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Effect of electroporation in a continuous flow system on bioaccumulation of magnesium, zinc and calcium ions in *Lactobacillus rhamnosus* B 442 cells

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ABSTRACT

Biomass of *Lactobacillus rhamnosus* B 442 was subjected to the continuous electroporation using an electroporator with a flow chamber (length of 10 cm, distance between electrodes 0.25 cm, stream width 0.25 cm, flow speed 10 mL/min) to improve accumulation of calcium, magnesium and zinc in the cells. For all tested ions, the following parameters were applied: voltage of 250 V (E = 1 kV/cm), 570 V (E = 2.28 kV/cm), 950 V (E = 3.8 kV/cm), and 1400 V (E = 5.6 kV/cm, the positive control), a frequency of 10 Hz, a pulse width of 100 μs and 30 electrical pulses. The use of PEF increased the accumulation of magnesium, zinc and calcium by 39, 73 and 162%, respectively, compared to the control. Positive correlation was found between ion accumulation and membrane permeability for zinc and magnesium. For calcium, the initial increase in permeability resulted in higher ion accumulation, but with a further increase of this parameter at 3.8 kV/cm, its decrease was observed caused by a drop in cell viability. Total number of bacteria ranged from 1.67×10^8 (for the cultures supplemented with calcium) to 1.34×10^{12} cfu/mL (for the cultures supplemented with magnesium).

1. Introduction

Biological membranes separating the cell's interior from the outside environment are complex and dynamic entities. They control which substances can enter and leave the cell thanks to their selective permeability. This permeability of biomembranes can be transiently and nonselectively increased by application of pulsed electric fields (PEF). This phenomenon is called electroporation and involves the formation of structural defects (pores) on membrane [1–5].

Permeabilization is controlled by PEF intensity [6,7]. In order to start the formation of transient membrane pores, the external electric field should reach a critical value which depends on the size and geometry of a cell [8]. For plant cells it is in the range of 1 – 2 kV/cm and for microbial cells – from 10 to 14 kV/cm [9]– it needs to be stressed that these exact values will depend also on duration and number of pulses applied [10]. If the external electric field is below the critical threshold, the cells can survive and the membrane can reseal resulting in reversible electroporation or, if the electric field exceed the critical value, this leads to cell death resulting in irreversible electroporation [11]. Electroporation is commonly used in medicine and molecular biology for introducing chemical species into cells, for which the cell membrane is normally impermeable to e.g. proteins, dyes, drugs, or nucleic acids, as well as for antitumor electrochemotherapy, tumor ablation, and even gene transfer in vivo, and it is used in food technology and biotechnology [7,12–14].

Evaluation of membrane integrity can be performed by the measurement of a fluorescent dye uptake by the cells. One of these dyes is propidium iodide (PI) which small (660 Da) and strongly hydrophilic molecules are unable to enter cells if the membrane is intact. PI is commonly used to assess the impact of PEF on biomembranes and the main advantage is rapid monitoring of the effect of treatment [15].

Magnesium, zinc and calcium belong to the most important micronutrients in people's diet. Magnesium and calcium are elements that are found in the largest quantities in the human body. The adult human body contains approximately 22–24 g of magnesium [16,17]. This element plays a number of important functions: it is a cofactor of enzymatic reactions, participates in carbohydrates metabolism, and it is necessary for a proper functioning of many organs and tissues [18–20]. Zinc is a component of over 200 metalloproteins and takes part in a number of biochemical

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reactions involving enzymes, structural proteins, and hormones [21]. The appropriate zinc concentration in cells is essential for the proper functioning of the system which protects the body from oxidative damage [22,23]. Zinc participates in the metabolic processes of the cell and in gene expression and it is considered to be safe for health (has no oxidizing capacity) [24,25]. Also calcium participates in physiological processes, e.g. in the contraction of skeletal, smooth and heart muscles and the conduction of nerve impulses and in blood clotting [26]. When the supply of the above mentioned elements with food or their absorption is insufficient, they must be supplemented.

Previous studies carried out in a small laboratory batch system showed that pulsed electric field can be used for enrichment of yeast and bacteria in zinc, magnesium, and calcium [27–32]. These elements are incorporated into complexes with amino acids, proteins, lipids, and polysaccharides [33]. Microorganisms supplemented in such a way could potentially serve as the source of these elements with high bioavailability in a diet. Several studies have shown that certain organic compounds of such elements as zinc and magnesium are more bioavailable than the inorganic forms, presumably because the mechanisms for absorption have adapted to these kinds of nutrients during species evolution [33,34]. Microorganisms are able to accumulate metal ions from aqueous solutions by different physico-chemical interactions (biosorption) or by a metabolism-dependent mechanism (bioaccumulation). In a given microbial system, several mechanisms of uptake may operate simultaneously and/or sequentially [35]. Probiotic bacteria used in food production seem to be the natural choice for this purpose. *Lactobacillus rhamnosus* is one of the best-studied *Lactobacillus* species evaluated as potential probiotics. This bacterium was originally considered to be a subspecies of *Lactobacillus casei*, but later genetic tests have proved to be a separate species. This genius produces antimicrobial substances against various pathogenic bacteria and inhibits harmful effects of bacterial enzymes that improve intestinal function [36,37]. To facilitate testing and experimentation that is easier to scale up to the large-scale application of PEF, a laboratory PEF flow system has been developed. Such a system was previously used for *E. coli* inactivation and lipid extraction from microalgae *Chlorella vulgaris* [38–40].

