Pulsed electric field for increasing intracellular trehalose content in Lactobacillus plantarum WCFS1

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Highlights

- PEF is used for increasing intracellular trehalose in *L. plantarum* WCFS1
- PEF at 7.5 kV/cm resulted in 100 mM intracellular trehalose and 75% survival
- Only 23% of the lactobacilli had a permeabilized membrane for PI at 7.5 kV/cm
- Resealing of membrane pores for PI uptake was very fast, in the order of seconds

Abstract

Pulsed electric field (PEF) processing has been developed and applied in food industry for several purposes. In this study we used PEF for increasing the intracellular trehalose content in *Lactobacillus plantarum* WCFS1. Our results indicated that it is possible to increase intracellular trehalose content in *Lactobacillus plantarum* WCFS1 to ~100 mM with 75 % survival when applying a PEF treatment with an electric field strength of 7.5 kV/cm. Fluorescence staining of PEF-treated cells with propidium iodide (PI) and SYTO 9 showed that at 7.5 kV/cm only a small fraction (23%) of the cells had a permeated membrane by this PEF treatment, of which approximately half had an irreversible permeated membrane. Resealing of the pores in the membrane for PI uptake was very fast, in the order of seconds. These results indicate that PEF treatment is promising for increasing intracellular trehalose, but further optimization is required to increase the trehalose content in all cells.

Industrial relevance

The market for probiotics is growing. Probiotic survival during processing steps such as spray drying is essential for their beneficial effect. We studied pulsed electric field treatment as a method to increase the intracellular trehalose content in *L. plantarum* WCFS1 which could enhance bacterial robustness during processing. This increased bacterial robustness may again contribute to more energy efficient processing routes of probiotic foods.

Keywords: probiotics, pulsed electric field, intracellular trehalose, propidium iodide, membrane permeability

1. Introduction

The application of pulsed electric fields (PEF) can be used in the pasteurization of fruit juices (Buckow, Ng, & Toepfl, 2013; Timmermans et al., 2014), extraction of components from plant cells (Corrales, Toepfl, Butz, Knorr, & Tauscher, 2008; Lopez, Puertolas, Condon, Raso, & Alvarez, 2009) and tissue softening for easier cutting (Toepfl, Heinz, & Knorr, 2005). PEF treatment induces the formation of pores in the cell membrane, which can be either reversible or irreversible depending on the applied PEF and resealing conditions. The formation of pores in the cell membrane facilitates exchange of components with cell surroundings. The concept of such facilitated transfer of extracellular components into bacterial cells is not yet used in food industry on a larger scale. Obviously, the concept is well known for its use to introduce foreign DNA into bacterial cells for research purposes.

In this research we focused on the uptake of small environmental molecules by PEF treatment in bacteria while maintaining culture viability. This may be important for its robustness during processing. Indeed, it has been shown previously that the insertion of trehalose facilitated by electroporation in mammalian cells led to much better robustness against freezing (Dovgan, Dermol, Barlič, Knežević, & Miklavčič, 2016; Shirakashi et al., 2002). To our knowledge, this method has not yet been used for increasing small molecule concentrations in bacterial cells. Trehalose is one of the protective small molecules that has been shown to enhance robustness during processing (Leslie, Israeli, Lighthart, Crowe, & Crowe, 1995; Termont et al., 2006). Therefore this study investigated the increase of intracellular trehalose content in *Lactobacillus plantarum* WCFS1 through electroporation while maintaining the culture viability.

Lactobacillus plantarum WCFS1 is a commonly studied model microorganism for the production of probiotic formulations (Perdana et al., 2013, 2014). Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Probiotics can be supplied to the consumer in dried formulation. Drying of probiotics enhances product shelf-life, and requires processing steps such as spray or freeze drying. Survival of the microorganisms during these processing

steps is essential for their beneficial function in the human gastrointestinal tract. However, especially for spray drying, their survival is relatively low, which is a big challenge in the production process (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). There are several methods to improve the survival of bacteria during these drying procedures, for example by encapsulation of the microorganisms. Another, less intensively studied approach for improved survival is intracellular protection. Intracellular trehalose could enhance survival during these processes (Termont et al., 2006). One way of increasing intracellular trehalose content could be electroporation in a solution of trehalose.

Electroporation is used commonly in biotechnology for inserting plasmid DNA into bacterial cells (transformation). For transformation, cells should be reversibly permeabilized to take up the plasmid and survive. The main difference between plasmid uptake and our aim is the size of the molecules and thus the size of the pores in the membrane required for the uptake of the molecules. Trehalose is a much smaller molecule than plasmid DNA and thus could be more facile to introduce in the cell by electroporation (Saulis, 2010). It is essential that the poration is reversible, and that the inactivation of the bacteria during the PEF treatment is as limited as possible.

