Bioelectrochemistry 137 (2021) 107680

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Short communication

Temperature and presence of ethanol affect accumulation of intracellular trehalose in *Lactobacillus plantarum* WCFS1 upon pulsed electric field treatment

E.M.J. Vaessen [a,b], H.A. Kemme [a,b], R.A.H. Timmermans [c], M.A.I. Schutyser [a], H.M.W. den Besten [b,*]

[a] Food Process Engineering, Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands
[b] Food Microbiology, Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands
[c] Wageningen Food and Biobased Research, Wageningen University and Research, P.O. Box 17, 6700 AA Wageningen, the Netherlands

ARTICLE INFO

Article history:
Received 12 June 2020
Received in revised form 19 September 2020
Accepted 24 September 2020
Available online 1 October 2020

Keywords:
Temperature
Ethanol
Pulsed electric field
Intracellular trehalose
Membrane fluidity

ABSTRACT

Pulsed electric field (PEF) treatment can be used to increase intracellular small molecule concentrations in bacteria, which can lead to enhanced robustness of these cells during further processing. In this study we investigated the effects of the PEF treatment temperature and the presence of 8% (v/v) ethanol in the PEF medium on cell survival, membrane fluidity and intracellular trehalose concentrations of *Lactobacillus plantarum* WCFS1. A moderate PEF treatment temperature of 21 °C resulted in a high cell survival combined with higher intracellular trehalose concentrations compared to a treatment at 10 and 35 °C. Interestingly, highest intracellular trehalose concentrations were observed upon supplementing the PEF medium with 8% ethanol, which resulted in more than a doubling in intracellular trehalose concentrations, while culture survival was retained. Overall, this study shows that treatment temperature and PEF medium optimization are important directions for improving molecule uptake upon PEF processing.

1. Introduction

Pulsed electric field (PEF) processing can lead to permeabilization of the cellular membrane [1]. This electropermeabilization is of interest for several applications in food and biotechnological industries, such as microbial inactivation and extraction of components from cells [2,3]. Another application is increasing intracellular concentrations of protective solutes such as trehalose in order to increase bacterial robustness during further processing. This increased robustness is relevant for the production of dried probiotics and starter cultures [4,5]. In earlier studies increased intracellular trehalose concentrations have been linked to increased survival of bacteria after freeze drying and spray drying [4,6], as well as to increased survival of mammalian cells after cryopreservation and freeze drying [7,8].

PEF employs the use of nano- to millisecond high voltage pulses that lead to permeabilization of the cellular membrane [3]. Due to this membrane permeabilization molecules that are present in the treatment medium can diffuse into the cell. The use of electropermeabilization for loading bacterial cells with specific molecules is not new; it is a commonly used approach to introduce plasmid DNA into cells [2]. Our study aims to increase the intracellular protective solute concentrations to increase bacterial robustness, while maintaining cell survival. This implicates that the permeabilization of the membrane should be temporary and that a large part of the permeabilized cells should be able to survive the treatment [1,9].

Many factors can influence survival and molecule uptake during PEF treatment. These factors include process parameters, microbial characteristics and treatment medium characteristics [10]. Temperature plays an important role in the inactivation effects of a PEF treatment. In general, more bacterial inactivation is observed at higher treatment temperatures, which are not lethal itself to the bacteria [11–13]. Despite that there is a lot of knowledge on the effects of temperature on survival of PEF-treated bacteria, the knowledge on the effects of PEF temperature on intracellular accumulation of small molecules is limited.

Temperature affects the fluidity of the cellular membrane, which is the target site of a PEF treatment. In general, short term exposure to a higher temperature results in an increase in membrane fluidity, while incubation for a longer time at higher temperature, for example during growth, will result in a more rigid cell membrane [11]. Not only temperature can influence the

* Corresponding author.
E-mail address: heidy.denbesten@wur.nl (H.M.W. den Besten).

https://doi.org/10.1016/j.bioelechem.2020.107680
1567-5394/

membrane fluidity, also the medium composition can. A component that is widely studied for its effects on membrane fluidity is ethanol [14,15]. Model simulations revealed that ethanol can easily pass through the lipid bilayer of a cell membrane, leading to a reduced order of the lipid hydrocarbon chains and therefore a higher membrane fluidity [16]. During long term exposure to ethanol, bacteria will adapt their membrane composition in order to control their optimal membrane fluidity, similar to long term incubation at non-optimal temperatures [14,15]. Some studies concluded that membrane fluidity can play a role in PEF resistance or electropermeabilization of cells [17,18], while others found that membrane fluidity was not the main factor determining bacterial PEF resistance [11].

