COMMUNICATION

TECHNOLOGY

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[1]

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 $(9.1 \pm 0.6) \cdot 10^7$ CFU ml^{-1} and $(7.1 \pm 0.3) \cdot 10^8$ CFU ml^{-1} for initial load of $(1.4 \pm 0.2) \cdot 10^3$ CFU ml^{-1} and $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-1} respectively; the final density in the IDPEF treated samples, however, was 120 ± 44 CFU ml^{-1} and $(1.1 \pm 0.3) \cdot 10^5$ 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 lactoperoxidaze system. However, to activate an endogenous milk lactoperoxidaze, an external substrate, thiocyanate, is needed. According to Codex Alimentarius, a critical drawback in using lactoperoxidaze system is the difficulty to control its use: lactoperoxidaze system may be misused 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 undernourished[1]. Most of the world's hungry live in the rural areas of South Asia and Sub-Saharian Africa and have insufficient income to adequately feed their families[1]. 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 unemployed[2]. 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 facilities[3].

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

[1]Porter School of Environmental Studies, Tel Aviv University, Tel Aviv, Israel, 6997801. Correspondence should be addressed to A.G. (agolberg@tauex.tau.ac.il).

Received 10 October 2014; accepted 5 February 2015; published online 17 March 2015; doi:10.1142/S2339547815200022

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[4]. 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[5]. 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[6]. The only additional method for milk preservation approved by Codex Alimentarius is a lactoperoxidaze system[4]. However, to activate an endogenous milk lactoperoxidaze, 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 lactoperoxidaze system is the difficulty to control its use. The control of use is needed because lactoperoxidaze system may be misused to disguise milk produced under poor hygienic conditions[4]. 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 lactoperoxidaze system is not available, there is a need for new technologies for milk preservation. In this work, we report on a new technology 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[7,8]. PEF technology has been previously reported for milk pasteurization with the goal to replace or enhance thermal pasteurization processes[9,10]. 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[12] and cell density control[13]. 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[12]. Furthermore, energy consumption analyses revealed the advantage of IDPEF over refrigeration for milk preservation[12]. 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 *Lysteria 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[14]. *L. monocytogenes* is found in raw, pasteurized and processed milk products[15]. Case reports show that humans may acquire listeriosis through consumption of pasteurized milk[16–20]. Previous studies show that *L. monocytogenes* may be resistant to traditional milk pasteurization methods[21,22]. Moreover, the committee investigating the Massachusetts 2007 outbreak of listeriosis concluded that the contamination took place, most likely, after milk pasteurization was performed successfully[17]. 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[23], or by unsterile transportation and storage[24]. 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[15,25]. 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 $(1.4 \pm 0.2) \cdot 10^3$ and high $(3.1 \pm 0.3) \cdot 10^6$ 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[12]. A general schematic of a possible IDPEF treatment planning protocol is shown on **Fig. 1**. The various parameters in **Fig. 1** are:

$C_{started}$ (CFU ml^{-1}) — initial microbial concentration before treatment or after recontamination.

C_{HL} (CFU ml^{-1}) — highest level of microbial concentration that does not lead to disease.

C_{LL} (CFU ml^{-1}) — lowest level of microal concentration detectible in the product.

C_{BT} (CFU ml^{-1}) — microbial concentration before a single PEF treatment.