There are many PEF process parameters that influence the effects of a PEF treatment such as the electric field strength, pulse shape, pulse duration, pulse frequency etc. (Raso et al., 2016). Besides the PEF process parameters, the treatment medium is an important factor for reversible or irreversible pore formation by PEF treatment. One of the main influencing parameters is the medium conductivity. Silve et al. (2016) found that a low medium conductivity (0.1 S/m) was more effective for reversible permeabilization of mammalian cells than a high medium conductivity (1.5 S/m). After the PEF treatment the cells need to reseal their membrane to maintain viability, and therefore the period after the treatment, referred to as resealing period, is considered an important parameter that can be influenced by the temperature (Teissie, Golzio, & Rols, 2005). In our study we varied the electric field strength to find optimal conditions to increase the intracellular

trehalose content while maintaining cell viability. Furthermore, staining with propidium iodide was used to study the reversibility of the pore formation.

2. Materials and Methods

2.1 Microorganism and pre-culture conditions

Fresh cultures of *Lactobacillus plantarum* WCFS1 were obtained by plating from frozen stocks on De Man Rogosa and Sharpe (MRS) agar plates (MRS: Merck, Germany; Bacteriological agar: Oxoid, United Kingdom). The plates were incubated at 30ºC for 60-70 hours under microaerophilic conditions in jars containing 6% oxygen (Anoxomat, Mart Microbiology, the Netherlands). After incubation the plates were stored at 4ºC until further use for a maximum of 3 days. For every experiment a culture was prepared by transferring a single colony into 10 mL MRS broth (Merck, Germany), growing for 24 ± 2 hours in 30ºC, followed by a 1:100 dilution in 10 mL MRS broth and growing overnight (16-18 hours) at 30ºC before starting the PEF experiment.

2.2 Culture preparation for PEF experiments

Five mL of an overnight culture was centrifuged (Thermo-Fischer Scientific, USA) at 13,500 × *g* for 10 minutes at room temperature. The pH of the supernatant was measured (Toledo Inlab Expert, Switzerland) and was 3.9 ± 0.1 for all experiments. The resulting pellet was washed once with 5 mL washing solution (Table 1) and after centrifugation dissolved in 5-mL PEF medium (Table 1). This resulted in a culture in PEF medium containing approximately $3\text{-}4 \cdot 10^9$ cells/mL.

2.3 PEF equipment and settings

PEF treatment was performed in disposable electroporation cuvettes with an electrode distance of 2 mm (Bio-Rad, USA) using Gene-Pulser Xcell equipment, including the PC module (Bio-Rad, USA). The PEF settings were as follows: for the voltage in the electric field screening experiments 500, 1000, 1500, 2000 and 2500 V were used. Two square wave pulses of 0.1-ms pulse duration were given with a pulse interval of 5 s as this was the minimum setting of the equipment. The specific energy input of these PEF treatments is presented in table 2. Other experiments were only performed at 1500 V with the same other settings. Droop values (average decay of the pulse height) were 5-6% for all experiments.

2.4 Electric Field screening experiments

From each culture in PEF medium, one part was taken aside as a control sample, for which all steps were similar except for skipping the PEF treatment. Per experiment, two electroporation cuvettes were filled with 400-µL culture in PEF medium and electroporated using the described equipment and settings. After the PEF treatment, the content of these two cuvettes was immediately pooled into one 1.5-mL vial to have enough sample volume for further analysis. The electroporated culture was left for resealing at room temperature (± 21ºC) for 30 minutes. After these 30 minutes, samples were taken for survival assessment and intracellular trehalose content measurements.

2.5 Survival assessment

Bacterial survival after the PEF treatment was based on plate counts. For each sample (control and electroporated) three dilution series were made by pipetting 50 µL of sample into 450 µL phosphate-buffered saline (PBS, table 1) and subsequently decimally diluting until 10^{-6}. This dilution was plated on MRS agar plates in duplicate, resulting in six plates in total per sample. Plates were incubated for 48-96 hours at 30ºC under microaerophilic conditions. After incubation, colony forming units (CFU) per plate were determined from plates containing between 30 and 300 colonies. The average of the six plates per sample

was taken for calculation of the survival. Survival was calculated by dividing the CFU/mL of electroporated samples over the CFU/ml of the control sample.

2.6 Intracellular trehalose content measurements

After 30 min of resealing time the control and PEF-treated samples were centrifuged for 10 minutes at 13,500 × *g* and 4ºC. The resulting pellet was washed three times with 1 mL PBS to remove all extracellular trehalose. The supernatant of the third washing step was stored at -20ºC for HPLC analysis to check the washing efficiency. After washing, the resulting pellet was dissolved in 0.75 mL of milliQ water and transferred into bead beater vials containing 0.1-mm silica beads (MP Biomedicals, USA). The cells were disrupted using a bead beater at 4 m/s (MP Biomedicals, USA) for 5 rounds of 1 minute, each followed with a 1-3 minute interval for cooling on ice to prevent excess heating of the samples. After cell disruption the samples were centrifuged again (10 minutes, 13,500 × *g*, 4ºC) and subsequently the supernatant was transferred to a 1.5-mL vial and stored in -20ºC until HPLC analysis. HPLC analysis was performed using a Rezex RSO-Oligosaccharide column (Phenomex, USA) at 80ºC with milliQ water as a mobile phase in a flow rate of 0.3 mL/min in combination with a RI detector (Shodex RI-201, Japan).