The aim of the work was to apply a continuous flow system for electroporation of *Lactobacillus rhamnosus* B 442 cells in the presence of selected metal ions in a medium in order to obtain bacteria biomass enriched with these ions.

2. Materials and methods

2.1. Materials

A bacteria strain of *Lactobacillus rhamnosus* B 442 from the Agricultural Research Service Culture Collection NRRL (WDCM97) maintained at the Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences in Lublin, Poland, was used in the experiment. The following components were used for the preparation of inoculum and culture medium: sterile MRS broth (Biocorp, Warszawa, Poland) 59.937 g/L, agar (DIFCO, Detroit, MI, USA) 15 g/L, NaCl 80 g/L, glycerol (TechlandLab, Tarnobrzeg, Poland), HNO_3 65% (Merck, Darmstadt, Germany) and in each medium different ions were added: $CaCl_2$, $ZnSO_4{\cdot}7H_2O$ or $MgCl_2{\cdot}6H_2O$ (Standard, Lublin, Poland), in fixed concentrations.

2.2. Biomass cultivation

Bacteria culture was carried out according to the procedure described elsewhere [31,32,41] with modifications. Bacteria were passaged three times in MRS broth and incubated for 19 h at 37 °C. Then the inoculum was prepared by transferring 15 mL of bacteria (5%) to 300 mL of sterile MRS broth in 1000- mL Erlenmeyer flasks. The flasks were incubated at 37 °C for 16 h. Bacterial cells were treated in stationary phase, because we wanted to see if the accumulation of respective ion of interest is feasible with continuous mode of operation as it was with previous electroporation system [27–32]. Due to the high conductivity of about 10 mS/cm of the bacterial medium alone after cultivation, the bacterial biomass was centrifuged. Then solution of Ca, Zn or Mg ions at concentration of 200 μg/mL of medium was added (10% of total volume) and the initial volume was restored by the addition of sterile deionized water. After centrifugation of the culture medium the conductivity dropped below 3 mS/cm. The conductivity was measured using conductometer Seve nCompact (Mettler Toledo, Ohio, USA).

2.3. Electroporation of bacteria cells in a continuous flow system

Bacterial biomass with a volume of 50 mL was subjected to the process of continuous electroporation using an electroporator with a flow chamber described by Flisar *et al.* [38] with the following parameters: chamber length of 10 cm, distance between electrodes 0.25 cm, stream width 0.25 cm, flow speed 10 mL/min. The flow speed through the chamber was adjusted as to expose each bacterial cell to 30 electrical pulses. In electroporation, series of pulses are most commonly used for the treatment. The use of longer pulses (in ms range) could possibly be more effective. But in our study shorter (100 μs pulses) were used, since the conductivity of the sample was quite high (approximately 2.3 mS/cm). With conductivity as high as this the usage of longer pulses would lead to unwanted sparkling. We therefore used higher number of shorter pulses (30 pulses) in order to achieve longer treatment time. For all tested ions, the following voltages were used: 250 V (E = 1 kV/cm), 570 V (E = 2.28 kV/cm), 950 V (E = 3.8 kV/cm) and 1400 V (E = 5.6 kV/cm) as positive control, a frequency of 10 Hz and a pulse width of 100 μs. At the same time, the negative control tests were performed with addition of selected ions but without electroporation. All determinations were performed in triplicate. During electroporation, the voltage and current was monitored using oscilloscope (LeCroy 9310C, New York, USA) with high voltage differential current and voltage probe (Tektronix, Capitol, United Kingdom).

As a representative case, recordings of a pulse and current obtained from oscilloscope are displayed in (Fig. 1).

The electric field (E) was estimated as presented in formula 1:

$$E = \frac{U}{d} \tag{1}$$

U – applied voltage,
d – electrode distance (0.25 cm).

