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Boosting microbial dextran production through moderate-intensity pulsed electric field (MI-PEF) treatments

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ABSTRACT

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB), particularly dextran from *Leuconostoc mesenteroides*, possess valuable technological and bioactive properties, supporting their wide industrial use. However, conventional fermentation often yields insufficient quantities to meet demand. This study assessed the potential of moderate-intensity pulsed electric fields (MI-PEF) as a novel approach to enhance dextran production by *Leuc. mesenteroides* DSA_O. Cells were harvested at mid-exponential phase and exposed to MI-PEF treatments before fermentation. Viability, colony morphology, EPS yield, and cell surface structure were evaluated. MI-PEF did not significantly reduce viability, though smaller colonies on solid media suggested a sub-lethal physiological effect. EPS yield increased proportionally to treatment intensity, reaching 40.19 ± 0.36 g/L, a more than sevenfold increase compared to the untreated inoculum. Confocal laser scanning microscopy revealed enhanced EPS accumulation at the cell surface, while scanning electron microscopy indicated marked alterations in cell envelope morphology, pointing to MI-PEF-induced structural remodeling. Preliminary NMR analysis confirmed that dextran branching degree and MW remained unchanged, ensuring preservation of its established functional and technological characteristics. These results indicate that MI-PEF can stimulate microbial metabolism to substantially increase EPS production without compromising cell viability or altering product quality. This study provides the first demonstration of MI-PEF as an effective tool to boost dextran biosynthesis in *Leuc. mesenteroides*, offering a cost-effective, scalable, and resource-efficient solution to meet industrial EPS demand.

1. Introduction

Leuconostoc mesenteroides is a lactic acid bacterium (LAB) recognized for its capacity to produce exopolysaccharides (EPS), particularly glucans. Among these, dextran stands out as a well-characterized polymer composed primarily of D-glucose units linked by α-(1 → 6) glycosidic bonds, with occasional α-(1 → 4), α-(1 → 3), or α-(1 → 2) branches (Van Cleve, Schaefer, & Rist, 1956). This unique structural versatility enables dextran to have a broad spectrum of industrial applications, ranging from the production of foods (as a texture enhancer or thickening agent), drugs, (as a cryoprotectant, osmotic agent, or antithrombotic agent) and the cosmetics (as a moisturizing and thickening agent) (Naessens,

Abbreviations: EPS, Exopolysaccharide/exopolysaccharide; LAB, Lactic acid bacteria; MI-PEF, Moderate-intensity pulsed electric fields; NMR, Nuclear magnetic resonance; MW, Molecular weight; PEF, Pulsed electric fields; US, Ultrasounds; HHP, High hydrostatic pressure; HI-PEF, High intensity pulsed electric fields; MRS, De Man Rogosa & Sharpe; ConA, Concanavalin-A; COSY, Correlation spectroscopy; DOSY, Diffusion ordered spectroscopy; MRD, Maximum recovery diluent; OD, Optical density; PBS, Phosphate buffered saline; HePS, Heteropolysaccharides; HoPS, Homopolysaccharides; CLSM, Confocal laser scanning microscopy; FEG-SEM, Field-Emission-Gun Scanning Electron Microscope; SEM, Scanning electron microscopy; DIC, Differential Interference Contrast; E, Electric field strength; W_T, Specific energy density; ^{1}H NMR, Proton nuclear magnetic resonance; DEPTQ, Distorsionless enhancement by polarization tranfer; n, Number of pulses; SE, Standard error; R^2, Coefficient of determination.

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Cerdobbel, Soetaert, & Vandamme, 2005). In addition, dextran possesses significant health-related bioactivities, including antimicrobial, antioxidant, and bifidogenic activities (Bisson et al., 2023, 2024).

Despite its growing importance, dextran yields achieved under standard fermentation conditions are often insufficient to meet increasing industrial demands, ranging from about 3 to 10 g/L for *Leuc. mesenteroides* under conventional batch fermentation (Aman, Siddiqui, & Qader, 2012; Bisson et al., 2023). Conventional strategies to optimize EPS production, involving the modification of growth media composition (e.g., sugar and organic/inorganic nitrogen concentration) and optimizing fermentation parameters (e.g., inoculum size, pH, temperature), while adequate, are typically time-consuming and resource-intensive (Ge et al., 2023; Wang et al., 2020).

