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Pulsed Electric Field Pre-treatment for Spray Drying of Probiotics

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Abstract

Pulsed electric field pre-treatment can be used for loading sugars into living cells, which may make cells more robust during subsequent spray drying. In this study we investigated the effect of the (change of) drying medium after PEF pre-treatment with trehalose for its impact on survival during subsequent spray drying for L. plantarum WCFS1. Our results showed that when trehalose was used as drying medium, no difference in survival with and without PEF pre-treatment was observed. However, when reconstituted skim milk and maltodextrin DE19 were used as drying medium an increased survival of the PEF-treated culture was observed compared to the control.

Keywords: *Pulsed electric field treatment; spray drying; trehalose, reconstituted skim milk, Lactobacillus plantarum WCFS1*

1. Introduction

Spray drying and freeze drying can be used to stabilize microorganisms such as probiotics and starter cultures for a prolonged shelf life. These heat sensitive microorganisms are often encapsulated in a carrier matrix consisting of carbohydrates and/or proteins to provide protection during the drying process and subsequent storage of the powders [1,2]. Components that are often used as carrier matrix are for example trehalose, lactose, maltodextrin, whey protein or reconstituted skim milk (RSM). Additionally, several (micro)organisms that survive well under dessication stress in nature are known to accumulate the disaccharide trehalose intracellular [3,4]. Also in processing applications, intracellular trehalose accumulation has been linked to enhanced robustness of several cell types. For example, trehalose has been found intracellularly in *Lactobacillus casei* and *Propionibacterium freudenreichii* after pre-conditioning of these bacteria towards osmotic stresses to increase their survival during spray drying [5,6]. Furthermore, the introduction of trehalose synthesis genes in *L. lactis* resulted in higher freeze drying survival [7]. A different approach to increase intracellular trehalose is pulsed electric field (PEF) treatment. Pulsed electric field employs the use of nano- to millisecond high voltage pulses to permeabilize the cellular membrane [8]. PEF pre-treatment to increase intracellular trehalose resulted in better survival of mammalian and plant cells after processing [9,10].

In previous work we showed that pulsed electric field treatment can also be used to increase intracellular trehalose in bacteria, namely in *Lactobacillus plantarum WCFS1* [11]. However, this did not lead to enhanced robustness of the PEF pre-treated cultures after drying and subsequent storage of the powders [12]. Remarkably, another recent study with a similar approach did find enhanced robustness of *Lactobacillus bulgaricus* upon spray drying with PEF pre-treatment to increase intracellular trehalose [13]. The main differences between these two studies are the microorganism investigated and the drying matrix. Gong et al. (2019) [13] dried the bacteria in RSM after PEF pre-treatment in trehalose, while in our study the same carbohydrate (i.e. trehalose) was used for PEF pre-treatment as well as for subsequent drying. Additionally, trehalose was also found intracellularly in *L. plantarum WCFS1* after drying and reconstitution of the powder, when trehalose was used as the drying matrix. This finding suggests that the intracellular accumulation of trehalose during drying could be the reason why the PEF pre-treatment had no added value in this case [12].

Based on these two recent studies, which seem to contradict each other, the further use of a PEF pre-treatment for enhanced bacterial robustness requires further understanding of the applicability as well as further understanding of the role of intracellular trehalose during drying. Therefore the effect of the (change of) drying medium should be further investigated for subsequent spray drying. The aim of this study is to thus to understand the role of intracellular trehalose during spray drying of lactic acid bacteria by evaluating the effect of the drying medium compared to the PEF medium on survival of *Lactobacillus plantarum WCFS1* after spray drying and storage.