2.4. Determination of bacterial cell membrane permeability

Electropermeabilization was detected spectrofluorometrically using fluorescent dye propidium iodide (PI) according to the methodology of Ganeva *et al.* [42].], with the presence of ion of interest. PI was added to bacterial cells at a concentration 100 μg/ml right before pulse application. Bacterial samples (5 mL each) were exposed to the same electric pulses as described above using prototype continuous pulse generator. After the pulses, bacterial cells were incubated for 15 min in the dark at room temperature (22 °C) in order for PI to enter the cells. Afterwards cells were centrifuged for 4 min at 12.000 × g to remove extracellular PI that did not enter the cells. Bacterial pellet was then re-suspended with 400 μl of deionized water and PI uptake was measured

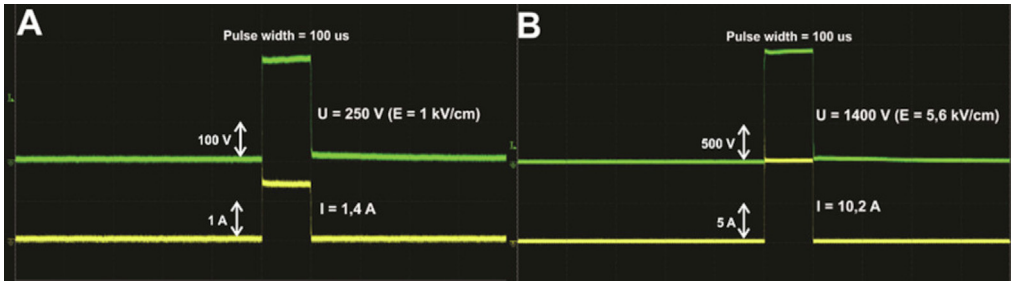

Fig. 1. Representative recordings of applied pulse (green line) and measured current (yellow line). (A) applied voltage and measured current were 250 V and 1,4 A, respectively; (B) applied voltage and measured current were 1400 V and 10,2 A, respectively.

spectrofluorometrically at 617 nm (Tecan Infinite M200, Tecan Austria GmbH).

The permeabilization (PI uptake) was expressed as presented in formula 2:

$$Permeabilization(\%) = \frac{PEF - PEF0(E=0)}{PEF(max) - PEF0(E=0)} \quad (2)$$

PEF - fluorescence intensity of cells exposed to electric pulses,

PEF 0 (E = 0) - fluorescence intensity of cells in control (no electric pulses applied),

PEF (max) - maximum fluorescence intensity, i.e. where saturation fluorescence is achieved.

For obtaining maximum fluorescence intensity pulses with amplitude of 1400 V (E = 5.6 kV/cm) were applied, causing 100% cell permeabilization (i.e. all the cells in the sample were pearmibilized). Namely, in order to achieve 100% permeabilization for our pulse protocol (30 pulses of 100 μs duration), the bacterium was treated with increasing electric field strength (from 250 V to 1400 V and higher) (data not shown). After the fluorescence intensity (i.e. accumulation of PI in the cells) reached maximum and did no longer increased (with increasing electric field), saturation fluorescence is achieved (at 5.6 kV/cm).

2.5. Determination of total number of microorganisms

Total number of microorganisms was determined by plate dilution method according to American Public Health Association [43]. Colonies of microorganisms were diluted 8 and 9 times with 0.8% sterile NaCl. Then, 1 mL of each was taken and placed in Petri dishes in two replicates. The diluted bacteria were flooded with sterile liquid agar (DIFCO Bacto-Agar Detroit Michigan USA). Cultures were incubated for 48 h at 37 °C. The Petri dishes, with two consecutive dilutions, on which from 25 to 250 colonies grew, were selected for reading. The total number of microorganisms (L) in 1 mL of the sample was calculated according to the formula (3):

$$L = \frac{C}{(N_1 + 0.1N_2)}\hat{A} \cdot d \quad (3)$$

C - sum of colonies on all dishes selected for counting,

N_1 - number of dishes from the first calculated dilution,

N_2 - number of dishes from second calculated dilution,

d - dilution ratio corresponding to the first (lowest) dilution

2.6. Determination of biomass

Biomass was determined spectrophotometrically (Spekol 11, Carl Zeiss, Jena, Germany) was carried out according to the procedure described by Góral *et al.* [32]. A sample of the culturing medium (2 mL) was centrifuged, supernatant was discarded, cells were rinsed three times with deionized water and brought to the original volume of 2 mL. Turbidimetric measurements were run against pure water at λ = 600 nm, in 2 mm measurement cell. Amount of biomass was calculated using equation for the standard curve $A_p = 1.1511c - 0.1053$, where A_p and c were apparent absorbance and concentration (mg/mL). The determination was performed in triplicate.

2.7. Determination of calcium, magnesium and zinc concentration

In order to determine the concentration of calcium, magnesium and zinc in cells, biomass was centrifuged (15 min, 3000 rpm, 1467 g), supernatant was discarded, and cells were rinsed three times with deionized water. Then biomass was lyophilized in a LABCONCO freeze dryer (model 64132, Kansas City, MO, USA), and mineralized in a MARS microwave oven (CEM Corporation, USA). Samples were prepared as follows: about 0.1 g of lyophilizate was transferred to a tube and 3 mL of HNO_3 was added. Then the samples were mineralized at 200 °C for 20 min. The obtained solutions were cooled down, transferred to 25 mL measuring flasks and topped up with deionized water. Concentration of calcium, magnesium and zinc ions in *L. rhamnosus* B 442 cells was determined using an electrothermal atomic absorption spectrophotometer (ET-AAS, VARIAN AA 280 FS) according to the procedure described by Góral *et al.* [32].