Consequently, there's significant interest in developing alternative strategies to enhance EPS yields. Inducing sublethal stress through various approaches, such as the application of non-thermal technologies like pulsed electric fields (PEF), ultrasound (US), and high-hydrostatic pressure (HHP), shows promise in achieving this goal (Kaletunç, Lee, Alpas, & Bozoglu, 2004; Liu, Yang, & Fang, 2018). Despite US and high-hydrostatic pressure HHP have also been explored to stimulate microbial activity and metabolite production, both methods show relevant limitations. US relies on acoustic cavitation, generating localized shear forces and temperature gradients that can cause uncontrolled stress responses or partial cell lysis (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012; Raso, Condon, & Álvarez, 2016; Vilkhu, Mawson, Simons, & Bates, 2008). HHP, in turn, requires very high pressures and expensive equipment, which reduce scalability and may irreversibly damage cell structures (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007; Yordanov & Angelova, 2010). In contrast, moderate-intensity PEF (MI-PEF) offers a tunable and energy-efficient approach, inducing uniform and reversible electroporation that could enhance microbial metabolism without compromising viability (Barba et al., 2015; Toepfl, Siemer, Saldaña-Navarro, & Heinz, 2014).

Based on the intensity of the treatment parameters, these non-conventional technologies can induce death or a sublethal stress to microbial cells (Kaletunç et al., 2004; Lytras et al., 2024). Sublethal stress on microbial cells often manifest as a reduced ability to grow on selective media or a delay in forming visible colonies, which can result in smaller colony sizes (Yang et al., 2021). Similarly, sublethal stress could also results in an extension of the lag phase or a reduction in the maximum specific growth rate (Fang et al., 2021; Rahbar, Mohammad-Rafiee, Santen, & Shaebani, 2024). Crucially, these observable changes in colony size and liquid culture growth parameters can serve as valuable sentinels, indicating a potentially interesting modification of microbial metabolism, particularly for the biosynthesis of valuable metabolites.

Among non-conventional technologies, PEF is based on the application of short pulses (μs–ms) of high voltage. When applied to microorganisms, PEF induced electrical break-down of cells membrane, a process known as electroporation, thus affecting the membrane permeability (Barba et al., 2015). Moreover, dielectric rupture may occur when charge accumulation induces a transmembrane potential sufficient to cause electro-compression, thinning the membrane and leading to its rupture (Toepfl et al., 2014). Traditionally, when PEF is performed at high intensity (HI-PEF, 20–80 kV/cm), irreversible and complete membrane damage is observed. For example, this technology was successfully applied to various food matrix (e.g., liquid whole egg, skim milk, and dairy products) (Cavalcanti et al., 2023; Monfort et al., 2010; Picart, Dumay, & Cheftel, 2002); whereas reversible phenomena with pore resealing occur when moderate conditions (MI-PEF, $<$10 kV/cm) are applied. This reversible damage can alter the biosynthetic behavior of microorganisms without inactivating them (Ewe, 2012; Lye, 2012), suggesting a significant potential to modulate bacterial fermentation dynamics. However, literature on modulating postbiotic production, including EPS, by LAB using MI-PEF treatments is minimal, with only one study exploring the effect of PEF on EPS production by *Lactococcus lactis* subsp. *cremoris* (Ohba, Uemura, & Nabetani, 2016).

This knowledge gap opens the opportunity to explore the potential of MI-PEF in increasing the production yields of microbial polymers known for their technological and functional features, thereby opening new avenues for industrial applications. Therefore, this study aims to investigate whether pre-treatments of the inoculum with MI-PEF influence dextran yield in *Leuc. mesenteroides*, providing valuable insights for optimizing biotechnological processes. Recognizing the importance of the microbial physiological state for effective treatment, MI-PEF treatments in this study were specifically carried out on cells collected in their mid-exponential growth phase, a period characterized by high metabolic activity and responsiveness. To highlight potential sublethal stress indicative of metabolic modifications, the size of colonies and the evaluation of the turbidimetric curve post-treatment were also assessed. Additionally, microscopic observations were conducted to reveal changes in cell morphology and surface characteristics.