2.7 Fluorescent staining experiments

The evaluation of the number of cells that were either reversibly or irreversibly permeabilized during PEF treatment was done using fluorescent staining. Two fluorescent stains for membrane integrity were used together in these experiments; SYTO 9 and propidium iodide (PI) (LIVE/DEAD kit, Invitrogen, USA). PI is a membrane impermeable stain which can only enter cells with a damaged membrane and a strong red fluorescence signal can be visualized by fluorescence microscopy upon binding of PI to nucleic acids. SYTO 9 is a membrane permeable stain which colours all cells green. The bacteria were stained either before the PEF treatment and 30 minutes after the PEF treatment (section 2.7.1), or at different time points after the PEF treatment to follow the resealing of the pores in time (section 2.7.2).

2.7.1 Addition of stains before and after PEF treatment

Staining the cells before the PEF treatment was done by the addition of PI and SYTO 9 to 1 mL of the culture in PEF medium, resulting in final stain concentrations of 40 µM PI and 3.3 µM SYTO 9. Subsequently, one part was taken aside as a control for initial membrane permeability (without PEF treatment) and the other part (400 µL) was pipetted into an electroporation cuvette for PEF treatment, performed as described before (section 2.3). After the PEF treatment, the PEF-treated and the control samples were kept in a dark environment for approximately 10-20 minutes before imaging using fluorescence microscopy (Axioskop 40FL Carl Zeiss, Germany). From the same biological sample, a second cuvette was PEF-treated without addition of any stains, after which the culture was transferred to a 1.5-mL vial and left for 30 minutes of resealing. After these 30 minutes of resealing 100 µL of the sample were mixed with 200-µL stain solution. The stain solution was made by dissolving PI and SYTO 9 in PBS in final concentrations of 40 µM and 3.3 µM respectively. This mixture was left for 10-20 minutes in the dark before fluorescence microscopic analysis. Approximately 20 images were captured per sample at a magnification of 630x using an Olympus XC30 camera (Olympus, Japan) and CellSens imaging software (Olympus, Japan). Image analysis was performed using a Matlab script to discriminate between red and green cells, as was described earlier by Perdana et al. (2012).

2.7.2 Addition of stains at different time points after PEF to follow membrane resealing

To analyse the resealing of the membrane in time after PEF treatment, stains were added to the PEF-treated bacteria at different time points after the PEF treatment. Again a staining solution was made by adding PI and SYTO 9 in PBS in final concentrations of 40 µM and 3.3 µM respectively. Immediately when the PEF treatment was finished, a stopwatch was set and at several time points (approximately at 10, 20, 30, 60 seconds, 5 and 30 minutes) the stains were added. These samples were left for 10-20 minutes in darkness before fluorescence microscopic analysis as described in section 2.7.1.

200 *2.8 Experimental set-up and statistical analysis*

201 All experiments were carried out at least three times with different biologically independent
202 samples, obtained from different pre-cultures of *L. plantarum* WCFS1. The results were
203 averaged or presented as single data points in the graphs. The significance was tested with
204 a Student's *t*-test, using a *P*-value of 0.05.

205

206 **3 Results and Discussion**

207 *3.1. PEF treatment for increasing intracellular trehalose content*

208 Electric field screening experiments were performed to find out whether it is possible to
209 increase the intracellular trehalose content in *Lactobacillus plantarum* WCFS1 while
210 maintaining culture viability. Electric field strengths from 2.5 to 12.5 kV/cm were
211 investigated by applying two square wave pulses of 100 µs. The field strengths used in this
212 experiment were higher than commonly used field strengths for similar experiments in
213 mammalian cells (Shirakashi et al., 2002; Silve et al., 2016) because bacteria are much
214 smaller than mammalian cells; therefore a higher field strength is required to affect the
215 bacteria with PEF (Saulis, 2010). However, to maintain the viability, the field strengths
216 that we used were lower than what is used to inactivate bacteria, which is generally above
217 15 kV/cm (Barba et al., 2015). Usually the temperature increase during the PEF process is
218 more when a higher field strength is applied, which may lead to additional inactivation
219 during the PEF treatment. In our case this effect was very small (maximum increase of
220 4ºC at 12.5 kV/cm), because of the low conductivity of the solution (0.15 S/m) in
221 combination with the relatively low electric field strengths.