2.8. Cell staining and microscopic observation

Visualization of ions in *L. rhamnosus* B 442 cells (the negative control samples and the samples electroporated at 5.6 kV/cm, i.e. the positive control) was performed by confocal laser microscope (Nikon eclipse, Amsterdam, Netherlands). Pigment solutions were prepared in accordance with the recommendations of the manufacturer. Calcium orange (2 mM) (Invitrogen, Waltham, USA) was dissolved in anhydrous DMSO, whereas morin hydrate (30 mg) and magnesium green (30 mg) were dissolved in 1 mL of methanol, respectively. A small amount of bacteria (approx. 0.5 mL) was placed in an Eppendorf tube filled with 0.5 mL of PBS and then the microorganisms were dissolved in the solution by shaking. 20 μL of the prepared calcium orange, morin hydrate or magnesium green solution was added to the bacterial suspension. Microorganisms were stained at room temperature in the dark for 20 min. The specimen for microscope observation was prepared by spreading the dyed bacterial suspension on a cover slide (without covering) and drying. An argon laser with wavelengths of 488 nm (morin hydrate), 549 nm (calcium orange) and 506 nm (magnesium green) was used to induce fluorescence.

2.9. Statistical analysis

All the assays were made in three replicates. Significant differences between particular groups were identified using the Student *t*-test applied to compare independent samples in pairs. Detailed

analysis was based on Tukey's confidence intervals. All statistical tests were carried out at significance level of $\alpha = 0.05$. Statistical processing of results was performed using R program version 3.1.2 (Free Software Foundation's GNU General Public License, Boston, USA).

3. Results and discussion

Table 1 presents the total number of microorganisms determined at different electric field strengths and different metal ions in the medium. In the case of magnesium, the increase of electric field strength from 2.28 to 3.8 kV/cm caused a significant rise in cell viability. Higher bacterial viability was also observed when the cells were treated in the presence of zinc ions and the filed strength increased from 1 to 2.28 kV/cm. In the case of calcium we observed the highest viability for the control sample. Exposure of cells to PEF at 1 kV/cm and 2.28 kV/cm caused a slight decrease of viability but at 3.8 and 5.6 kV/cm the number of living cells was, respectively, 25 and 47 times lower than in the control sample. Divalent metal ions such as Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} are important factors stimulating the production of enzymes in lactobacilli [44]. Lew *et al.* [45] reported that only magnesium and manganese had a significant effect on the growth of *L. rhamnosus*. We can confirm that among the investigated metal ions magnesium had the highest impact on cell viability. According to Góral and Pankiewicz [31] viability of *L. rhamnosus* cells treated with PEF in medium containing magnesium did not change significantly when field strength ranged from 0.1 to 1 kV/cm or it was slightly reduced in the range of 1.5 to 3.5 kV/cm. The authors used a kind of laboratory batch system in which 100 mL of cells suspension were mixed continuously during PEF treatment. They also reported the similar effect for zinc but the opposite trend was observed when the treatment was carried out in the medium containing calcium ions. In our studies on bioaccumulation of calcium we noted that the total number of microorganisms only slightly decreased when lower values of field strength (1 and 2.28 kV/cm) were applied but at 5.6 kV/cm this drop was almost 50-fold in comparison to the control culture. In order to determine the bacterial biomass, a standard curve (Fig. 2) was prepared showing the dependence of apparent absorbance on the concentration of biomass. Bacterial biomass enriched with zinc, magnesium or calcium was, respectively, 0.203, 0.189, and 0.250 g/100 mL.

The growth and metabolism of *Lactobacilli* can be stimulated by low intensity pulsed electric field. In the study of Seratlic et. al. pulses bellow 12 J/cm^3 caused positive effect on the growth of *Lactobacilli* [46]. Nevertheless, in our study, the applied energies were much higher (approximately 4.5 J/cm^3, 26 J/cm^3, 84 J/cm3 and 168 J/cm^3); therefore, we conclude that the electric field alone did not cause the rise in cell viability in our study. Since the main objective of this study was to achieve maximum accumulation of respective ion of interest in the bacteria with new continuous electroporation system, we did not further investigate the individual impact on *Lactobacilli* viability.

An important step during electroporation is to monitor applied pulses and delivered currents. Namely, the electrical properties of the sample (different conductivity) might affect the current delivered. Furthermore, pulses and currents have to be measured in each experiment in order to ensure that pulses were properly applied and suitable reproduction and comparison of results is possible [47]. Therefore, we measured applied pulses and delivered currents and Fig. 1 shows representative recordings. Since the electric current is proportional to the voltage [48], with higher voltage (1400 V), higher current (10.2 A) and with lower applied voltage (250 V), lower (1.4 A) current was measured.

Table 1
Effect of electroporation in the presence of selected ions on viability of bacteria cells.