2. Materials and Methods

2.1. Microorganism

Lactobacillus plantarum WCFS1 was used in this study and obtained from the Food microbiology strain collection. Originally, this strain is isolated from human salive [14]. It was grown on de Man Rogosa and Sharpe (MRS, Merck, USA) medium and pre-cultured as follows. Bacterial culture was taken from the -80°C stock, plated on MRS and incubated under microaerobic conditions at 30°C for approximately 70 hours. After incubation plates were stored at 4°C until further use, though for a maximum of 4 days. Subsequently, a single colony was inoculated into 10 ml broth (MRS) and this culture was incubated for 24±2 hours without shaking at 30 °C. After incubation this culture was transferred into fresh broth in a 1:100 dilution in the required volume for the following experiments and incubated under the same conditions as the previous step. This final pre-culture step was 16-18 hr to obtain the culture in the stationary growth phase.

2.2. Pulsed Electric Field Treatment

For every experiment the pre-cultured bacteria were centrifuged for 10 minutes at 13,500 x g and subsequently washed with washing solution as described in previous research [11]. Subsequently, the bacterial pellet was resuspended in the trehalose electroporation medium containing 0.3 M trehalose dihydrate (Sigma-Aldrich, USA) (for rest of composition, see:[11]. The culture in electroporation medium had a conductivity of 0.15 S/m at room temperature.

Lactobacillus plantarum WCFS1 was PEF treated in an IXL batch PEF system with custom made treatment chamber as described in previous research [12]. The PEF treatment consisted of 10 square wave pulses of 6.3 kV/cm with a pulse duration of 100 µs and pulse interval of 5 s. For every experiment, part of the bacterial culture was kept as a control (without PEF). PEF treated and control samples were used for survival analysis and intracellular trehalose measurements.

2.3. Spray drying and storage experiments

Drying solutions of 20 w/w% solids concentration were prepared by combining demineralized water with maltodextrin DE19 (MD19, Roquette, France), reconstituted skim milk (RSM, Arla Food Ingredients, Denmark) or trehalose (Merck, USA). Five mL of bacterial culture in trehalose electroporation medium with or without PEF were added to 50 mL of the prepared drying solutions. Samples were taken for survival analysis before spray drying. The culture in drying medium was subsequently spray dried in a Büchi B-290 laboratory spray dryer (Büchi Labortechnik AG, Switzerland) with an inlet temperature of 140°C. The flow rate was adjusted to obtain an outlet temperature of 80±1°C. After spray drying, the powder was collected and part of the powder was distributed in samples of 200 ± 3 mg, which were subsequently used for survival analyses and storage tests. Additionally, the moisture content was measured using a moisture analyzer (Sartorius, Germany), and was 3-5 w/w% for all experiments. For survival analysis during storage, the samples were stored at room temperature (21± 2 °C) in desiccators containing an oversaturated potassium acetate solution (a_w=0.23). Samples for survival analysis were taken approximately every 21 days.

2.4. Survival assessment

Samples for survival analysis were decimally diluted in peptone physiological salt solution (PPS, Tritium Microbiologie, the Netherlands). Dilution series were made at least in duplicate and the appropriate dilutions were subsequently plated on MRS agar in duplicate. Plates were incubated microaerobically at 30°C for 2-4 days. After incubation colonies were counted and survival was calculated by dividing the cfu/ml of the sample after processing (after PEF or spray drying) by the cfu/ml of the sample before processing. Dried samples were first rehydrated in 10 mL PPS, resulting in the -1 dilution and subsequently further diluted and plated as described above.

2.5. Intracellular trehalose measurements

Intracellular trehalose extraction and subsequent measurement with HPAEC analysis was performed according to the method described in Vaessen et al. (2019) [12]. Briefly, extracellular trehalose was washed away with three washing steps in phosphate buffered saline (PBS). Subsequently the cell pellet was suspended in milliQ water and homogenized in a bead beater to extract intracellular trehalose. Trehalose concentrations in these samples were measured using high pressure anion exchange chromatography with PAD detection.

2.6. Experimental set-up

All experiments were carried out at least in duplicate, with each replicate being biologically independent, pre-cultured on another day.