222 The results show that an increasing electric field strength led to a decrease in survival after
223 PEF treatment (Fig. 1A). At field strengths of 10 and 12.5 kV/cm the survival was below
224 50%. At the same time, a higher intracellular trehalose concentration after PEF was
225 observed at 7.5, 10 and 12.5 kV/cm (Fig. 1B). This indicated that it is possible to use PEF
226 for increasing the intracellular trehalose concentration while limiting the microbial

227 inactivation. At 7.5 kV/cm there was both an enhanced intracellular trehalose content and
228 a high survival after the PEF treatment (62-93%). A critical electric field strength was
229 observed between 5 and 7.5 kV/cm for trehalose diffusion into the cell. At 2.5 and 5 kV/cm,
230 no increase was observed in intracellular trehalose compared to the control (0 kV/cm)
231 samples. Note that the trehalose content of the cells is presented in µg/mL sample. The
232 trehalose concentrations in the samples at 7.5, 10 and 12.5 kV/cm were in a similar range,
233 while the survival after PEF decreased with increasing field strength from 7.5 to 12.5
234 kV/cm. When calculating the intracellular trehalose concentration per viable cell, an
235 increase in intracellular trehalose concentration was found from 7.5 to 12.5 kV/cm.
236 However, the intracellular trehalose could be in all cells, or may be inserted at high
237 concentrations in only a small portion of these cells. It is known that PEF treatment does
238 not affect all cells in the medium in the same way, which can be due to their orientation in
239 the medium, to shielding of the electric field by other cells present and/or variations in the
240 membrane or biological state of the individual bacteria (Pucihar, Kotnik, Teissié, &
241 Miklavčič, 2007; Toepfl, Heinz, & Knorr, 2007).

242 *3.2. Propidium iodide staining to study solute uptake during PEF treatment*

243 To find out how many cells were affected by the PEF treatment, propidium iodide was used
244 as a marker molecule. Experiments were performed at 7.5 kV/cm, because this electric
245 field strength resulted in an increase in intracellular trehalose content while the culture
246 viability was still high. Other pulse parameters were the same as for the electric field
247 screening experiments.

248 Only a small part, approximately 23%, of the bacterial population exhibited a permeable
249 membrane for PI using these PEF conditions (Fig. 2). Because when PI and SYTO 9 were
250 added to the culture in PEF medium before the PEF treatment, only 23% of the cells was
251 stained red after the PEF treatment. This indicated that 77% of the cells may not be
252 affected by the PEF treatment (Fig. 2). From the fraction of cells that was affected,
253 approximately half had a reversibly permeabilized membrane (approximately 13%). This
254 fraction was the difference in red (PI) stained cells between addition of the stains before

the PEF treatment and 30 minutes afterwards. This may imply that the trehalose concentration that was measured before in fact might originate from a high trehalose concentration achieved in a limited fraction of the bacterial population, while other cells may not have been affected.

The other 10% had an irreversibly damaged membrane, as these cells were stained red when the stains were added 30 minutes after the PEF treatment. This fraction may well be considered to be dead. However, comparing membrane damage with bacterial survival can be complicated because of the different viability states of bacteria (culturable/metabolically active/intact membrane) (Davis, 2014; Sträuber & Müller, 2010). Garcia et al. (2007) found a good correlation between *L. plantarum* cells that were stained with PI after PEF treatment and cells that were not culturable anymore. Ulmer et al. (2002) found however no significant decrease in plate counts at electric field strengths below 19 kV/cm with energy inputs until 42 kJ/kg, but did find an increase in membrane permeability at these field strengths. The energy input and electric field strength in this study (16.2 kJ/kg at 7.5 kV/cm) were lower, but resulted in a slight decrease in plate counts and permeability (Fig. 1 & 2). Variations between studies may be due to differences in PEF settings or other factors such as treatment medium composition, but also due to the presentation of the results (log-scale versus percentage) of the plating method.

Assuming that the increase in intracellular trehalose concentration that we measured with HPLC analysis (Fig. 1) is in the bacteria that were reversible permeable for PI, the amount of intracellular trehalose in these cells can be calculated from the results of section 3.1 using equation 1.

$$C_{tre}^{i} = \frac{C_{tre}^{s}}{N_{cells} \cdot x_{rev} \cdot V_{cell} \cdot x_{cytosol}} \quad (1)$$

In which C_{tre}^{i} is the intracellular trehalose concentration in the reversible permeabilized cells in mM, C_{tre}^{s} the trehalose concentration in the sample in mM, which can be obtained from Fig. 1 by dividing by the molecular weight of trehalose, N_{cells} the total number of cells per

mL sample, x_{rev} the reversible permeabilized fraction, V_{cell} the bacterial cell volume and $x_{cytosol}$ the fraction cytosol of the total cell volume.