Electric field (kV/cm)	Total number of microorganisms (CFU/mL)		
	Mg (200 μg/mL)	Zn (200 μg/mL)	Ca (200 μg/mL)
0 (control)	$1.36 \cdot 10^9$	$2.96 \cdot 10^9$	$7.86 \cdot 10^9$
1.0	$1.43 \cdot 10^9$	$2.63 \cdot 10^9$	$1.24 \cdot 10^9$
2.28	$1.45 \cdot 10^9$	$2.48 \cdot 10^{10}$	$4.51 \cdot 10^9$
3.8	$1.34 \cdot 10^{12}$	$1.71 \cdot 10^{10}$	$3.10 \cdot 10^8$
5.6	$1.03 \cdot 10^{12}$	$5.96 \cdot 10^{10}$	$1.67 \cdot 10^8$

Figs. 3-5 present relationships between electric field strength and accumulation of magnesium, zinc and calcium in *L. rhamnosus* cells as well as membrane permeability determined by measuring the amount of fluorescent PI uptake. The permeability was studied in the presence of respective ion of interest. As shown in Fig. 3, a significant increase of membrane permeability in comparison to the control sample was observed at 3.8 kV/cm. In the case of zinc and calcium distinctively higher uptake of PI was already noted at 2.28 kV/cm (see Figs. 4 and 5). Permeabilization of cell membrane can be reversible or irreversible. If the exposure of a cell to the electric field is neither too long nor too intense, the membrane reseals and permeabilization is thus reversible. However, if a cell is exposed to a sufficiently high electric field, its membrane becomes permanently permeable; cell loses its homeostasis, which leads to cell death [49]. In our study, we did not use electrical parameters that would damage our cells in a way that bacteria would die (see also Table 1). On the contrary, we used electric parameters, which would reversibly permeabilize the membrane, so that respective ion of interest could accumulate in the cell at the highest levels possible. Therefore, no correlation was observed between bacterial viability and permeabilization of their membrane.

The study of Góral and Pankiewicz [31] showed that under optimized conditions (15 min exposure of the 20 h grown culture to PEF of the 2.0 kV/cm, at frequency of 1 Hz and 20 μs pulse width at concentration 400 μg Mg^{2+}/mL medium) accumulation of magnesium in the *L. rhamnosus* biomass reached 4.28 mg/g d.m. In the case of zinc, Góral *et al.* [32] reported that the highest bioaccumulation of zinc in *L. rhamnosus* was achieved when electroporation was performed at optimal parameters: field strength of 3.0 kV/cm, pulse width of 20 μs, electroporation time of 15 min after 20 h of culturing and at zinc concentration of 500 μg/mL medium. Bacterial cells accumulated 2.85 mg Zn/g d.m. which was almost 51% lower than in our experiment. However, the authors carried out the experiment in a totally different system. The PEF treatment chamber consisted of four paralel plexiglas plates which had stainless stell electrodes of an area equal to 4 cm^2, facing each other with a gap of 5 mm. The culture (100 mL) was agitated in a chamber during PEF treatment with a magnetic stirrer. This system did not guaranteed the treatment of all cells and the results are thus difficult to compare.

In the case of calcium the increase of membrane permeability led initially to the higher accumulation of this element reaching maximum at 2.28 kV/cm but then over 2-fold drop in accumulation was observed, most likely due to a decrease in cell viability (Fig. 5). It is known that an excess of the cytosolic calcium concentration can be toxic for the cell [50]. Therefore, cells have developed a specific system of pumps and exchangers to keep low concentrations of Ca^{2+} for proper cell functioning [51,52]. It is not known if the presence of ions affected bacterial membrane electropermeabilization, but it was shown on mammalian cells that calcium ions enhances the permeabilization of cells compared to control [53].

In order to confirm the influence of PEF on accumulation of metal ions in *L. rhamnosus* we observed the cells under a confocal laser microscope. Microscopic observations of the cells from the samples not exposed to PEF (A, C, and E) and the cultures electroporated at the highest field strength (B, D, and F) are presented in Fig. 6. In the case of magnesium and zinc, the cells treated with PEF