3. Results & Discussion

3.1. Effect of carrier matrix during drying after PEF pre-treatment on survival

The results of two recent studies on survival of *Lactobacilli* after drying with a pulsed electric field pre-treatment leading to increased intracellular trehalose concentrations seem to contradict each other [12,13]. One of the major differences between these two studies is the drying medium that was used after the PEF treatment. This difference suggests that the type of drying medium might play a role. We dried *L. plantarum WCFS1* with and without PEF pre-treatment in trehalose electroporation medium in several drying matrices, i.e. maltodextrin DE19, reconstituted skim milk (RSM), whey protein and trehalose. Indeed, our results show that when drying in trehalose, no difference in survival with and without PEF pre-treatment was found (Fig. 1A), similar to what was observed after freeze drying in our previous study [12]. Interestingly, PEF pre-treated cultures survived better after spray drying when performing the PEF treatment in trehalose electroporation medium and subsequently spray drying the culture in reconstituted skim milk (Fig. 1). The average survival of the PEF treated culture almost was doubled compared to the culture without PEF pre-treatment. This difference indicates that PEF pre-treatment is promising for increasing survival after spray drying, though that the drying medium plays an important role in this effect. Furthermore, these results of the effect of increased intracellular trehalose on bacterial survival during drying are in agreement with the study of Termont et al. [7]. In this study the intracellular trehalose concentrations in *L. lactis* were increased by a genetic modification approach. These increased intracellular trehalose concentrations also led to enhanced robustness of this strain during freeze drying in RSM and subsequent incubation in gastric juice.

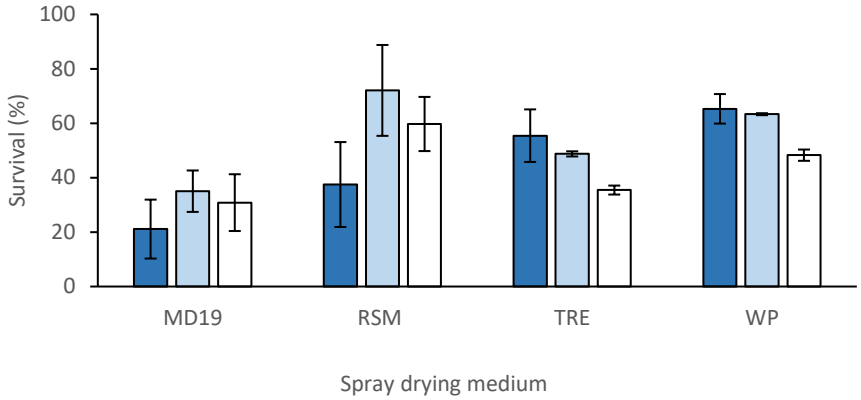

Figure 1 Survival of L. plantarum WCFS1 after spray drying in different drying media with and without PEF pre-treatment leading to increased intracellular trehalose. Different colours represent survival after spray drying of control cultures (blue), PEF treated cultures (light blue) and PEF pre-treated cultures corrected for PEF survival (white). Error bars indicate standard deviations of biologically independent replicates (n≥2).

The spray drying experiments with maltodextrin DE19 as drying matrix also showed an increasing trend in average survival of the PEF-treated culture compared to the control, though not as clear as for RSM (Fig. 1). For spray drying in whey protein no difference was observed between the survival of the PEF pre-treated culture and the control culture. These findings raise the question why PEF pre-treatment, leading to increased intracellular trehalose (Fig. 2), only has an added value when a specific drying matrix is used. For trehalose as drying matrix we could explain this by the increase in intracellular trehalose during drying processes itself when this sugar is used as a carrier matrix [12]. However, for whey proteins a similar effect is not expected based on the much larger size of these molecules compared to trehalose. Reconstituted skim milk is a mixture of proteins (mainly casein and whey proteins), lactose and minerals. Why PEF pre-treatment has an added value upon drying in this matrix specifically remains to be elucidated.