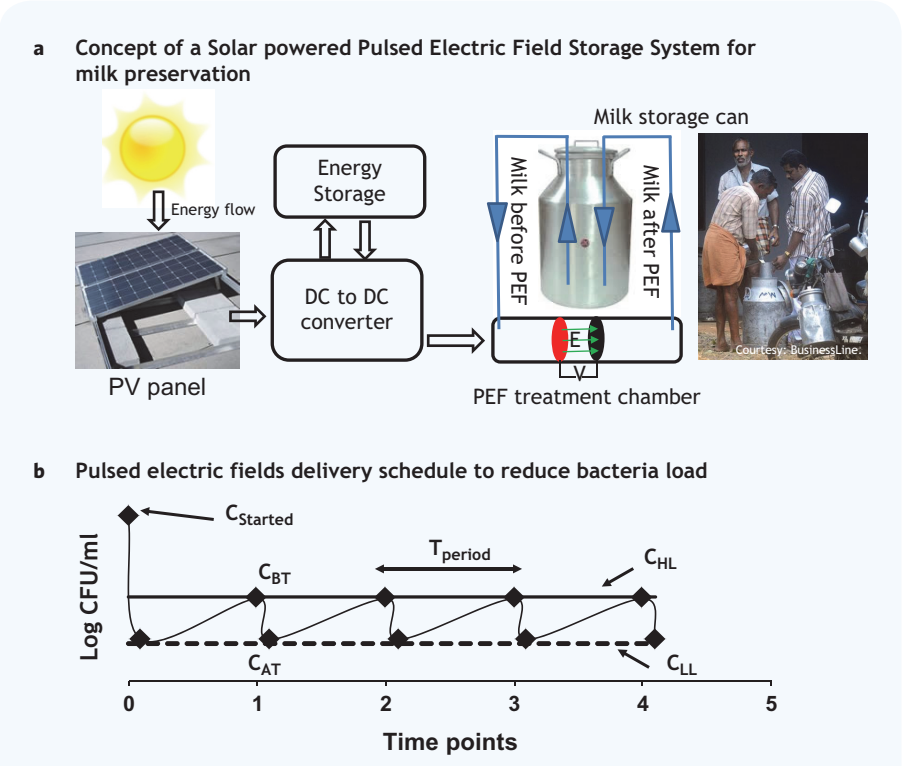

Figure 1 (**a**) The concept of solar powered pulsed electric field system for milk preservation for the small farmers in the low-income countries. (**b**) Schematic diagram of microbial growth under intermittently delivered pulsed electric fields (IDPEF) mediated storage protocol. The scheme describes 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_{AT} to C_{BT}).

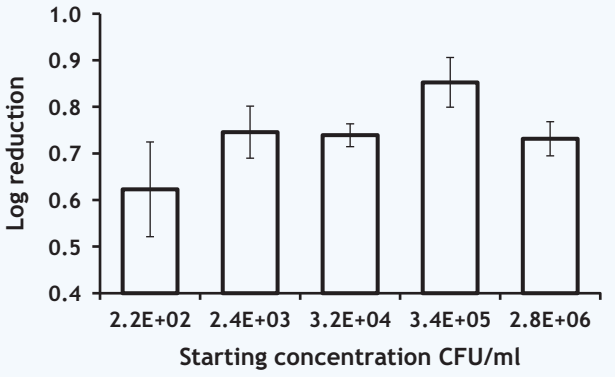

Figure 2 The effect of contamination load on *L. monocytogenes* PEF treatments. The treatment consisted of 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm^{-1} field, delivered at 2 Hz with 1 min pause between the sequences. Cuvettes with a 1 mm gap between electrodes were used. $p < 0.001$ in comparison with control, $p > 0.05$ between groups. Error bars represent ±1 standard deviation.

C_{AT} (CFU ml^{-1}) — microbial concentration after a single PEF treatment.
T_{period} (h) — time interval between intermittent PEF treatments.

The objective function of an IDPEF treatment is to maintain a microbial concentration below 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}. 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^{-1}), and that the time interval between treatments is T_{period} (h), the following equation is relevant to treatment design:

$$C_{BT} = C_{AT} \cdot \exp(kT)$$
$$T_{period} = (\ln C_{BT}/C_{AT})/k \quad (1)$$