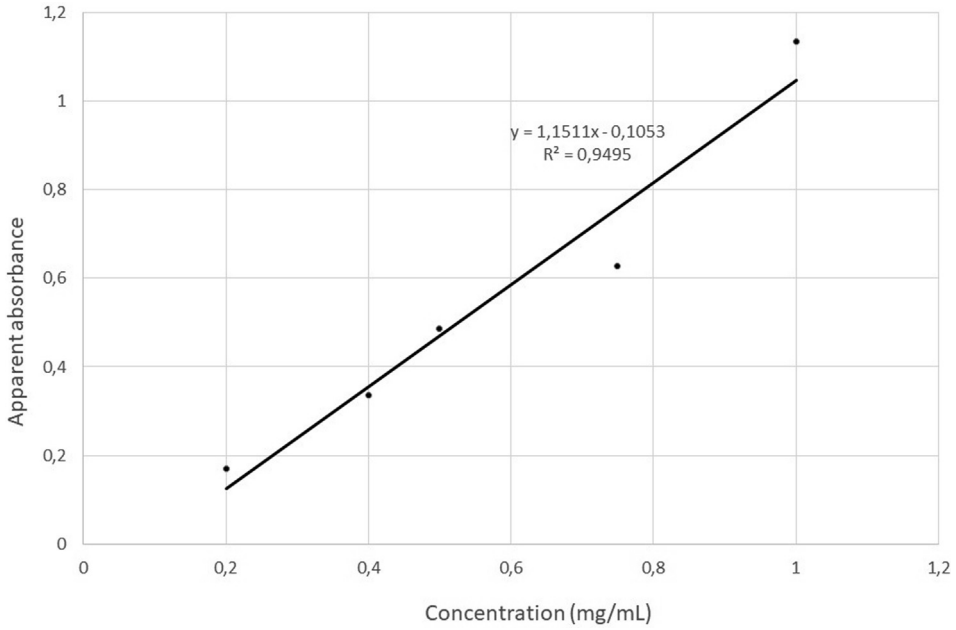

Fig. 2. The standard curve for determination of the bacteria biomass.

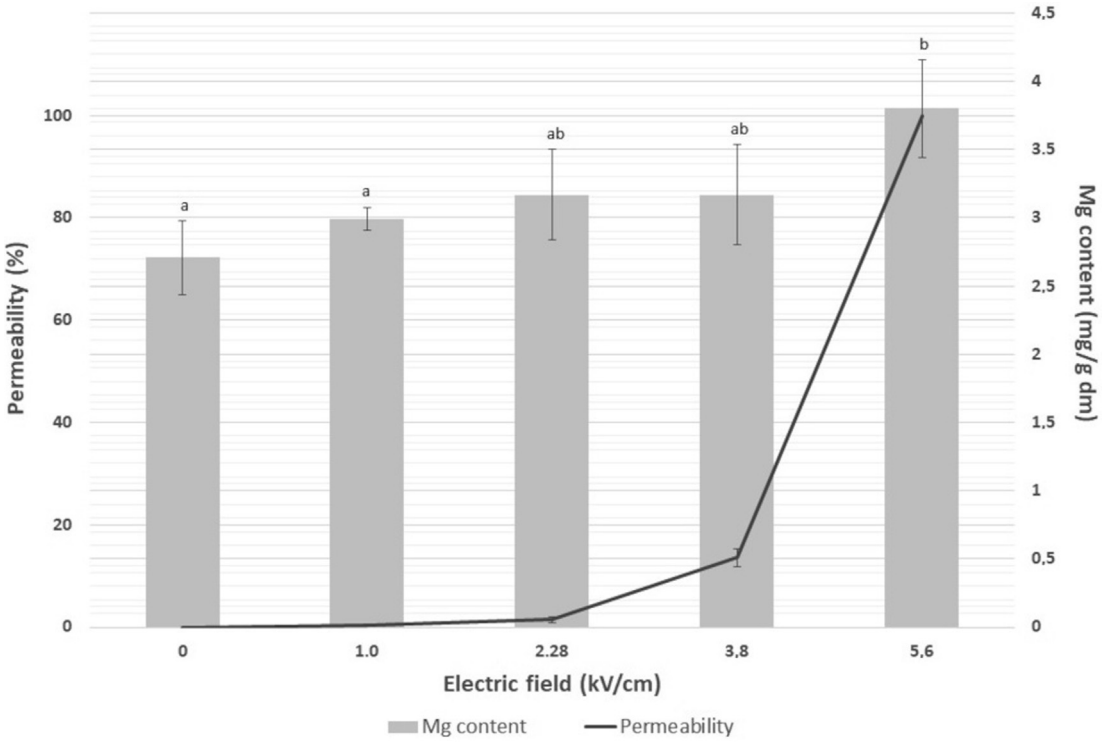

Fig. 3. Effect of electroporation on membrane permeability (line) and magnesium accumulation (bars) in *L. rhamnosus* cells. Means with the same letters do not differ significantly ($p < 0.05$; $n = 3$).

show much higher yellow-green or green fluorescence than those from the untreated cultures. The cells containing calcium showed intensive red fluorescence. As it is seen from the Fig. 6E and 6F this fluorescence was slightly higher after electroporation but at the same time the number of the observed cells was much lower. This is in line with the results of elements concentration in the cells determined by electrothermal atomic absorption spectrophotometry and discussed above.

4. Conclusion

The results of this study provide new perspectives on the use of a PEF continuous flow system for enrichment of probiotics with magnesium, zinc and calcium, and potentially also of other trace elements essential for the proper functioning of human body. The undoubted advantage of such a system is controlled and scalable PEF treatment at the selected pulse