2. Materials and methods

2.1. Microorganisms and chemicals

Leuc. mesenteroides DSA_O was used in this study (Bisson et al., 2024) and stored as a 30 % (*v*/v) glycerol stock culture at −80 °C. Overnight culture was prepared in De Man, Rogosa, and Sharpe (MRS) broth at 30 °C under microaerophilic conditions. Lab-Lemco powder, Maximum Recovery Diluent (MRD), MRS broth, yeast extract, and technical agar were purchased from Oxoid (Milan, Italy). Absolute ethanol, D-(+)-sucrose, dipotassium hydrogen phosphate, manganese sulphate tetrahydrate, and sodium acetate trihydrate were acquired from Carlo Erba (Milan, Italy). Lactic acid, magnesium sulphate heptahydrate, phosphate buffered saline (PBS), and triammonium citrate were purchased from Sigma-Aldrich (Milan, Italy). Tween-80 was obtained from Liofilchem (Milan, Italy).

2.2. Assessment of mid-exponential phase

The MI-PEF treatments were performed on cells collected during the mid-exponential phase. To establish the mid-exponential phase, 200 mL of MRS-S (peptone 10 g/L, Lab-Lemco powder 8 g/L, yeast extract 4 g/L, sucrose 20 g/L, dipotassium hydrogen phosphate 2 g/L, sodium acetate trihydrate 5 g/L, triammonium citrate 2 g/L, magnesium sulphate heptahydrate 0.2 g/L, manganese sulphate tetrahydrate 0.05 g/L, Tween 80 1 mL/L; pH 6.2 ± 0.2) were inoculated at 1 % (*v*/v) with the overnight culture of *Leuc. mesenteroides* and incubated at 25 °C for 38 h in aerobiosis (Biolog AG-System, Fratelli Galli, Milan, Italy). At the beginning of incubation and after 8, 12, 16, 20, 24, 32, and 38 h, viable counts were assessed onto MRS agar plates incubated at 30 °C for 48 h (Herigstad, Hamilton, & Heersink, 2001). Data were modelled using Online DMFit (https://combasebrowser.errc.ars.usda.gov/DMFit.aspx) to estimate the mid-exponential phase and the corresponding viable count. Cells at the mid-exponential phase from a 200-mL MRS-S culture were collected by centrifugation (13,000 xg at 4 °C for 5 min) (Dlab D3024, Dlab Scientific co. ltd., Beijing, China), washed three times with PBS, and resuspended at about 10^4 CFU/mL before MI-PEF treatments.

2.3. MI-PEF treatments

Cell suspension was treated with MI-PEF at an electric field strength (E) of 1.18 ± 0.02 kV/cm generated by an M100 ScandiNova generator (Uppsala, Sweden) using a square pulse of 5 μs in a parallelepiped treatment chamber (16.8-mL volume capacity), with a 1.5-cm gap between the two stainless electrodes. Processing parameters were controlled by LabVIEW4PEF software (LabVIEW4PEF_B-618-01 9.0, ProdAl, Fisciano, Italy). Based on preliminary trials and on reported literature (Najim & Aryana, 2013; Zimmermann, 1986), the treatments were carried out at 125 V in six different combinations (T1-T6) of frequency (Hz), and number of pulses (n) (Table 1).

The specific energy density (W_T) was computed in accordance with Eq. (1) (Plazzotta, Ibarz, Manzocco, & Martín-Belloso, 2021):

$$W_T = \frac{V^2 t}{Rm} \quad (1)$$

Where V is the voltage (V), t is the time (s) calculated as pulse duration (5 μs) multiplied by pulse number, R is the electrical resistance (Ω) and m is the sample mass (kg).

Untreated cells served as the control. On the treated and control microbial suspensions, an assessment of microbial viability was made as previously described. The temperature before and after the treatments was measured by a thermocouple probe (Hanna Instruments, Tersid s.r.l., Milano, Italy).