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 $(1.4 \pm 0.2) \cdot 10^3$ CFU ml^{-1} and $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-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, consisted of 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, on milk contaminated with various initial loads of *L. monocytogenes*. Five initial concentrations were tested: (1) $2.2 \cdot 10^2$; (2) $2.4 \cdot 10^3$; (3) $3.2 \cdot 10^4$; (4) $3.4 \cdot 10^5$; (5) $2.8 \cdot 10^6$ CFU ml^{-1}. The applied PEF protocol led to the following *Log* reduction in the bacteria load in the milk for the tested groups of initial concentrations: (1) 0.62 ± 0.10; (2) 0.75 ± 0.06; (3) 0.74 ± 0.02; (4) 0.85 ± 0.05; (5) 0.73 ± 0.04 (**Fig. 2**). ANOVA[26] 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 disninfection 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 kVcm^{-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 $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-1}. In the untreated samples of milk, *L. monocytogenes* proliferated without interruption and reached the "plateau" stage $(7.4 \pm 0.3) \cdot 10^8$ CFU ml^{-1} already 9 hours after contamination. In contrast, 12 hours after the contamination, the concentration of *L. monocytogenes* in the IDPEF samples was $(1.1 \pm 0.3) \cdot 10^5$ CFU ml^{-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 $(1.4 \pm 0.2) \cdot 10^3$ CFU ml^{-1}. In the untreated samples of milk, *L. monocytogenes* proliferated without interruption and reached $(9.1 \pm 0.6) \cdot 10^7$ CFU ml^{-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^{-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^{-1}) for $(1.4 \pm 0.2) \cdot 10^3$ CFU ml^{-1} and

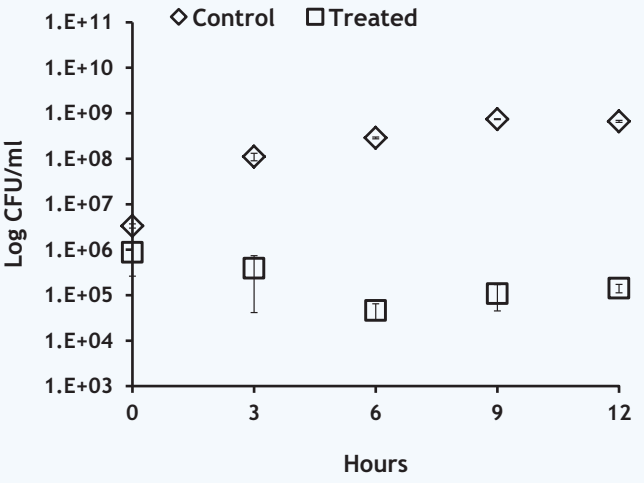

Figure 3 The effect of IDPEF on milk with high initial load of bacteria. The milk with initial concentration of $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-1} of *L. monocytogenes* was treated every 1.5 h by 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm^{-1} field, delivered at 2 Hz with 1 min pause between the sequences every 1.5 h 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. $p < 0.001$ in comparison with control. Error bars represent ±1 standard deviation.

Table 1 IDPEF effect on *L. monocytogenes* concentration in milk at an initial concentration of $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-1}.

Time Points	Untreated milk (CFU ml^{-1})	IDPEF milk (after PEF) (CFU ml^{-1})
Time 0	$(3.1 \pm 0.3) \cdot 10^6$	$(8.8 \pm 0.4) \cdot 10^5$
3 hours	$(1.1 \pm 0.2) \cdot 10^8$	$(3.9 \pm 0.3) \cdot 10^5$
6 hours	$(2.8 \pm 0.1) \cdot 10^8$	$(4.6 \pm 1.8) \cdot 10^4$
9 hours	$(7.4 \pm 0.2) \cdot 10^8$	$(1.1 \pm 0.6) \cdot 10^5$
12 hours	$(7.5 \pm 0.3) \cdot 10^8$	$(1.1 \pm 0.3) \cdot 10^5$