The cell volume of *L. plantarum* WCFS1 was estimated, based on cell size measurements from microscopic pictures, to be around $9.2 \cdot 10^{-13}$ mL, which is in a similar range of what was found in literature (Dumont, Marechal, & Gervais, 2004). The total number of cells was approximately $3.5 - 4 \cdot 10^{9}$ cells per mL based on plate counting results. By assuming x_{rev} to be 0.1 (Fig. 3), $x_{cytosol}$ to be 0.7 (Luby-Phelps, 1999), and taking a trehalose content in the sample of 8 µg/mL from figure 1, the possible amount of trehalose in these cells is calculated to be approximately 100 mM, or around 35% of the concentration that was imposed from the outside during PEF treatment. This amount is similar to what was earlier estimated for trehalose uptake during PEF treatment in mammalian cells (Shirakashi et al., 2002). Termont et al. (2006) found that intracellular trehalose concentrations of approximately 30-50 mg/g wet cell weight (wcw) in *Lactococcus lactis* protected the bacteria during freeze drying and enhanced resistance against bile salts and gastric acid. By assuming a wet cell weight of approximately 1 pg per cell (the biggest part of the cell consists of water), our method resulted in an intracellular trehalose content of approximately 25 mg/g wcw, which is slightly below the concentrations of Termont et al. (2006) that were obtained via genetic modification.

Of course, when making these estimations based on PI uptake, we need to take into account that trehalose is another molecule than PI; trehalose has a molecular weight of 342 Da while PI has a molecular weight of 668 Da. Saulis (2010) described that molecular uptake by PEF treatment can be different for molecules of different sizes, e.g. small ions compared to mannitol and sucrose. This strongly depends on the pore size, which can be affected by several pulse parameters such as electric field strength and pulse width. Also after the pulse, during the resealing phase, a pore may stay open longer for small molecules such as ions than for molecules like sugars and PI (Saulis, 2010).

To study the time that the pores stayed open for PI uptake after PEF treatment, PI was added at different time points after the PEF treatment. The results indicated that the pores

created during the PEF treatment, closed or reduced in size really fast after the treatment, i.e. in the range of several seconds (Fig. 3). This is much shorter than what Shirakashi et al. (2004) described for mammalian cells: they found resealing times for PI uptake in the order of several minutes. All the samples that were stained before the PEF treatment (t=0 in Fig. 3) showed significantly higher PI uptake than the samples stained at different time points after PEF treatment. This indicated that for *L. plantarum* WCFS1, resealing was very fast after the PEF treatment.

Given the relatively small fraction of the bacterial population currently affected by the PEF treatment, it is important to increase the reversibly electroporated fraction of the bacterial population for future applications of this process in food industry. A bigger fraction of reversible electroporated cells, containing trehalose, is required for the potential beneficial effect during processing. For this beneficial effect trehalose should be present in the cells for internal protection of the membrane and other molecules such as proteins in the cytosol. To further develop the proposed PEF method, it should be evaluated to other bacteria as well. However, the optimal PEF conditions for trehalose transfer probably differ between bacterial species or even strains as was demonstrated before for inactivation of bacteria (Saldaña et al., 2009). Moreover, further studies are required to critically test the benefits of an additional PEF treatment to improve survival during subsequent processing.

4. Conclusions

Pulsed electric field treatment can increase the intracellular trehalose content of *Lactobacillus plantarum* WCFS1 while maintaining culture viability. Electric field screening experiments with two pulses of 100 µs indicated a critical electric field strength for trehalose diffusion into the cell between 5 and 7.5 kV/cm. Two square wave pulses of 100 µs at 7.5 kV/cm led to an increase in intracellular trehalose while maintaining cell viability after the PEF treatment. Study of the membrane permeability during and after this PEF treatment showed that only a small fraction of the bacterial population was reversible affected by the current PEF treatment, but these cells acquired a high internal concentration of trehalose. The pores in the cell membrane of *L. plantarum* WCFS1 closed

very fast after the PEF treatment for the uptake of PI, which indicates that resealing time for PI uptake is in the order of seconds. Optimization of trehalose uptake by PEF treatment should focus on a higher fraction of reversible electroporated cells, containing trehalose, in order to potentially enhance bacterial robustness during processing.