2.4. Sublethal effect of MI-PEF on microbial growth in liquid and solid media

To detect sublethal damage of MI-PEF, the diameter of the colonies (growth on solid medium) and the turbidimetric growth kinetics (growth in liquid medium) of the cultures were investigated and compared to those of control cells.

(i) Growth onto solid medium. Cell suspensions were serially diluted in MRD, and 100 μL of each dilution was spread plated onto MRS agar and incubated at 30 °C for 48 h. After incubation, colonies were counted and images of the plates were acquired through a photo booth (Images & Computers, Bareggio, Italy) equipped with a digital camera (EOS 550D, Canon, Milan, Italy). The camera was positioned 50 cm above the specimen, facing a black background, with light provided by four 100 W photographic reflectors. The following parameters were set to acquire the images: exposure time, 1/25 s; F-stop, f/5; and focal length, 60 mm. The pictures were saved in 3456 × 2304-pixel JPEG format, converted to 8-bit grayscale, and software calibration applied. The diameter of the colonies was determined using Image-Pro Plus 6.3 (Media Cybernetics Inc., USA).

(ii) Growth in liquid medium. 10 μL of cell suspensions were grown in duplicate wells of a U-bottomed 96-well microtiter plate (Corning Life Science, Corning, NY, USA) with 190 μL of MRS broth with 5 % NaCl (*w/v*). Non-inoculated wells were used as blanks. The microtiter plate was incubated for 48 h at 30 °C, and the OD at 630 nm (OD_{630}) was recorded at 30 min intervals by a Sunrise microplate reader (Tecan, Milan, Italy). Data were modelled using the Baranyi model (Baranyi & Roberts, 1994) implemented in DMfit (Eq. 2):

$$OD(t) = OD_0 + \left(OD_{max} - OD_0 \times \frac{e^{\mu max A(t)} - 1}{e^{\mu max A(t)} - 1 + e^{\mu max \lambda}} \right) \quad (2)$$

The values of lag phase (λ; h), maximum growth rate (μmax; log OD/h), and maximum OD (OD_{max}) were estimated.

2.5. Electron microscopy (FEG-SEM)

The observation of cell surface morphology was carried out using Field-Emission-Gun Scanning Electron Microscope FEG-SEM (Jeol JSM7600F, JEOL, Tokyo, Japan). Briefly, 10 μL of cell suspension was fixed to a stainless-steel support and treated with 2.5 % glutaraldehyde (*v*/v) for one hour. Then, they were washed three times with PBS and gradually dehydrated in ethanol (50–100 %) for 15 min. All samples were sputter-coated (Cressington, Watford, UK) with a thin (2-5 nm) layer of gold to improve their electrical conductivity. FEG-SEM images were acquired at different magnification with an acceleration voltage of 5 kV and a working distance of 15 mm. To prevent the formation of artifacts, samples were stored in a desiccator with silica beads and taken immediately before analyses to avoid hydration. To ensure the images were representative, at least three different areas of each sample were acquired.

Table 1
MI-PEF treatments applied in this study.

Parameter	T1	T2	T3	T4	T5	T6
f (Hz)	250	250	250	700	700	700
n	300	600	900	300	600	900
W_T (kJ/kg)	326.3	655.4	980.6	322.7	645.0	970.8

2.6. Confocal laser scanning microscopy (CLSM)

Control and MI-PEF-treated (T6) *cells* were stained with Alexa Fluor 488-conjugated Concanavalin A (ConA) to label EPS. Cell suspensions in PBS were added to a ConA stock solution (1 mg/mL in sodium bicarbonate 0.1 M at pH 8.3) at a 1:1 ratio and then incubated in the dark at room temperature for 30 min. Then cells were washed twice with PBS at 8000 x *g* for 2 min and observed on a Leica TCS SP8 confocal system (Leica Microsystems, Wetzlar, Germany) equipped with a 63×/1.40 oil immersion objective. Single-plane confocal images (pinhole size, 2 Airy Units) and corresponding Differential Interference Contrast (DIC) images were acquired at a zoom factor of 3.0, using a pulsed white light laser set to 488 nm.