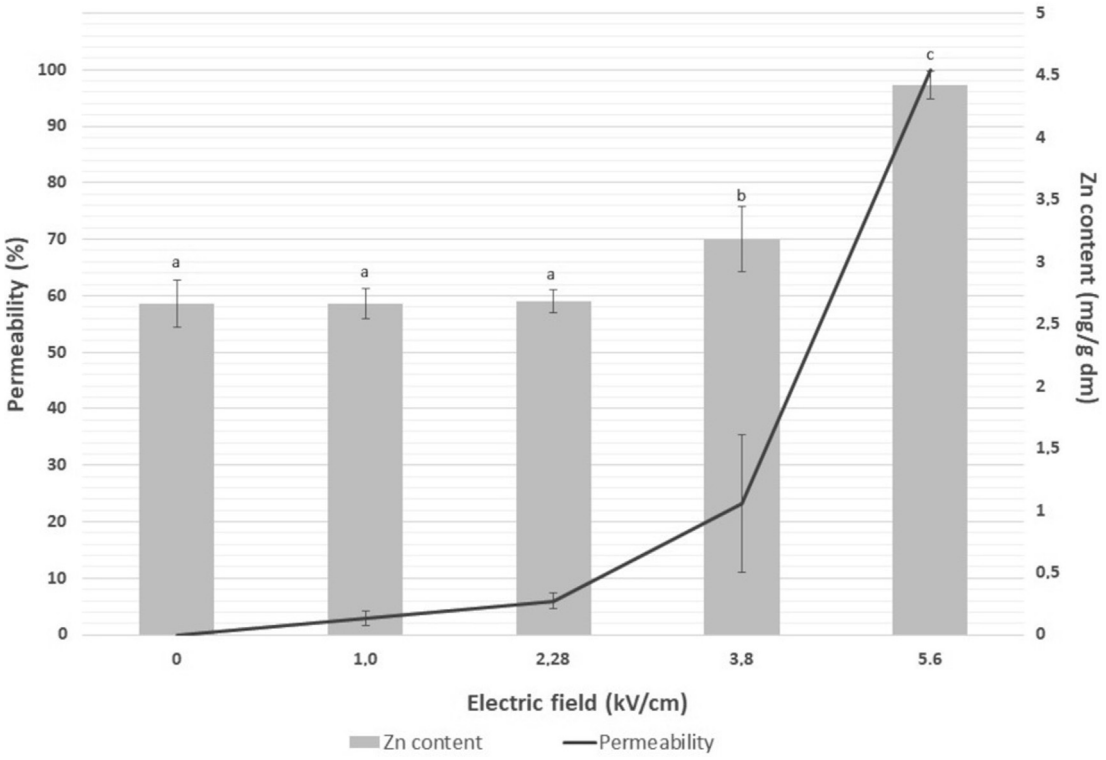

Fig. 4. Effect of electroporation on membrane permeability (line) and zinc accumulation (bars) in *L. rhamnosus* cells. Means with the same letters do not differ significantly ($p < 0.05$; $n = 3$).

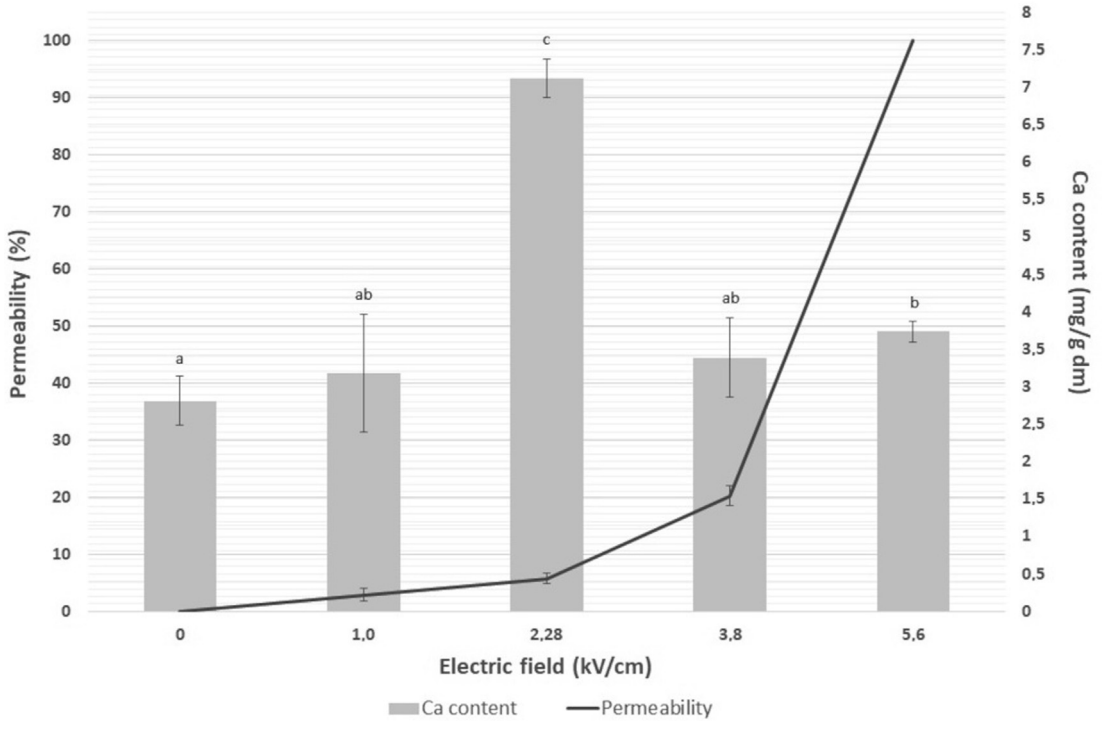

Fig. 5. Effect of electroporation on membrane permeability (line) and calcium accumulation (bars) in *L. rhamnosus* cells. Means with the same letters do not differ significantly ($p < 0.05$; $n = 3$).