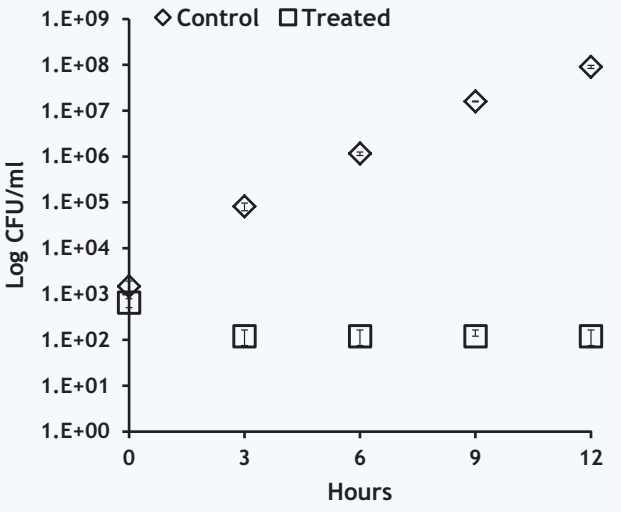

Figure 4 The effect of IDPEF on milk with low initial load of bacteria. The milk with initial concentration of $(1.4 \pm 0.2) \cdot 10^3$CFU ml^{-1} of *L. monocytogenes* was treated every 1.5 h by 2 sequences of 10 square wave pulses, each 50 μs duration, 12.5 kV cm^{-1} field, delivered at 2 Hz with 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. $p < 0.001$ in comparison with control. Error bars represent ± 1 standard deviation.

Table 2 IDPEF effect on *L. monocytogenes* concentration in milk at an initial concentration of $(1.4 \pm 0.2) \cdot 10^3$ CFU ml^{-1}.

Time Points	Untreated milk (CFU ml^{-1})	IDPEF milk (after PEF) (CFU ml^{-1})
Time 0	$(1.4 \pm 0.2) \cdot 10^3$	649 ± 142
3 hours	$(8.1 \pm 0.2) \cdot 10^4$	120 ± 44
6 hours	$(1.2 \pm 0.1) \cdot 10^6$	120 ± 44
9 hours	$(1.6 \pm 0.4) \cdot 10^7$	120 ± 34
12 hours	$(9.1 \pm 0.6) \cdot 10^7$	120 ± 45

$(3.1 \pm 0.3) \cdot 10^6$ 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:

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

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 $T9$ h data points. For the lower dose group we used $T3$ h and $T12$ h data points.

We found that for $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-1} starting concentration, the growth rate of *L. monocytogenes* in milk at 32 °C was 0.3 h^{-1} ($k1$), while for $(1.4 \pm 0.2) \cdot 10^3$ 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):

$$g = \ln 2/k \quad (3)$$

The calculated average generation time for *L. monocytogenes* in milk at 32 °C was approximately 1.5 hours:

$$\langle g \rangle = \frac{\ln 2/k1 + \ln 2/k2}{2}. \quad (4)$$

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*[15,21]. 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} [27]. 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[28]. The family uses 50% of the produce, while another 50% is sold to the local milkman[28]. 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.

The FDA/FSIS study suggests that in the affected products the concentration of *L. monocytogenes* varies between 0.04 to 250 CFU ml^{-1} [29]. In the reported investigations, up to 10^9 CFU ml^{-1} were found in products, most probably due to severe post contamination and storage regime abuse[30]. 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} [15]; however, this critical concentration is much lower for immunosuppressed immune-system individuals, children or elder people. The proposed IDPEF protocol prevented the proliferation of *L. monocytogenes* in the very high initial load of bacteria $(3.1 \pm 0.3) \cdot 10^6$ CFU ml^{-1} leading to the final concentration of $(1.1 \pm 0.3) \cdot 10^5$ CFU ml^{-1}, which is, however, higher than the critical concentration of $7.7 \cdot 10^4$ CFU (**Table 1, Fig. 3**). We showed, however, that once applied during the storage, IDPEF prohibits the proliferation of *L. monocytogenes* to the levels that may cause a disease at the samples with low initial concentration of $(1.4 \pm 0.2) \cdot 10^3$. Indeed, after 12 hours of incubation at 32°C, 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 storage the concentration was only 120 ± 44 CFU ml^{-1} (**Table 2, Fig. 4**). This tested low initial concentration is an order of magnitude higher than the suggested initial concentration of *L. monocytogenes* in the contaminated samples[29].