2.7. EPS production, isolation, and quantification

EPS production was carried out by inoculating 30 mL of MRS-S with 300 μL of each MI-PEF-treated or control cell suspension. MRS-S was incubated at 25 °C for 24 h. The cultures were then diluted 1:1 with MilliQ water and centrifuged at 13,000 x*g* at 4 °C for 10 min in an Avant J-25 centrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was collected, and EPS were precipitated overnight by adding three volumes of cold (4 °C) absolute ethanol. Then, the pellet was washed three times with absolute ethanol, and the supernatant was removed by centrifugation at 3000 xg for 15 min at 4 °C. The pellet was then solubilized in deionized water and dried overnight in a vacuum oven at 105 °C (Vuotomatic 50, Bicasa, Milan, Italy). The EPS yield was then determined by gravimetry (Bisson et al., 2024).

2.8. Chemical characterization of the EPS

The obtained EPS was dissolved in water (2.5 mg/mL) and shaken with Vortex 1 (Ika, Milan, Italy) until complete solubilization. The chemical nature of the isolated EPS was analyzed by Nuclear Magnetic Resonance (NMR) spectroscopy on a Bruker Avance III 400 NMR spectrometer (Bruker, Karlsruhe, Germany). Five mg of EPS were dissolved in 0.6 mL of D_2O, and a series of 1D and 2D experiments were acquired at 25 °C, including ^1H and ^{13}C spectra, DEPTQ (Distorsionless Enhancement by Polarization Transfer Including Quaternary Carbon Detection), homonuclear COSY (Correlated Spectroscopy), and heteronuclear HSQC (Heteronuclear Single Quantum Coherence) NMR experiments were referred to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate), used as an external standard, and expressed as part per million (ppm). To estimate the molecular weight (Mw) of the EPS, DOSY (Diffusion Ordered Spectroscopy) NMR experiments were used (Maina et al., 2014). A calibration curve was constructed using dextran standards (2 mg in 0.6 mL of D_2O by varying, in each sample, the pulse sequence parameters (δ, Δ). The molecular diffusion coefficient (D) was calculated using the Stejskal-Tanner Eq. (3):

$$I = I_0 \exp\left[(\gamma \times g \times \delta)^2 \times \left(\Delta - \frac{\delta}{3} \right) \times D \right] \quad (3)$$

where g is the gradient strength (g/cm), Δ is the diffusion delay (s), D is the diffusion coefficient (cm^2/s), δ is the gradient length (s), I is the observed spin-echo intensity, I_0 is the intensity of the analog experiment performed without gradients and γ the magnetogyric constant (rad/Gs) of the nucleus whose phase is encoded/decoded by the gradients. The diffusion coefficients of the dextran standards (D, m^2/s) were plotted against their Mw according to the following Eq. (4):

$$D = KMw^{\alpha}D \quad (4)$$

The exponential equation was $D = 7.283 \times 10^{-9}\ M_w^{-0.496}$ in agreement with a previous report (Viel et al., 2003). Applying the same protocol, the diffusion coefficient of the EPS was estimated, and the Mw was calculated using the exponential equation from the calibration curve.

2.9. Statistical analysis

All trials were carried out at least in duplicate, and values are expressed as the means ± standard deviation (S·D). One-way ANOVA was performed to evaluate the significance of differences among means ($p < 0.05$) using R v.4.1.2 for Windows (The R Foundation for for statistical computing, Vienna, Austria), and Tukey's HSD *post-hoc* test was used to assess significant differences between means. The relationship between the specific energy density and EPS yield at 250 and 700 Hz was assessed using the linear correlation coefficient (R^2) by Origin Pro 9 software (OriginLab, Northampton, MA, UK).

3. Results and discussion

It is well-established that the physiological state of microbial cells significantly influences their susceptibility and response to physical treatments (Abee, 1999). In the exponential growth phase cells are highly metabolically active, making them sensitive to external stress, compared to cells in lag or stationary phases, which often exhibit higher stress resistance (Wang & Levin, 2009). Consequently, a thorough determination of this growth phase was performed at the outset of the study to ensure consistent and effective application of treatments. Under the experimental conditions applied in this study, the culture was observed to be in the mid-exponential phase after 18 h of incubation (Fig. S1). Therefore, for subsequent MI-PEF treatments, the cells were harvested at this time.