A common way to investigate membrane fluidity is by measuring the fluorescence anisotropy of a lipid soluble membrane probe, such as 1,6- diphenyl-1,3,5-hexatriene (DPH), which is inversely related to membrane fluidity [19]. Studies that investigated the relation between PEF and membrane fluidity focussed mainly on bacterial inactivation [11,17]. In contrast, we studied the effects of temperature and the presence of ethanol in the PEF medium on bacterial survival and measured the trehalose accumulation upon PEF treatment in our model probiotic *Lactobacillus plantarum* WCFS1. The focus of our study is on short-term exposure to these process conditions with the aim of maximizing intracellular trehalose concentrations and PEF survival, which might contribute to increased robustness of these bacteria. In addition, membrane fluidity was measured by the DPH fluorescence anisotropy method to evaluate whether or not the observed effects could be related to membrane fluidity.

2. Materials and methods

2.1. Microorganism and pre-culture conditions

Lactobacillus plantarum WCFS1, originally isolated from human saliva [20], was obtained from the Wageningen Food Microbiology strain collection. This strain was pre-cultured in the same way as described in previous studies [5]. Briefly, liquid cultures of *L. plantarum* WCFS1 were obtained by inoculating a single colony from a De Man Rogosa and Sharpe (MRS) agar plate (MRS: Merck, USA, Bacteriological agar: Oxoid, United Kingdom) into 10 mL MRS broth. This culture was incubated without shaking at 30 °C for 24 ± 2 h. Subsequently, these cultures have been diluted 1:100 into fresh MRS broth and incubated again under the same conditions for 17 ± 1 h. After this overnight incubation the pH of the supernatant was measured and was 3.9 ± 0.1 for all cultures.

2.2. Culture preparation for PEF experiments

Overnight cultures were centrifuged at 13,500 × *g* for 10 min at room temperature. The resulting pellet was washed once with washing solution (composition similar to the PEF medium (see Table 1) without addition of trehalose [exact composition of the washing solution can be found in Vaessen et al. [5]]) and after a second centrifugation step suspended into the PEF medium. The PEF media were prepared by adding the amounts of salts and trehalose described in Table 1 to 1 L of demi-water for the standard PEF medium, or to 920 mL demi-water and 80 mL 99.9% ethanol (Merck, USA) for the PEF medium containing 8% (v/v) ethanol. The amounts of the added salts slightly differed between the two PEF media in order to obtain a similar final treatment medium conductivity of 0.15 S/m at room temperature.

Table 1
Amounts of salts and trehalose in the PEF media used in this study. These amounts were added to 1 L demi-water without (standard) or with 8% (v/v) ethanol, respectively.

Component	Amount added for standard PEF medium (g)	Amount added for ethanol containing PEF medium (g)
NaCl[a]	0.29	0.38
$Na_2HPO_4{\cdot}2H_2O$[b]	0.89	1.18
$NaH_2PO_4{\cdot}H_2O$[b]	0.69	0.91
$MgCl_2{\cdot}6H_2O$[a]	0.095	0.13
Trehalose[b]	99.3	99.3

Chemicals were obtained from [a]Sigma Aldrich, USA and [b]Merck, USA.

2.3. PEF experiments

PEF experiments were carried out in a batch PEF equipment (IXL, The Netherlands) with a custom made treatment chamber consisting of two parallel titanium plate electrodes separated with a silicon spacer with a thickness of 3 mm, as elaborately explained in previous work [21]. The electrodes had a treatment area of approximately 430 cm^2 each, resulting in a treatment volume of 13 mL. The temperature in the treatment chamber was controlled at a temperature of 10, 21 or 35 °C via a water bath that was connected as a thermostatic cooling/heating block that was mounted to the grounded electrode. For all experiments in this study the treatment chamber was filled with approximately 13 mL of culture suspension, after which a PEF treatment with two pulses of 100 μs at 6.3 kV/cm was applied. Subsequently, approximately 5 mL (~40%) of sample (i.e. two pulses sample) was taken out of the treatment chamber for analysis and the treatment chamber was filled again up to 13 mL by adding untreated culture. Then, 10 more pulses of 100 μs at 6.3 kV/cm with a pulse interval of 5 s were given, resulting in a PEF treatment of 10–12 pulses in total for these samples. The control samples without PEF treatment, samples with two pulses and with 10–12 pulses were subsequently used for survival and intracellular trehalose analysis within 20 min after the PEF treatment. The pulse wave form of the monopolar applied pulses is shown in Fig. 1. The specific energy input per pulse was 5.7 kJ/kg. This might lead to a maximum theoretical temperature increase of 16 °C for the PEF treatment with 10–12 pulses. However, due to the large pulse interval (5 s) and large electrode area connected to the water bath, this temperature increase was by far not reached.