In the results presented in the light blue bars in Fig. 1 the decrease in survival during the PEF treatment itself was not taken into account. Survival of *L. plantarum WCFS1* after a PEF treatment with ten pulses at 6.3 kV/cm was approximately 80% (Fig. 2). When taking into account this survival, the differences in survival after spray drying between the PEF pre-treated and control cultures become smaller (white bars). For trehalose and whey protein as drying matrices even a decrease in overall survival is observed when taking the survival of the PEF treatment into account. The average survival of the whole process increased approximately from 37 to 60% in RSM as drying matrix when PEF pre-treatment was applied. This increase is similar to what Gong et al. (2019) [13] observed, they found a maximum survival increase over the whole process from 38 to 61% upon applying PEF pre-treatment. Overall, these results confirmed our hypothesis that the efficacy of a PEF pre-treatment, leading to increased intracellular trehalose (Fig. 2), depends on the drying medium that is used.

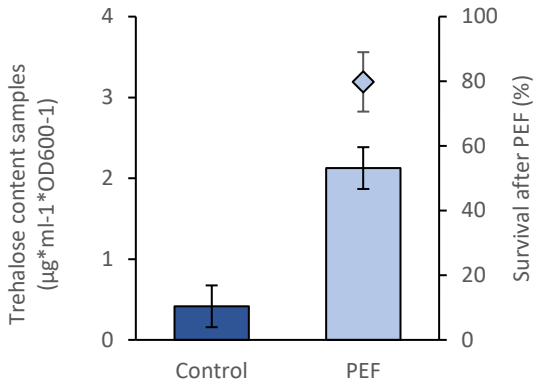

Figure 2 Intracellular trehalose content (bars) and survival (blue diamond) after PEF treatment of Lactobacillus plantarum WCFS1 in trehalose electroporation medium. Intracellular trehalose is presented as trehalose concentration of the cell extract per OD-value of the culture. Error bars represent standard deviations of independent replicates (n=8).

In addition to the results of the PEF treated cultures compared to the control cultures, also differences were observed in survival of *L. plantarum WCFS1* after spray drying in the different matrices. Drying the culture (without PEF) in trehalose and whey protein resulted in the highest survival under these drying conditions, followed by RSM and maltodextrin DE19. Higher survival of trehalose after spray drying compared to maltodextrin is in line with previous research with trehalose and maltodextrin DE16 [15]. Not only the survival differed between these drying matrices, also the final morphology of the dried powder particles varried (Fig. 3). Reconstituted skim milk and maltodextrin particles were wrinkled, while trehalose particles had a smooth surface. Compared to the RSM particles, the maltodextrin particles exhibited smaller wrinkles (Fig. 3). Possibly, there might also be a relation between particle morphology/skin formation and bacterial survival [16,17].

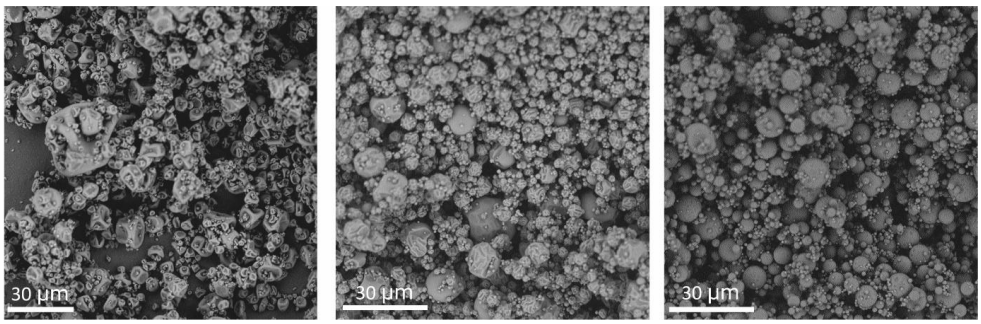

Figure 3 SEM images showing the morphology of dried powder particles after spray drying, from left to right: reconstituted skim milk (RSM), maltodextrin DE19 and trehalose.

3.2. Survival during storage after drying

In addition to survival during the spray drying process itself, bacterial survival during storage is important for prolonged shelf life of dried probiotics and starter cultures. Therefore we also monitored survival of spray dried *L. plantarum WCFS1* cultures in RSM and trehalose with and without PEF pre-treatment for a period of 9 to maximum 12 weeks (Fig. 4).