parameters, as well as optimization of process parameters. In comparison to a laboratory batch system with mixing [31,32], electroporation of *L. rhamnosus* cells B 442 in a continuous flow system is also less time consuming. Nevertheless, the main limitation of this technology in order to be practically competitive is optimization of parameters that could achieve highest bacterial viability and accumulation of ions of interest.

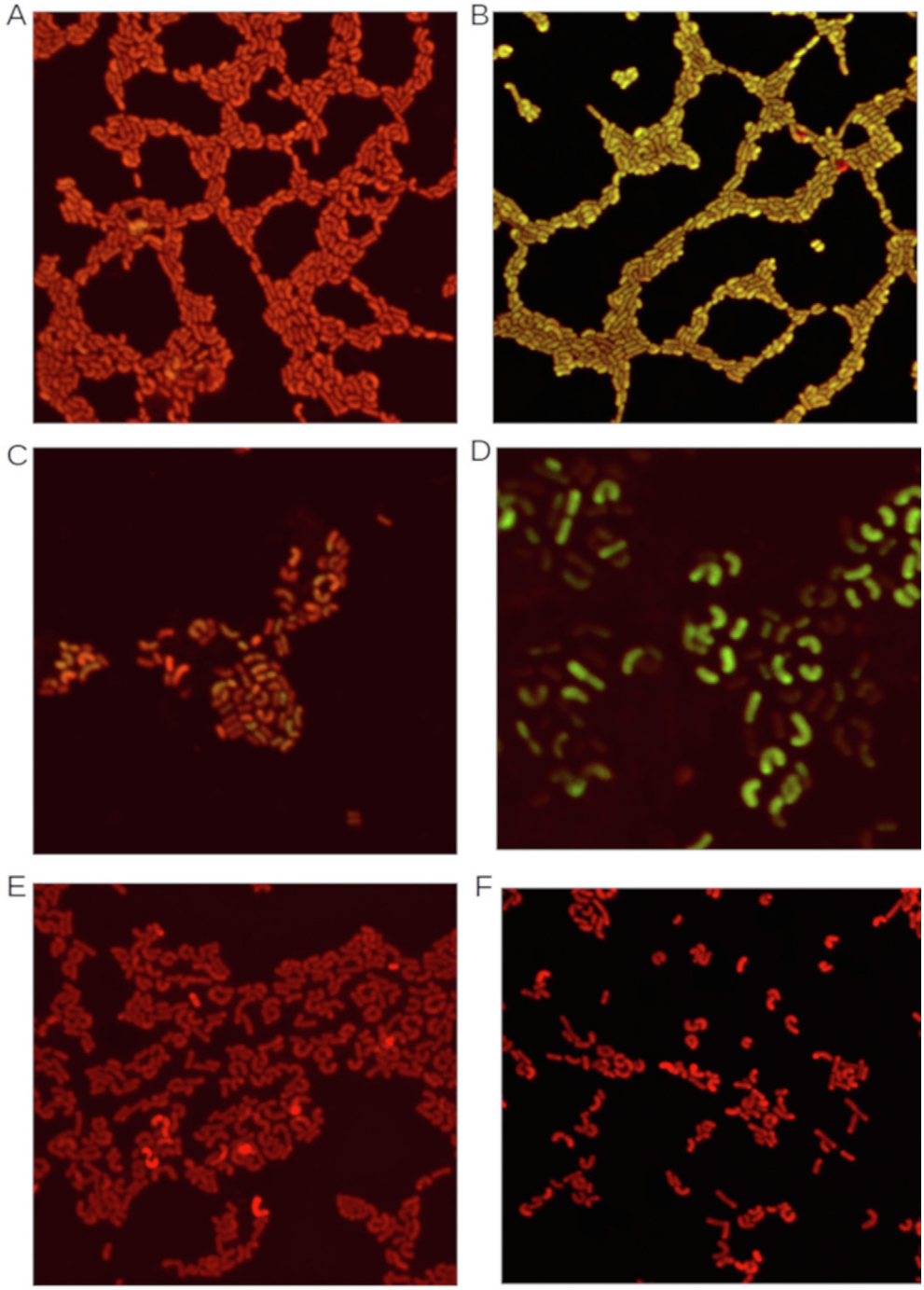

Fig. 6. Visualization of metal ions accumulated in bacterial cells: A (control) and B – magnesium (yellow-green fluorescence), C (control) and D – zinc (green fluorescence), E (control) and F – calcium (red fluorescence). Magnification: 300x (A, B, C, E, F); 500x (D).

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.

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