2.4. Survival analysis

Survival analysis was performed in the same way as described in Vaessen et al. [5]. Briefly, control and PEF-treated samples were decimally diluted in phosphate buffered saline (PBS) after which the appropriate dilution was plated on MRS agar. For each sample dilution series were made in triplicate and per dilution series two plates were made, resulting in six plates per sample. These plates

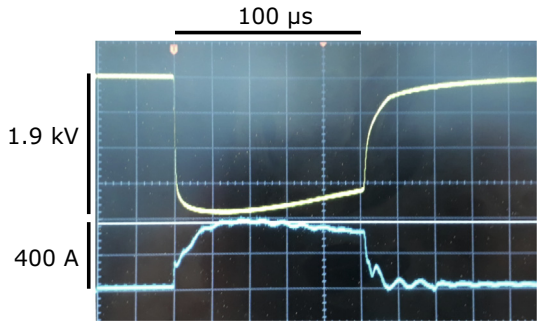

Fig. 1. Typical pulse shape, voltage and current applied in this study, leading to an energy input of approximately 5.7 kJ/kg per pulse.

were incubated for 2–4 days after which the colony forming units (cfu) were counted and the average number of the six plates per sample was taken and used to calculate the number of cells (cfu/mL). Survival percentages were calculated by dividing the cfu/mL of the PEF-treated sample by the cfu/mL of the control sample and subsequently multiplying with 100%.

2.5. Intracellular trehalose analysis

Intracellular trehalose concentrations were determined according to the method described in Vaessen et al. [21]. Briefly, extracellular trehalose was washed away by three washing steps in PBS, and subsequently the pellet was suspended in milliQ water and homogenized in a bead beater to extract intracellular trehalose. Trehalose concentrations in cell extracts were measured using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [21].

2.6. Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were carried out to measure membrane fluidity in a similar way as described previously [11,14,22]. Fluorescence anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Merck, Germany) was measured in a fluorophotometer RF-6000 with a UV–VIS polarizer (Schimadzu Corporation, Japan), where the temperature of the cuvette holder was controlled by a water bath and the sample in the cuvette was stirred in order to have a homogenous temperature and cell concentration. A stock solution of 7.5 mM DPH in DMSO (Merck, Germany) was kept in the freezer at −20 °C. Overnight cultures of *L. plantarum* WCFS1 were washed in the same way as described in Section 2.2 and subsequently suspended in the PEF medium to obtain an OD_{600} of 0.6. DPH from the stock solution was added to this culture in PEF medium in a final concentration of 3 μM. Furthermore the following control samples were prepared using the same concentrations: PEF medium, PEF medium supplemented with DPH, PEF medium with inoculated culture. All samples were incubated for 15 min at 30 °C before measuring. Fluorescence intensities I_{VV}, I_{VH}, I_{HV} and I_{HH} with polarisation directions vertical (V) and horizontal (H) were measured for all samples with an excitation wavelength of 358 nm and emission wavelength of 430 nm, after which fluorescence anisotropy (r) was calculated according to equation (1) [23].

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \quad \text{with } G = \frac{I_{HV}}{I_{HH}} \tag{1}$$

In which the fluorescence intensities were corrected for the influence of culture concentration, medium and DPH using equation (2).

$$I_{VV} = I_{VVculture+medium+DPH} - I_{VVculture+medium} - I_{VVmedium+DPH} + I_{VVmedium} \tag{2}$$

This equation was also used to calculate I_{VH}, I_{HV}, and I_{HH}.

2.7. Experimental set-up

All PEF experiments were carried out using biologically independent replicates, with each replicate pre-cultured and PEF-treated on another day. Significance of the treatment effect was tested with a Students *t*-test and trend analysis was done using linear regression in Excel with significance testing of the slope value. P-values below 0.05 were considered significant.

3. Results and discussion

3.1. Effects of PEF temperature on survival and intracellular trehalose content

Lactobacillus plantarum WCFS1 was PEF-treated at 10, 21 and 35 °C with an electric field strength of 6.3 kV/cm. Survival after PEF treatment with 10–12 pulses decreased when the PEF temperature was increased from 10 to 21 °C and further to 35 °C (Fig. 2A). This inactivation trend was similar for the PEF treatment with 2 pulses, although in this case the survival decreased from 97 to 85% and only differed significantly between 10 °C and 35 °C (Fig. 2A). This decrease in survival after PEF treatment when increasing the PEF temperature is similar to the effect of temperature observed in other studies on bacterial inactivation with PEF treatment [11–13]. These studies describe a synergistic effect between temperature and PEF treatment on bacterial inactivation. In order to obtain a similar level of inactivation, PEF treatments with lower energy inputs are required when the treatment temperature is higher. Interestingly, the largest increase in intracellular trehalose was observed at a moderate temperature of 21 °C, where the concentration was 16 μg/mL when 10–12 pulses were applied (Fig. 2B). This amount is similar to what was found in our previous study for a PEF treatment with 10 pulses of 6.3 kV/cm [21]. At a lower temperature of 10 °C up to 11 μg/mL intracellular trehalose was found in the cell extract samples together with a high culture survival (Fig. 2A). However, at 35 °C the trehalose contents of the cell extract samples upon the applied PEF treatments only increased to 5 μg/mL and 3 μg/mL when 2 or 10–12 pulses were applied, respectively. For the PEF treatment with 10–12 pulses at 35 °C culture survival also decreased considerably to 54%, which might partly explain the smaller increase in intracellular trehalose for this PEF condition. Based on these results, we propose a PEF temperature of 21 °C being optimal for increasing intracellular trehalose concentrations while maintaining a high culture viability. To our knowledge, the influence of temperature on small molecule uptake during PEF treatment has not been studied before, though some studies report similar trends on the effect of electroporation on plasmid uptake. For example, Rivas et al. [24] found that the transformation efficiency of *E.coli* was higher upon PEF treatment at 24 °C compared to 7 °C, 16 °C and 30 °C. It should be noted here that the mechanism for plasmid uptake during electroporation differs from small molecule uptake due to stronger interactions between the membrane and the plasmid [25].