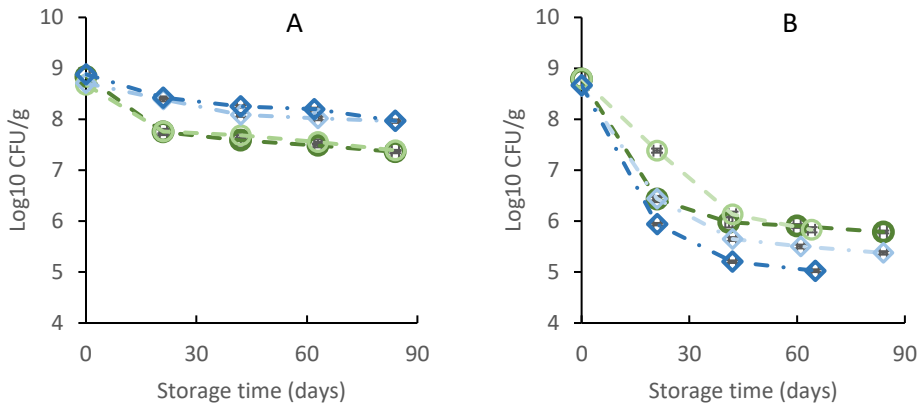

Figure 4 Survival of spray dried L. plantarum WCFS1 during storage (room temperature, aw=0.23). Control (green circles) and PEF pre-treated (blue diamonds) cultures were spray dried in reconstituted skim milk (A) and trehalose (B). Each experiment has been performed in independent duplicates, as presented by the slightly different colours.

Interestingly, the PEF pre-treated cultures that were spray dried in RSM survived better during storage compared to cultures without PEF pre-treatment (Fig. 4A). The CFU counts of the PEF pre-treated cultures remained approximately 0.5 log higher than the control cultures at every time point. This difference shows that increased intracellular trehalose concentrations upon PEF not only contributes to increased survival after drying in RSM, but also during subsequent storage.

Also here, such a difference was not observed for cultures dried in trehalose (Fig. 4B). Both the PEF treated and control cultures decreased in survival over time, there might even be a slight negative effect of the PEF pre-treatment on survival. Though, the deviation between the duplicate storage experiments was larger for cultures dried in trehalose compared to cultures dried in RSM, which makes it difficult to draw conclusions about this observed trend. During 12 weeks of storage the cultures dried in trehalose reduced much more in viability compared to the cultures dried in RSM. This difference indicates that RSM is the best matrix of these two for protection of *L. plantarum WCFS1* during storage of the spray dried powders. This protective effect of RSM is in line with results of Ananta et al. (2005) [18], who observed a very limited decrease in survival during storage of spray dried *L. rhamnosus GG* with RSM as a carrier. They found a negative effect on survival during storage upon replacing part of the RSM with pre-biotic substances [18]. Furthermore, Broeckx et al. (2017) [2] found that in addition to the presence of carbohydrates, the presence of phosphates in the carrier matrix contributes to better survival of *L. rhamnosus GG* during storage in these powders. They suggest that the observed increase in survival can be because of the buffering capacity of these phosphates [2]. This buffering capacity might also play a role in the better survival observed for storage of *L. plantarum WCFS1* in RSM compared to trehalose.

4. Conclusions

The effect of the (change of) drying medium after PEF pre-tratement with trehalose was investigated for impact on survival during subsequent spray drying for *L. plantarum WCFS1*.

Similar to what was observed after freeze drying in our previous study, our results showed that when trehalose was used as drying medium, no difference in survival with and without PEF pre-treatment was observed. However, the spray drying experiments with RSM and maltodextrin DE19 as drying matrix showed an increased survival of the PEF-treated culture compared to the control, although the difference was larger for RSM. For spray drying in whey protein no difference was observed. Why PEF pre-treatment has speifically added value upon drying in RSM requires further investigation. Interestingly, for RSM the difference in viability with and without PEF pre-treatment remained also during storage. Possibly, this may be related to the buffering capacity of phosphates in RSM compared to the other matrices trehalose and maltodextrin.

5. Acknowledgements

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