (k2). The generation half time \(g\) (h) is directly related to growth rate constant, and is given by Equation (3):

\[
g = \ln 2/k
\]  

(3)

The effect of IDPEF on milk with low initial load of bacteria. The milk with initial concentration of (1.4 ± 0.2) · 10⁴ CFU ml⁻¹ of *L. monocytogenes* was treated every 1.5 h by 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm⁻¹ field, delivered at 2 Hz with 1 min pause between the sequences during 12 h of storage. Cuvettes with a 1 mm gap between electrodes were used. Points after the single PEF are shown for the treated group. Error bars represent ± 1 standard deviation.
that can be used for the IDPEF storage chambers. Finally, the impact of IDPEF on the organoleptic and enzymatic activity of the milk should be further investigated. Nevertheless, given the potential advantages of IDPEF technology, we believe that under proposed operation and continuous development of storage chambers, IDPEF storage could empower millions of small farmers in the low-income countries by providing them a simple and energy efficient technology for milk preservation.

 MATERIALS AND METHODS

 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). 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.

 Bacterial count
 The bacteria were counted with the spread counting method. The samples were diluted 10 fold 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.

 Single 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. In this work we treated 85 μl of milk immersed in 1 mm gap parallel electrodes cuvette (Genesee Scientific, San Diego, CA) by applying 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm–1 field, delivered at 0.5 Hz with 1 min pause between the sequences using an ECM 830 square pulse generator (BTX, Harvard Apparatus, MA, USA). Twenty pulses were delivered in total. The temperature after the treatment was immediately measured in the cuvette using a Neoptix Signal conditioner with a 0.7 mm probe covered with polyimide (Neoptix, Quebec, Canada). Electric field amplitude and pulse duration were measured by a high impedance Tektronix TDS 210 oscilloscope (Tektronix Inc., OR, USA). The bacterial numbers were counted before and after each the treatment by the spread counting method.

 The effect of L. monocytogenes starting concentration on PEF efficiency
 The level of possible L. monocytogenes concentration in food varies. We tested the effect of a certain pulse electric field protocol in the milk contaminated with 5 levels of concentration from 105 to 107 CFU ml–1. The samples were treated by applying 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm–1 field, delivered at 0.5 Hz with 1 min pause between the sequences using an ECM 830 square pulse generator (BTX, Harvard Apparatus, MA, USA). Three repeats were performed for each experimental point.

 Intermittently delivered pulsed electric field storage
 We tested IDPEF on (1.4 ± 0.2) × 105 CFU ml–1 and (3.1 ± 0.3) × 106 CFU ml–1, starting concentrations. Fifteen cuvettes (three repeats for each plating point) were used for each starting concentration. Every 1.5 hours, the samples were subjected to 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm–1 field, delivered at 0.5 Hz with 1 min pause between the sequences using an ECM 830 square pulse generator (BTX, Harvard Apparatus, MA, USA), and stored without agitation at 32 °C in the intervals between treatments. After the incubation samples were cooled to the Room Temperature (24 °C) and the PEF treatment was delivered. Bacterial counting was performed every 3 hours. Each counting point the whole volume of the three cuvettes for each starting concentration were taken for plating; the rest of the samples were stored at 32 °C. The total experiment lasted 12 hours.

 Statistics
 Three repeats were performed for each experimental point. Statistical analyses, 1 way ANOVA and Student tests with unequal variances were performed with Matlab (ver. R2014b, MathWorks, MA, USA) Statistical Toolbox.

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