3.2. Effects of temperature and ethanol on membrane fluidity

Membrane fluidity can be influenced by temperature [11,22]. In Fig. 3 the fluorescence anisotropy of the probe DPH is shown, which is inversely related to membrane fluidity. The slope of the curve in Fig. 3A was significantly decreasing ($P < 0.05$) based on linear regression analysis, which indicates that membrane fluidity increased with increasing processing temperature. This is in line with the results of Meneghel et al. [22], who found a similar relation between temperature and fluorescence anisotropy for *Lactobacillus bulgaricus*.

In addition to the effect of temperature, the effect of the presence of 8% ethanol in the PEF medium was evaluated. A concentration of 8% ethanol was chosen based on previous research, where this concentration was found to be sublethal for *L. plantarum* WCFS1 [26] and induced membrane fluidity changes in another Gram-positive bacterium [15]. The effect of the presence of 8% ethanol on membrane fluidity was evaluated at 10 and 21 °C, but no difference in DPH fluorescence anisotropy between the samples with and without addition of ethanol was observed at both

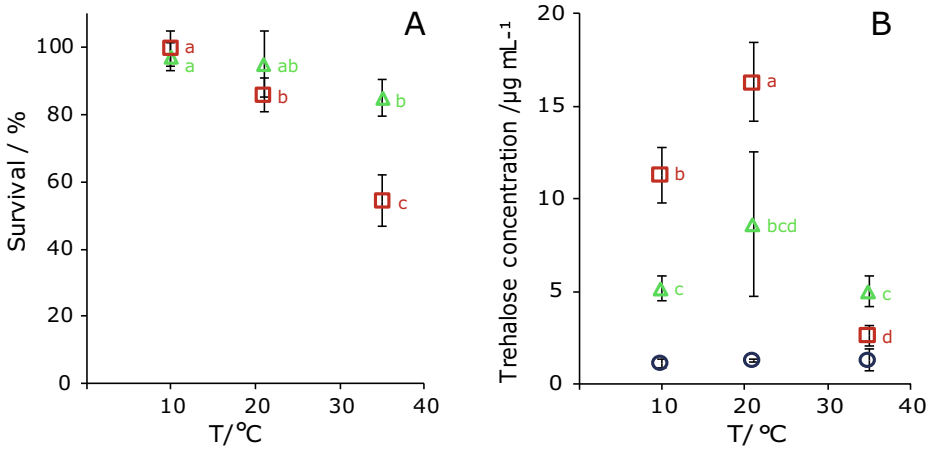

Fig. 2. Effect of temperature on survival after PEF treatment (A) and intracellular trehalose content (B) of *L. plantarum* WCFS1. Different symbols represent control (not PEF treated) (○) and PEF treated cultures with 2 (□) and 10–12 pulses (△) of 100 µs at 6.3 kV/cm. Intracellular trehalose contents are presented in µg/mL sample, with each sample containing ~ $4*10^9$ CFU/mL before PEF treatment. Error bars show standard deviations of biologically independent replicates (n = 3) and different letters indicate significant differences.

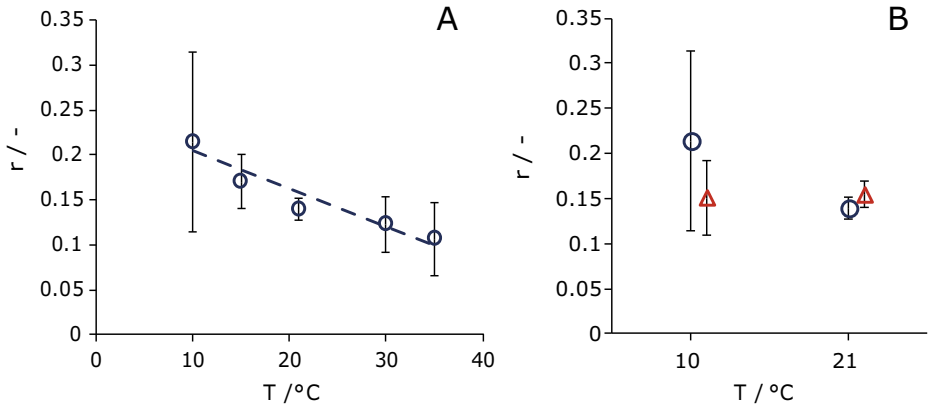

Fig. 3. Effect of temperature (A) and presence of 8% ethanol in the PEF medium (B) on membrane fluidity of *L. plantarum* WCFS1. Fluorescence anisotropy values (r) of DPH are presented without (○) and with (□) the presence of ethanol. The linear regression line is shown in figure A. Error bars show standard deviations of biologically independent replicates (n ≥ 3).