3.1. Effect of MI-PEF on microbial viability and growth in liquid and solid media

Table 2 shows the kinetic parameters and the viability of *Leuc. mesenteroides* DSA_O after MI-PEF treatments.

The turbidimetric growth curves obtained (Fig. S2) were modelled and the growth parameters, i.e., the length of the lag phase and the values of maximum growth rate and maximum optical density, were estimated and reported in Table 2. Indeed, sub-lethal damage to bacteria can be detected by turbidimetry in both liquid cultures and cultures grown on solid media and can be indicative of metabolic modulation (Bisson, Marino, Poletti, Innocente, & Maifreni, 2021; Krieger, Litzmann, Mathys, Ananta, & Knorr, 2013; Zhang et al., 2021). However, in our study, the MI-PEF treatments did not cause any significant changes ($p > 0.05$) in the growth pattern of *Leuc. mesenteroides* DSA_O with respect to the control.

Table 2
Kinetic parameters (mean ± S.D.) of control and treated (T1-T6) *Leuc. mesenteroides* DSA_O.

Sample	Lag (λ; h)	μmax (Log OD/h)	OD_{max}	SE	R^2
control	21.16 ± 0.09	0.06 ± 0.00	1.15 ± 0.01	0.00836	1.00
T1	16.44 ± 2.18	0.05 ± 0.01	1.14 ± 0.03	0.01580	0.999
T2	17.14 ± 2.76	0.05 ± 0.01	1.16 ± 0.01	0.00984	1.00
T3	20.14 ± 0.28	0.06 ± 0.00	1.14 ± 0.00	0.0112	0.999
T4	19.38 ± 0.60	0.06 ± 0.00	1.15 ± 0.01	0.01010	1.00
T5	20.35 ± 0.18	0.06 ± 0.00	1.14 ± 0.03	0.00874	1.00
T6	21.29 ± 0.28	0.06 ± 0.00	1.13 ± 0.01	0.00869	1.00

OD, optical density; SE, standard error of fit; R^2, coefficient of determination.

Moving to the growth on solid medium (agar plates), it is evident that, in this study no lethal effects were observed in the treated cells compared to the control, as evidenced by the similar viability values between the samples ($p > 0.05$) obtained on MRS agar plates (Table S1). On the other hand, the colony diameter on MRS agar plates was different in the treated samples compared to the control (Fig. 1). MI-PEF treatments resulted in a significant reduction ($p < 0.05$) in colony diameter compared to the control culture, suggesting that the treated cells may have suffered sub-lethal damage. As an example, the colony appearance of both control and MI-PEF (T6) treated *Leuc. mesenteroides* DSA_O is reported in Fig. S3. Only minor differences were observed among treated cells, suggesting the treatments may have reached a saturation point for the sublethal effect on agar growth.

In summary, sub lethally damaged cells showed no significant changes in growth kinetics in liquid medium, however a reduction in colony size on solid medium was observed. This apparent discrepancy is related to the different growth conditions and recovery mechanisms in the two environments. In a liquid medium, nutrients are constantly available, and waste products are diluted or dispersed more effectively. This more forgiving environment may allow sublethal damaged cells to recover more quickly or compensate for initial damage without resulting in a significant impact on the overall growth curve. On agar, cells grow in a more static environment. Nutrient diffusion is limited, and waste product removal is less efficient than in a liquid environment. A sublethal damaged cell may initially have difficulty absorbing or metabolizing nutrients effectively. A reduced metabolic efficiency or a slowdown in cell division, even if slight, results in less accumulated biomass over time and, consequently, smaller diameter colonies. Damaged cells may also require a more extended repair period before they begin to divide actively, which delays colony formation and limits their final size (Schottroff et al., 2018). The increased availability of resources may mask a slight initial slowdown (Fang et al., 2021). Furthermore, the reduction in colony size confirms that the treatments indeed induced effective sublethal stress, a prerequisite for expecting a change in metabolism (Zhang et al., 2021).

3.2. Effect of MI-PEF treatments on EPS yield

The impact of MI-PEF treatments on EPS production by *Leuc. mesenteroides* DSA_O was then investigated (Fig. 2).