In summary, we demonstrated the application of IDPEF on milk storage and showed that IDPEF can control *L. monocytogenes* proliferation in milk. The IDPEF method advantages for food preservation are as follows. First, PEFs actually kill the bacteria, in comparison with thermal methods that slow the bacteria metabolism. Second, PEF treatment does not require a continuously operating electrical power based infrastructure as refrigeration. Finally, to the best of our knowledge, PEF is insensitive to temperature or chemical resistance of bacteria. However, we have shown previously that PEF is sensitive to the bacteria surface charge[31]. Additional research is needed 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 coupling to the solar energy systems in the rural areas. In additional, research is needed for the materials

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[32]. 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 $kVcm^{-1}$ 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 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, 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[15]. We tested the effect of a certain pulse electric field protocol in the milk contaminated with 5 levels of concentration from 10^6 to 10^2 CFU ml^{-1}. The samples were treated 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 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 $(1.4 \pm 0.2) \cdot 10^3$ CFU ml^{-1} and $(3.1 \pm 0.3) \cdot 10^6$ 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 $kVcm^{-1}$ 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.

ACKNOWLEDGEMENTS

The author acknowledges Prof. Boris Rubinsky from UC Berkeley for the active discussions about the work and for provision of part of laboratory equipment for pulse electric field treatment. The author acknowledges Prof. Daniel Portnoy and Dr. Chris Rae from UC Berkeley for providing the bacteria strains.

REFERENCES

1. FAO. *The State of Food Insecurity in the World: The Multiple Dimensions of Food Security 2013*. Web report, pp. 1–56 (2013).
2. Shefner-Rogers, C.L., Rao, N., Rogers, E.M. & Wayangankar, A. The empowerment of women dairy farmers in India. *J. Appl. Commun. Res.* **26**, 319–337 (1998).
3. Moran, J. *Business Management for Tropical Dairy Farmers* (Landlinks Press, 2009), http://www.publish.csiro.au/pid/6054.htm
4. FAO. *Benefits and Potential Risks of the Lactoperoxidase System of Raw Milk Preservation* (2005).
5. FAO. *The Technology of Traditional Milk Products in Developing Countries* (1990).
6. IEA. *Energy Poverty and Health* (WHO Collaboration, 2010), http://www.iea.org/publications/worldenergyoutlook/resources/energydevelopment/energypovertyhealthwhocollaboration/
7. Golberg, A., Fischer, J. & Rubinsky, B. The use of irreversible electroporation in food preservation. In *Irreversible Electroporation* (ed.) Rubinsky, B. (Springer, Berlin–Heidelberg) pp. 273–312.
8. Wan, J., Coventry, J., Swiergon, P., Sanguansri, P. & Versteeg, C. Advances in innovative processing technologies for microbial inactivation and enhancement of food safety — pulsed electric field and low-temperature plasma. *Trends Food Sci. Technol.* **20**, 414–424 (2009).
9. Odriozola-Serrano, I., Bendicho-Porta, S. & Martín-Belloso, O. Comparative study on shelf life of whole milk processed by high-intensity pulsed electric field or heat treatment. *J. Dairy Sci.* **89**, 905–911 (2006).
10. Bendicho, S., Barbosa-Cánovas, G.V. & Martín, O. Milk processing by high intensity pulsed electric fields. *Trends Food Sci. Technol.* **13**, 195–204 (2002).
11. Garcia-Amezquita, L.E., Primo-Mora, A.R., Barbosa-Cánovas, G.V. & Sepulveda, D.R. Effect of nonthermal technologies on the native size distribution of fat globules in bovine cheese-making milk. *Innov. Food Sci. Emerg. Technol.* **10**, 491–494 (2009).
12. Golberg, A., Kandel, J., Belkin, M. & Rubinsky, B. Intermittently delivered pulsed electric fields for sterile storage of turbid media. *IEEE Trans. Plasma Sci.* **38**, 3211–3218 (2010).
13. Golberg, A., Bei, M., Sheridan, R.L. & Yarmush, M.L. Regeneration and control of human fibroblast cell density by intermittently delivered pulsed electric fields. *Biotechnol. Bioeng.* **110**, 1759–1768 (2013).
14. Marnissi, E. Presence of Listeria monocytogenes in raw milk and traditional dairy products marketed in the north-central region of Morocco. *African J. Food Sci.* **7**, 87–91 (2013).
15. Ryser, E.T. & Marth, E.H. *Listeria, Listeriosis, and Food Safety*, 3rd Ed. (CRC Press, 2007), 896 pp.
16. Fleming, D.W. *et al.* Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N. Engl. J. Med.* **312**, 404–407 (1985).
17. Cumming, M. *et al.* Outbreak of Listeria monocytogenes infections associated with pasteurized milk from a local dairy — Massachusetts, 2007. *Morb. Mortal. Wkly. Rep.* **57**, 1097–1100 (2008).
18. Dalton, C.B. *et al.* An outbreak of gastroenteritis and fever due to Listeria monocytogenes in milk. *N. Engl. J. Med.* **336**, 100–105 (1997).
19. Xanthiakos, K., Simos, D., Angelidis, A.S., Nychas, G.J.E. & Koutsoumanis, K. Dynamic modeling of Listeria monocytogenes growth in pasteurized milk. *J. Appl. Microbiol.* **100**, 1289–1298 (2006).
20. Lundén, J., Tolvanen, R. & Korkeala, H. Human Listeriosis outbreaks linked to dairy products in Europe. *J. Dairy Sci.* **87**, E6–E12 (2004).
21. Doyle, M.P. *et al.* Survival of Listeria monocytogenes in milk during high-temperature, short-time pasteurization. *Appl. Environ. Microbiol.* **53**, 1433–1438 (1987).
22. Doyle, M.E., Mazzotta, A.S., Wang, T., Wiseman, D.W. & Scott, V.N. Heat resistance of Listeria monocytogenes. *J. Food Prot.* **64**, 410–429 (2001).