temperatures (Fig. 3B). This may be due to the inaccuracy of the DPH measurements or related to the effect of prolonged exposure of the cells to ethanol and/or to the effect of ethanol on membrane proteins. In the case of prolonged exposure the cells start to adapt their membrane to the presence of ethanol, amongst others by cis–trans isomerisation of monounsaturated fatty acids, to retain their initial membrane fluidity [14,27]. This adaptation can already result in a retention of the initial membrane fluidity after 30 min exposure to ethanol [14]. Indeed, in our membrane fluidity experiments cells were exposed to ethanol for more than 30 min, because membrane fluidity measurements (including 15 min of pre-incubation time with DPH) at several conditions took 30–90 min. On the other hand, in the PEF experiments the cells were only 10–15 min in the ethanol medium before the PEF treatment. In order to more accurately quantify the effect of ethanol exposure on membrane fluidity it is important to strictly control the ethanol exposure time during the membrane fluidity experiments and to perform these experiments for a broader range of temperatures and ethanol concentrations. Additionally, cell fixation with formaldehyde before membrane fluidity measurements, as was done by some studies that used the DPH method [11,28], could help to prevent effects of prolonged exposure to ethanol during these measurements. Ethanol exposure might also have an impact on membrane proteins. The conformational changes of these proteins might counterbalance the fluidizing effect of ethanol on the membrane phospholipids by increasing the molecular order of the lipids in the cellular membrane [19,29]. However, more research is needed to investigate the role of lipid-protein interactions in bacterial membrane fluidity.

3.3. Effects of ethanol on PEF results

Although the DPH fluorescence anisotropy measurements in the presence of ethanol did not result in clear conclusions regarding the membrane fluidity, the presence of ethanol in the PEF medium significantly affected the accumulation of intracellular trehalose during PEF treatment (Fig. 4B). Intracellular trehalose accumulation upon PEF treatment was significantly increased; up to 42 µg/mL trehalose was found in the samples that were PEF-treated (10–12 pulses) in the presence of ethanol, whereas up to 16 µg/mL in samples without ethanol in the PEF medium was observed (Fig. 4B). At the same time, survival of the cultures upon PEF treatment was not affected by the addition of ethanol to the PEF medium (Fig. 4A). The effect of ethanol addition on accumulation of intracellular trehalose was more pronounced at the PEF condition of 10–12 pulses than at 2 pulses (Fig. 4B).

The limited effect of the presence of ethanol on survival after PEF treatment is similar to what was observed by Cébrian et al.

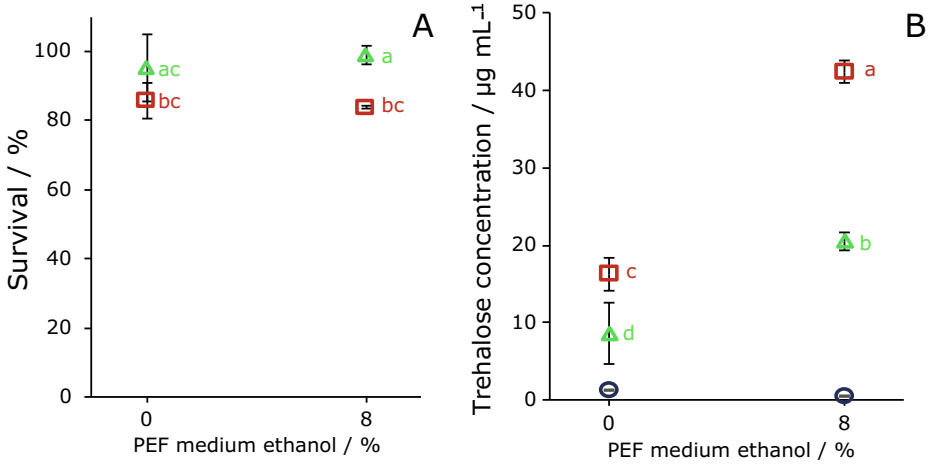

Fig. 4. Survival (A) and intracellular trehalose content after PEF treatment (B) with and without the addition of 8% ethanol to the PEF medium at 21 °C. Control (○) and PEF-treated cultures with 2 (□) and 10–12 pulses (△) of 100 µs at 6.3 kV/cm. Intracellular trehalose contents are presented in µg/mL sample, with each sample containing ~ $4*10^9$ CFU/mL before PEF treatment. Error bars show standard deviations of biologically independent replicates (n = 3) and different letters indicate significant differences.