It's interesting to note that the EPS yield significantly increased ($p < 0.05$) even under the mildest conditions applied (T1) compared to the control, rising from 5.96 ± 0.3 g/L to 25.34 ± 0.81 g/L for the control and T1, respectively. The maximum EPS yield of 40.19 ± 0.36 g/L was reached in correspondence of T6, a more than sevenfold increase in

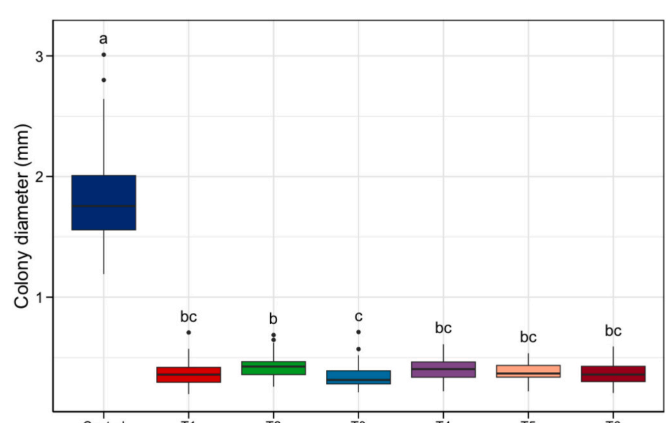

Fig. 1. Distribution of colony diameters of control and treated (T1-T6) *Leuc. mesenteroides* DSA_O on MRS agar. Different letters indicate a statistically significant difference ($p < 0.05$) between samples.

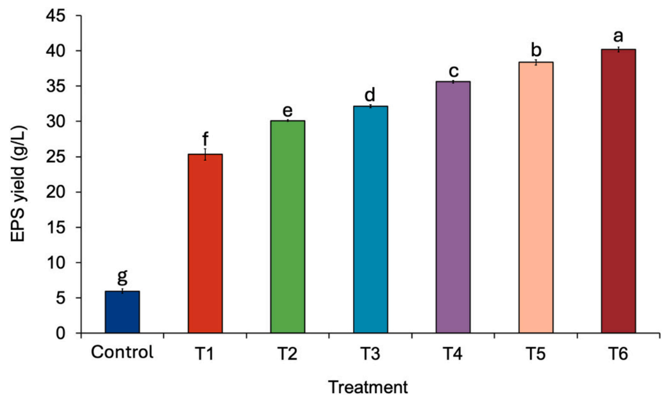

Fig. 2. EPS yields (mean ± S.D.) by control (0) and MI-PEF-treated (T1-T6) cells. Different letters indicate a statistically significant difference ($p < 0.05$) between samples.

comparison to the control. Interestingly, following PEF treatments, the temperature rise was negligible (1 °C in the case of T1-T5 and 3 °C in the case of T6 treatment), which suggests that the pronounced response in terms of EPS yield observed in this study would be best explained by non-thermal PEF effects. These findings demonstrated that the application of MI-PEF to the inoculum before fermentation could be a valuable strategy for making EPS production more yield-efficient at the industry level.

Acquired data were further processed to investigate the correlation between EPS yield and specific energy density (W_T) (Plazzotta et al., 2021). As the frequency is not considered in W_T computation, correlation analysis was performed separately for samples obtained at 250 and 700 Hz (Fig. 3). Results highlighted a strong positive correlation among W_T and EPS yield as confirmed by $R^2 > 0.93$ ($p < 0.05$) corroborating the effectiveness of MI-PEF in provoking EPS release. It is interesting to note that the estimated slope value (m) of the regression was higher ($p < 0.05$) when PEF treatments were conducted at 250 Hz as compared to those at 700 Hz suggesting a more effective permeabilization of cells at low frequencies. This is likely because cells are more sensitive at lower frequencies: the electric pulses are more effective in creating transient or permanent pores in the membrane, with reduced interference from pulse overlap and biological adaptation. This can lead to more efficient permeabilization and, in this context, a greater EPS yield by slightly increase the energy density (Potočnik, Miklavčič, & Maček Lebar, 2019). This suggests that low-frequency MI-PEF treatments may offer a broader