23. Latorre, A.A. *et al.* Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with Listeria monocytogenes. *J. Dairy Sci.* **93**, 2792–2802 (2010).
24. Reij, M.W. & Den Aantrekker, E.D. Recontamination as a source of pathogens in processed foods. *Int. J. Food Microbiol.* **91**, 1–11 (2004).
25. Walker, S.J., Archer, P. & Banks, J.G. Growth of Listeria monocytogenes at refrigeration temperatures. *J. Appl. Bacteriol.* **68**, 157–162 (1990).
26. Montgomery, D. C. *Design and Analysis of Experiments*, 5th Ed. (Wiley, New York, 2001).
27. Guerrero-Beltrán, J.Á., Sepulveda, D.R., Góngora-Nieto, M.M., Swanson, B. & Barbosa-Cánovas, G.V. Milk thermization by pulsed electric fields (PEF) and electrically induced heat. *J. Food Eng.* **100**, 56–60 (2010).
28. Hemme, T., Garcia, O. & Saha, A. A review of milk production on India with particular emphasis on small-scale producers. *FAO, PPLPI Working Paper No. 2, 20031–58* (2003).
29. FDA/FSIS. *Quantitative Assessment of Relative Risk to Public Health from Foodborne Listeria Monocytogenes Among Selected Categories of Ready-to-Eat Foods* (2003).
30. FDA. *FAO. Risk Assessment of Listeria Monocytogenes in Ready-to-Eat Foods: Technical Report. FDA* (2004).
31. Golberg, A., Rae, C.S. & Rubinsky, B. Listeria monocytogenes cell wall constituents exert a charge effect on electroporation threshold. *Biochim. Biophys. Acta Biomembr.* **1818**, 689–694 (2012).
32. Golberg, A., Belkin, M. & Rubinsky, B. Irreversible electroporation for microbial control of drugs in solution. *AAPS PharmSciTech* **10**, 881–886 (2009).