[11], who observed similar survival of *Staphylococcus aureus* with and without addition of benzyl alcohol to the PEF medium. Based on their PEF-treatment results in medium supplemented with benzyl alcohol and their PEF-treatment results at different temperatures, these authors concluded that membrane fluidity is not decisive for the viability of PEF-treated cells. Both process modifications, addition of alcohol and temperature increase, increased the membrane fluidity, but had different effects on survival upon PEF treatment. Although we could not differentiate between the membrane fluidity of the culture in medium with ethanol or without ethanol as explained in Section 3.2, our data point in the same direction as their conclusion. We observed a similar survival and a large increase in trehalose accumulation upon PEF-treatment with ethanol supplementation and on the other hand a decrease in both survival and trehalose accumulation upon increasing the PEF temperature from 21 to 35 °C. Both process modifications may lead to increased membrane fluidity, but had different effects on cell viability and therefore show the same trend as the data of Cébrian et al. [11]. However, to confirm that membrane fluidity is not decisive for the viability of PEF-treated cells further research into these aspects using more PEF conditions is required.

Interestingly, some studies reported an increased transformation efficiency when ethanol was present in the electroporation medium. Pyne et al. [30] reported a 1.6 fold increase in transformation efficiency for *Clostridium pasteurianum* in the presence of 5% (v/v) ethanol, though the pulse duration of the exponential decay pulse was not controlled in this study and slightly differed between the condition with and without ethanol. Other researchers found that supplementation of the electroporation medium with 10% (v/v) ethanol resulted in transformed *Oenococcus oeni* cells, whereas electroporation without ethanol did not provide any transformants, which also indicates a positive effect of ethanol on molecule uptake during PEF treatment [31].

Our results regarding PEF treatment in the presence of ethanol, with a combination of a high culture viability and a large increase in intracellular trehalose concentrations, are very interesting for PEF processes aiming at uptake of protective molecules, for example with the aim of enhancing robustness towards processing. Whether or not this large increase in intracellular trehalose will result in further enhancement of robustness remains to be investigated, as increased intracellular trehalose concentrations do not necessarily lead to enhanced robustness under all process conditions [32]. The intracellular trehalose data presented in Figs. 2 and 4 are concentrations found in the cell extract of the whole culture and these values do not provide information on whether the trehalose was present in all cells or only in a fraction of the cell population. The effect of a specific PEF treatment is not the same for every cell in a culture; part of the culture will maintain an intact cell membrane, while other cells of the culture will experience reversible or irreversible membrane damage due to the PEF treatment [1,9,18]. To further elucidate the impact of ethanol on reversible membrane permeabilization during PEF treatment at single cell level, we applied a fluorescence staining approach using the membrane impermeable probe propidium iodide (supplementary information). Based on the applied fluorescent staining approach, PEF treatment in the presence of 8% ethanol resulted in a larger reversible permeabilized fraction of the cell population compared to a PEF treatment without ethanol in the PEF medium. For one of the tested PEF conditions the reversible fraction increased significantly from 9% without ethanol to 40% with ethanol (Figs. S.1 and S.2). This increase might indicate that it is likely that the higher trehalose concentrations measured in ethanol-exposed cells in Fig. 4B is due to a larger fraction of the cell population with increased intracellular trehalose concentrations. These results also pointed out that modification of the PEF medium composition, like we did with ethanol supplementation, is promising for applications aiming for reversible permeabilized cells, while maintaining the same medium conductivity and thus the same energy input of the PEF treatment.

4. Conclusions

This study shows that for the aim of increasing small molecule uptake upon PEF treatment a moderate temperature of 21 °C is more efficient than 10 or 35 °C. Moreover, the presence of 8% ethanol in the PEF medium led to a significant increase in molecule uptake and a larger fraction of reversible permeabilized cells without any negative effect on culture survival. These increased intracellular trehalose concentrations may lead to increased bacterial robustness during processing. Furthermore, these findings can benefit optimization of other PEF processes aiming at molecule uptake and/or reversible cell permeabilization.

CRediT authorship contribution statement

E.M.J. Vaessen: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **H.A. Kemme:**

Methodology, Investigation, Formal analysis. **R.A.H. Timmermans:** Conceptualization, Writing - review & editing. **M.A.I. Schutyser:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing. **H.M.W. den Besten:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was carried out within the framework of the Institute for Sustainable Process Technology (ISPT), The Netherlands (project DR-20-06). Partners in this project are Danone, DSM, Cosun, NIZO Food Research, Winclove and Wageningen University and Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2020.107680.