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References

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., … Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, *77*, 773–798. https://doi.org/10.1016/j.foodres.2015.09.015

Buckow, R., Ng, S., & Toepfl, S. (2013). Pulsed electric field processing of orange juice: A review on microbial, enzymatic, nutritional, and sensory quality and stability. *Comprehensive Reviews in Food Science and Food Safety*, *12*(5), 455–467. https://doi.org/10.1111/1541-4337.12026

Corrales, M., Toepfl, S., Butz, P., Knorr, D., & Tauscher, B. (2008). Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison. *Innovative Food Science and Emerging Technologies*, *9*(1), 85–91. https://doi.org/10.1016/j.ifset.2007.06.002

Davis, C. (2014). Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *Journal of Microbiological Methods*. https://doi.org/10.1016/j.mimet.2014.04.012

Dovgan, B., Dermol, J., Barlič, A., Knežević, M., & Miklavčič, D. (2016). Cryopreservation of human umbilical stem cells in combination with trehalose and reversible electroporation. In *IFMBE Proceedings* (Vol. 53, pp. 307–310). https://doi.org/10.1007/978-981-287-817-5_68

Dumont, F., Marechal, P.-A., & Gervais, P. (2004). Cell size and water permeability as determining factors for cell viability after freezing at different cooling rates. *Applied and Environmental Microbiology*, *70*(1), 268–272. https://doi.org/10.1128/AEM.70.1.268

García, D., Gómez, N., Mañas, P., Raso, J., & Pagán, R. (2007). Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated. *International Journal*

372 *of Food Microbiology*, *113*(2), 219–27.
373 https://doi.org/10.1016/j.ijfoodmicro.2006.07.007

374 Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., … Sanders, M. E.
375 (2014). Expert consensus document: The international scientific association for
376 probiotics and prebiotics consensus statement on the scope and appropriate use of
377 the term probiotic. *Nature Reviews Gastroenterology and Hepatology*, *11*(8), 506–
378 514. https://doi.org/10.1038/nrgastro.2014.66

379 Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and
380 sucrose protect both membranes and proteins in intact bacteria during drying.
381 *Applied and Environmental Microbiology*, *61*(10), 3592–3597.

382 Lopez, N., Puertolas, E., Condon, S., Raso, J., & Alvarez, I. (2009). Enhancement of the
383 extraction of betanine from red beetroot by pulsed electric fields. *Journal of Food*
384 *Engineering*, *90*(1), 60–66. https://doi.org/10.1016/j.jfoodeng.2008.06.002

385 Luby-Phelps, K. (1999). Cytoarchitecture and physical properties of cytoplasm: volume,
386 viscosity, diffusion, intracellular surface area. *International Review of Cytology*, *192*,
387 189–221. https://doi.org/10.1016/S0074-7696(08)60527-6

388 Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., & Ross, R. P. (2008).
389 Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry*, *106*(4
390 SPEC. ISS.), 1406–1416. https://doi.org/10.1016/j.foodchem.2007.04.076

391 Perdana, J., Bereschenko, L., Fox, M. B., Kuperus, J. H., Kleerebezem, M., Boom, R. M.,
392 & Schutyser, M. A. I. (2013). Dehydration and thermal inactivation of *Lactobacillus*
393 *plantarum* WCFS1: Comparing single droplet drying to spray and freeze drying. *Food*
394 *Research International*, *54*(2), 1351–1359.
395 https://doi.org/10.1016/j.foodres.2013.09.043

396 Perdana, J., Bereschenko, L., Roghair, M., Fox, M. B., Boom, R. M., Kleerebezem, M., &
397 Schutyser, M. A. I. (2012). Novel method for enumeration of viable *Lactobacillus*
398 *plantarum* WCFS1 cells after single-droplet drying. *Applied and Environmental*

399 *Microbiology*, *78*(22), 8082–8088. https://doi.org/10.1128/AEM.02063-12

400 Perdana, J., den Besten, H. M. W., Aryani, D. C., Kutahya, O., Fox, M. B., Kleerebezem,
401 M., … Schutyser, M. A. I. (2014). Inactivation of *Lactobacillus plantarum* WCFS1
402 during spray drying and storage assessed with complementary viability
403 determination methods. *Food Research International*, *64*, 212–217.
404 https://doi.org/10.1016/j.foodres.2014.06.029

405 Pucihar, G., Kotnik, T., Teissié, J., & Miklavčič, D. (2007). Electropermeabilization of
406 dense cell suspensions. *European Biophysics Journal*, *36*(3), 173–185.
407 https://doi.org/10.1007/s00249-006-0115-1

408 Raso, J., Frey, W., Ferrari, G., Pataro, G., Knorr, D., Teissie, J., & Miklavčič, D. (2016).
409 Recommendations guidelines on the key information to be reported in studies of
410 application of PEF technology in food and biotechnological processes. *Innovative*
411 *Food Science and Emerging Technologies*, *37*, 312–321.
412 https://doi.org/10.1016/j.ifset.2016.08.003

413 Saldaña, G., Puértolas, E., López, N., García, D., Álvarez, I., & Raso, J. (2009).
414 Comparing the PEF resistance and occurrence of sublethal injury on different strains
415 of *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes* and
416 *Staphylococcus aureus* in media of pH 4 and 7. *Innovative Food Science & Emerging*
417 *Technologies*, *10*(2), 160–165.
418 https://doi.org/https://doi.org/10.1016/j.ifset.2008.11.003