References

[1] G. Saulis, Electroporation of cell membranes: the fundamental effects of pulsed electric fields in food processing, Food Eng. Rev. 2 (2010) 52–73, https://doi.org/10.1007/s12393-010-9023-3.
[2] T. Kotnik, W. Frey, M. Sack, S. Haberl Meglič, M. Peterka, D. Miklavčič, Electroporation-based applications in biotechnology, Trends Biotechnol. 33 (2015) 480–488, https://doi.org/10.1016/j.tibtech.2015.06.002.
[3] J. Raso, W. Frey, G. Ferrari, G. Pataro, D. Knorr, J. Teissie, D. Miklavčič, Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes, Innov. Food Sci. Emerg. Technol. 37 (2016) 312–321, https://doi.org/10.1016/j.ifset.2016.08.003.
[4] P. Gong, K. Lin, J. Zhang, X. Han, L. Lyu, H. Yi, J. Sun, L. Zhang, Enhancing spray drying tolerance of *Lactobacillus bulgaricus* by intracellular trehalose delivery via electroporation, Food Res. Int. 127 (2019), https://doi.org/10.1016/j.foodres.2019.108725 108725.
[5] E.M.J. Vaessen, H.M.W. den Besten, T. Patra, N.T.M. van Mossevelde, R.M. Boom, M.A.I. Schutyser, Pulsed electric field for increasing intracellular trehalose content in *Lactobacillus plantarum* WCFS1, Innov. Food Sci. Emerg. Technol. 47 (2018) 256–261, https://doi.org/10.1016/J.IFSET.2018.03.007.
[6] S. Termont, K. Vandenbroucke, D. Iserentant, S. Neirynck, L. Steidler, E. Remaut, P. Rottiers, Intracellular accumulation of trehalose protects *Lactococcus lactis* from freeze-drying damage and bile toxicity and increases gastric acid resistance, Appl. Environ. Microbiol. 72 (2006) 7694–7700, https://doi.org/10.1128/AEM.01388-06.
[7] B. Dovgan, A. Barlič, M. Knežević, D. Miklavčič, Cryopreservation of Human Adipose-Derived Stem Cells in Combination with Trehalose and Reversible Electroporation, J. Membr. Biol. 250 (2017) 1–9, https://doi.org/10.1007/s00232-016-9916-z.
[8] T. Chen, J.P. Acker, A. Eroglu, S. Cheley, H. Bayley, A. Fowler, M. Toner, Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells, Cryobiology. 43 (2001) 168–181, https://doi.org/10.1006/cryo.2001.2360.
[9] E.M.J. Vaessen, R.A.H. Timmermans, M.H. Tempelaars, M.A.I. Schutyser, H.M.W. den Besten, Reversbility of membrane permeabilization in *Lactobacillus plantarum* WCFS1 after pulsed electric field treatment, Sci. Rep. (2019) 19990, https://doi.org/10.1038/s41598-019-56299-w.
[10] P. Wouters, I. Alvarez, J. Raso, Critical factors determining inactivation kinetics by pulsed electric field food processing, Trends Food Sci. Technol. 12 (2001) 112–121, https://doi.org/10.1016/S0924-2244(01)00067-X.
[11] G. Cebrián, S. Condón, P. Mañas, Influence of growth and treatment temperature on Staphylococcus aureus resistance to pulsed electric fields : Relationship with membrane fluidity, Innov. Food Sci. Emerg. Technol. 37 (2016) 161–169, https://doi.org/10.1016/j.ifset.2016.08.011.
[12] K. Aronsson, U. Rönner, Influence of pH, water activity and temperature on the inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* by pulsed electric fields, Innov. Food Sci. Emerg. Technol. 2 (2001) 105–112, https://doi.org/10.1016/S1466-8564(01)00030-3.
[13] R.A.H. Timmermans, M.N. Nierop Groot, A.L. Nederhoff, M.A.J.S. van Boekel, A. M. Matser, H.C. Mastwijk, Pulsed electric field processing of different fruit juices: Impact of pH and temperature on inactivation of spoilage and pathogenic micro-organisms, Int. J. Food Microbiol. 173 (2014) 105–111, https://doi.org/10.1016/j.ijfoodmicro.2013.12.022.
[14] S. Chu-Ky, R. Tourdot-Marechal, P.A. Marechal, J. Guzzo, Combined cold, acid, ethanol shocks in *Oenococcus oeni*: Effects on membrane fluidity and cell viability, Biochim. Biophys. Acta - Biomembr. 1717 (2005) 118–124, https://doi.org/10.1016/j.bbamem.2005.09.015.
[15] M. Graca Da Silveira, E.A. Golovina, F.A. Hoekstra, F.M. Rombouts, T. Abee, Membrane fluidity adjustments in ethanol-stressed *Oenococcus oeni* cells, Appl. Environ. Microbiol. 69 (2003) 5826–5832, https://doi.org/10.1128/AEM.69.10.5826.
[16] M. Patra, E. Salonen, E. Terama, I. Vattulainen, R. Faller, B.W. Lee, J. Holopainen, M. Karttunen, Under the influence of alcohol: The effect of ethanol and methanol on lipid bilayers, Biophys. J. 90 (2006) 1121–1135, https://doi.org/10.1529/biophysj.105.062364.
[17] O. Yun, Z. Liu, X. Zeng, Z. Han, *Salmonella typhimurium* resistance on pulsed electric fields associated with membrane fluidity and gene regulation, Innov. Food Sci. Emerg. Technol. 36 (2016) 252–259, https://doi.org/10.1016/j.ifset.2016.06.013.
[18] M. Kanduser, M. Šentjurc, D. Miklavcic, The temperature effect during pulse application on cell membrane fluidity and permeabilization, Bioelectrochemistry. 74 (2008) 52–57, https://doi.org/10.1016/j.bioelechem.2008.04.012.
[19] F. Fonseca, C. Pénicaud, E.E. Tymczyszyn, A. Gómez-Zavaglia, S. Passot, Factors influencing the membrane fluidity and the impact on production of lactic acid bacteria starters, Appl. Microbiol. Biotechnol. (2019) 6867–6883, https://doi.org/10.1007/s00253-019-10002-1.
[20] M. Kleerebezem, J. Boekhorst, R. van Kranenburg, D. Molenaar, O.P. Kuipers, R. Leer, R. Tarchini, S.A. Peters, H.M. Sandbrink, M.W.E.J. Fiers, W. Stiekema, R.M. K. Lankhorst, P.A. Bron, S.M. Hoffer, M.N.N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W.M. de Vos, R.J. Siezen, Complete genome sequence of *Lactobacillus plantarum* WCFS1, PNAS. 100 (2003) 1990–1995, https://doi.org/10.1073/pnas.0337704100.
[21] E.M.J. Vaessen, H.M.W. den Besten, E.D.C. Esveld, M.A.I. Schutyser, Accumulation of intracellular trehalose and lactose in *Lactobacillus plantarum* WCFS1 during pulsed electric field treatment and subsequent freeze and spray drying, LWT. 115 (2019), https://doi.org/10.1016/j.lwt.2019.108478 108478.
[22] J. Meneghel, S. Passot, S. Dupont, F. Fonseca, Biophysical characterization of the *Lactobacillus delbrueckii* subsp. *bulgaricus* membrane during cold and osmotic stress and its relevance for cryopreservation, Appl. Microbiol. Biotechnol. 101 (2017) 1427–1441, https://doi.org/10.1007/s00253-016-7935-4.
[23] M. Shinitzky, Membrane fluidity and cellular functions, Physiol. Membr. Fluidity. 1 (1984) 1–51.
[24] A. Rivas, M.C. Pina-Pérez, S. Rodriguez-Vargas, M. Zuñiga, A. Martinez, D. Rodrigo, Sublethally damaged cells of *Escherichia coli* by Pulsed Electric Fields: The chance of transformation and proteomic assays, Food Res. Int. 54 (2013) 1120–1127, https://doi.org/10.1016/j.foodres.2013.01.014.
[25] J.M. Escoffre, T. Portet, L. Wasungu, J. Teissié, D. Dean, M.P. Rols, What is (Still not) known of the mechanism by which electroporation mediates gene transfer and expression in cells and tissues, Mol. Biotechnol. 41 (2009) 286–295, https://doi.org/10.1007/s12033-008-9121-0.
[26] H. van Bokhorst-van, T. de Veen, M. Abee, P.A. Tempelaars, M. Bron, M.L. Marco Kleerebezem, Short- and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*, Appl. Environ. Microbiol. 77 (2011) 5247–5256, https://doi.org/10.1128/AEM.00515-11.
[27] B. Loffeld, H. Keweloh, cis/trans isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8, Lipids. 31 (1996) 811–815, https://doi.org/10.1007/BF02522976.
[28] B. Aricha, I. Fishov, Z. Cohen, N. Sikron, S. Pesakhov, I. Khozin-Goldberg, R. Dagan, N. Porat, Differences in membrane fluidity and fatty acid composition between phenotypic variants of *Streptococcus pneumoniae*, J. Bacteriol. 186 (2004) 4638–4644, https://doi.org/10.1128/JB.186.14.4638-4644.2004.
[29] M.E. Tóth, L. Vígh, M. Sántha, Alcohol stress, membranes, and chaperones, cell stress chaperones. 19 (2014) 299–309, https://doi.org/10.1007/s12192-013-0472-5.
[30] M.E. Pyne, M. Moo-Young, D.A. Chung, C.P. Chou, Development of an electrotransformation protocol for genetic manipulation of *Clostridium pasteurianum*, Biotechnol. Biofuels. 6 (2013), https://doi.org/10.1186/1754-6834-6-50.
[31] J.S. Assad-García, M. Bonnin-Jusserand, D. Garmyn, J. Guzzo, H. Alexandre, C. Grandvalet, An improved protocol for electroporation of *Oenococcus oeni* ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent, Lett. Appl. Microbiol. 47 (2008) 333–338, https://doi.org/10.1111/j.1472-765X.2008.02435.x.
[32] E.M.J. Vaessen, H.M.W. den Besten, K.M.N. Leito, M.A.I. Schutyser, Pulsed electric field pre-treatment for enhanced bacterial robustness during drying: Effect of carrier matrix and strain variability, Innovative Food Science and Emerging Technologies, 2020 102515.