419 Saulis, G. (2010). Electroporation of cell membranes: the fundamental effects of pulsed
420 electric fields in food processing. *Food Engineering Reviews*, *2*(2), 52–73.
421 https://doi.org/10.1007/s12393-010-9023-3

422 Shirakashi, R., Köstner, C. M., Müller, K. J., Kürschner, M., Zimmermann, U., &
423 Sukhorukov, V. L. (2002). Intracellular delivery of trehalose into mammalian cells by
424 electropermeabilization. *Journal of Membrane Biology*, *189*(1), 45–54.
425 https://doi.org/10.1007/s00232-002-1003-y

426 Shirakashi, R., Sukhorukov, V. L., Tanasawa, I., & Zimmermann, U. (2004).
427 Measurement of the permeability and resealing time constant of the electroporated
428 mammalian cell membranes. *International Journal of Heat and Mass Transfer*,
429 *47*(21), 4517–4524. https://doi.org/10.1016/j.ijheatmasstransfer.2004.04.007

430 Silve, A., Leray, I., Poignard, C., & Mir, L. M. (2016). Impact of external medium
431 conductivity on cell membrane electropermeabilization by microsecond and
432 nanosecond electric pulses. *Scientific Reports*, *6*, 19957.
433 https://doi.org/10.1038/srep19957

434 Sträuber, H., & Müller, S. (2010). Viability states of bacteria-Specific mechanisms of
435 selected probes. *Cytometry Part A*. https://doi.org/10.1002/cyto.a.20920

436 Teissie, J., Golzio, M., & Rols, M. P. (2005). Mechanisms of cell membrane
437 electropermeabilization: A minireview of our present (lack of?) knowledge.
438 *Biochimica et Biophysica Acta - General Subjects*.
439 https://doi.org/10.1016/j.bbagen.2005.05.006

440 Termont, S., Vandenbroucke, K., Iserentant, D., Neirynck, S., Steidler, L., Remaut, E., &
441 Rottiers, P. (2006). Intracellular accumulation of trehalose protects *Lactococcus*
442 *lactis* from freeze-drying damage and bile toxicity and increases gastric acid
443 resistance. *Applied and Environmental Microbiology*, *72*(12), 7694–7700.
444 https://doi.org/10.1128/AEM.01388-06

445 Timmermans, R. A. H., Nierop Groot, M. N., Nederhoff, A. L., van Boekel, M. A. J. S.,
446 Matser, A. M., & Mastwijk, H. C. (2014). Pulsed electric field processing of different
447 fruit juices: Impact of pH and temperature on inactivation of spoilage and
448 pathogenic micro-organisms. *International Journal of Food Microbiology*, *173*, 105–
449 111. https://doi.org/10.1016/j.ijfoodmicro.2013.12.022

450 Toepfl, S., Heinz, V., & Knorr, D. (2005). Overview of pulsed electric field processing of
451 foods. In *Emerging technologies for food processing* (pp. 69–97). Academic Press:
452 London, UK.

453 Toepfl, S., Heinz, V., & Knorr, D. (2007). High intensity pulsed electric fields applied for
454 food preservation. *Chemical Engineering and Processing: Process Intensification*,
455 *46*(6), 537–546. https://doi.org/10.1016/j.cep.2006.07.011

456 Ulmer, H. M., Heinz, V., Gänzle, M. G., Knorr, D., & Vogel, R. F. (2002). Effects of pulsed
457 electric fields on inactivation and metabolic activity of *Lactobacillus plantarum* in
458 model beer. *Journal of Applied Microbiology*, *93*(2), 326–335.
459 https://doi.org/10.1046/j.1365-2672.2002.01699.x

460

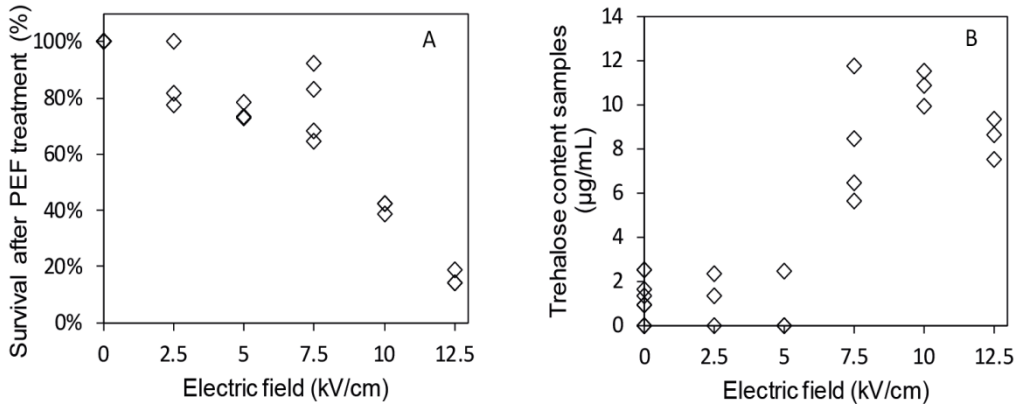

Figure 1 **Survival (A) and intracellular trehalose content (B) after PEF treatment at various electric field strengths (2.5 - 12.5 kV/cm).** A: Survival is based on plate counts of the PEF treated samples versus the control samples. B: Measurements at 0 kV/cm are the control (not PEF treated) samples. Data points at 0 µg/mL were measured but below the detection limit of the HPLC method (~1 µg/ml trehalose). Each data point at a certain electric field strength represents one biological replicate. At least three biological replicates were measured for each electric field strength.

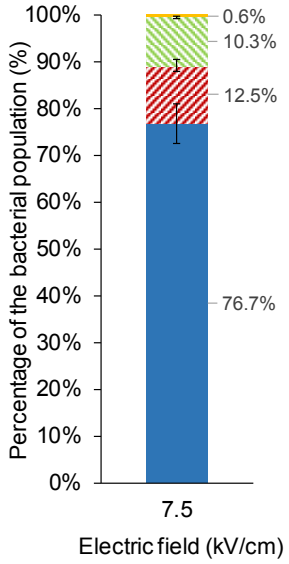

Figure 2 **Membrane permeability due to PEF treatment measured using PI staining.** PEF treatment was performed with 2 pulses of 100 µs at 7.5 kV/cm. Percentage of the population with a permeable membrane for PI before PEF treatment (■), an irreversibly permeable membrane 30 minutes after the PEF treatment (▨), a reversibly permeable membrane 30 minutes after PEF treatment (▨) and without membrane permeability for PI during PEF treatment (■). Error bars indicate standard deviations of 3 biological replicates.

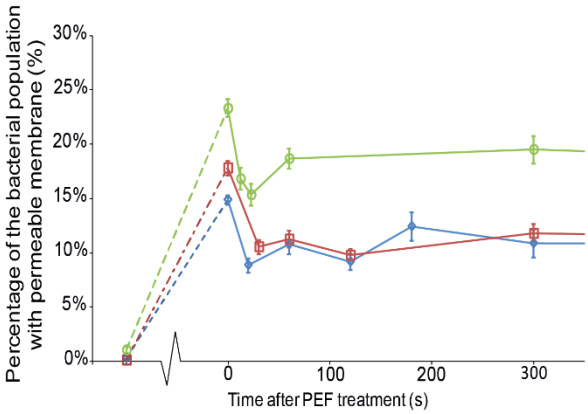

Figure 3 Cell membrane permeability for PI before, during and after PEF treatment over time. Time points on the x-axis represent the moment at which the stain was added to the culture. t = 0 s is the moment of the PEF treatment. The first data point (before the axis break) is the control (without/before PEF) and the data points at t = 0 s are with stains added before PEF treatment. Data points after t=0 indicate the membrane permeability at the specific time at which the stains were added. One data point at t=1800 or 2400 s for each replicate is not presented, this data point was similar to the t= 300 s data point. Each symbol represents one biological replicate, error bars indicate the standard error of the mean of different pictures, n=10-20.

Table 1 Composition, pH and conductivity of solutions used in the PEF experiments. All solutions were autoclaved for 15 minutes at 121ºC before use.

	Washing solution	**PEF medium[1]**	**PBS**
Composition	0.29 g NaCl[b] 0.89 g $Na_2HPO_4 \cdot 2 H_2O$[a] 0.69 g $NaH_2PO_4 \cdot H_2O$[a] 0.095 g $MgCl_2 \cdot 6 H2O$[b] 1000 g demineralized water	99.3 g Trehalose[a] 0.29 g NaCl[b] 0.89 g $Na_2HPO_4 \cdot 2 H_2O$[a] 0.69 g $NaH_2PO_4 \cdot H_2O$[a] 0.095 g $MgCl_2 \cdot 6 H2O$[b] 1000 g demineralized water	1.93 g $Na_2HPO_4 \cdot 2H_2O$[a] 0.35 g $NaH_2PO_4 \cdot H2O$[a] 8.20 g NaCl[b] 1000 g demineralized water
pH	6.9	6.8	7.2
Conductivity	0.21 S/m	0.15 S/m (with bacteria)	n.a.

[1]Adapted from Silve et al. (2016) ; salts from SNM medium, with addition of trehalose

Chemicals obtained from: [a] Merck, Germany, [b] Sigma Aldrich, USA

Table 2 Specific energy of the PEF treatments with 2 pulses of 100 μs pulse duration at various electric field strengths.

Electric field (kV/cm)	**Specific energy input[1] (kJ/kg)**
2.5	1.8
5.0	7.2
7.5	16.2
10.0	28.9
12.5	45.1

[1]Calculated assuming a liquid density similar to that of a 10% sucrose solution (Asadi, 2005)