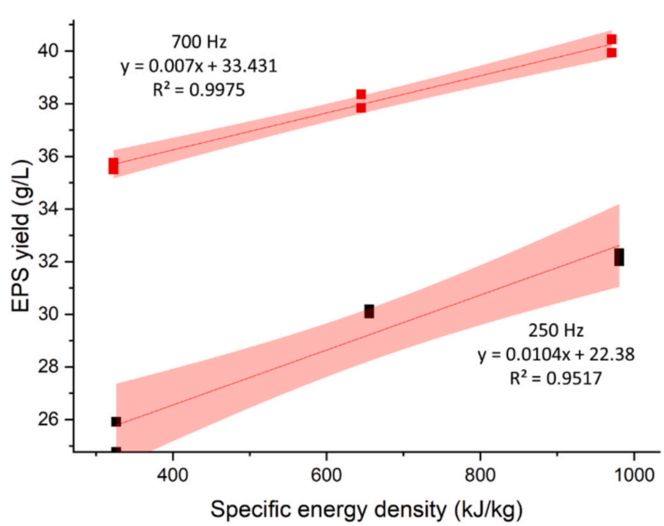

Fig. 3. Relation between the specific energy density and EPS yield at 250 and 700 Hz.

window of opportunity to positively modulate microbial metabolism, potentially enhancing the production of other microbial metabolites as well.

MI-PEF (at levels of 1 kV/cm or less) can alter the metabolism of LAB, enhancing acid tolerance and EPS production (Kanafusa, Uhlig, Uemura, Gómez Galindo, & Håkansson, 2021; Najim & Aryana, 2013). As far as EPS is concerned, the literature is scarce, and reports on the effect of MI-PEF on *Lc. lactis* subsp. *cremoris* in a single scientific paper. In that case, PEF enhanced the EPS yield by 32 %, reaching 16 mg/L, much lower than what was observed in this study. This was likely because, in that case, it was a heteropolysaccharide (HePS), i.e., EPS composed of different monosaccharide units, whose microbial synthesis is quite complex (Ohba et al., 2016). To the best of our knowledge, no experimental data are available on this topic for LAB species that produce homopolysaccharides (HoPS), such as the dextran by *Leuc. mesenteroides*. The authors tried to unravel the mechanisms and concluded that that MI-PEF induced electro-condensation of the cell membrane, causing localized pH drop. This acidic environment may have cleaved immature EPS, enabling more efficient lipid carrier recycling and resulting in a twofold increase in EPS yield (Ohba, Uemura, & Nabetani, 2017). Due to the differences in the biosynthesis of HoPS and HePS, this theory could not be applied to our case study. Alternatively, mild MI-PEF treatments may enhance membrane permeability and trigger sub-lethal stress response, leading cells to increase EPS production as a protective strategy.

To assess whether MI-PEF treatment could induce structural modifications in the EPS, we characterized the polymer using NMR spectroscopy. Previous studies have shown that alterations in LAB fermentation conditions can influence EPS structure (Wang et al., 2020). Given that EPS bioactivities are closely related to their chemical structure, we explored the potential impact of MI-PEF on the EPS produced by *Leuc. mesenteroides* DSA_O. The NMR and the DOSY spectra (Fig. S4) revealed that the EPS obtained after MI-PEF treatment (Fig. S4 A) retained the same structural features as the control sample (Fig. S4 B) (Bisson et al., 2024). The ^{1}H NMR spectrum confirmed that the polymer is a linear dextran with approximately 3 % degree of branching. Confirmation of the dextran nature of the polymer was achieved by comparing the ^{1}H NMR spectrum of the EPS obtained after MI-PEF treatment (Fig. S4 B) with that of a dextran reference standard (Fig. S5). Additionally, DOSY experiments showed that its molecular weight was consistent with that of the untreated EPS. Overall, these findings indicate that MI-PEF can enhance EPS production without altering its chemical structure, thereby preserving its functional properties.

3.3. SEM and CLSM observations

To more deeply investigate the mechanisms leading to enhanced dextran production by *Leuc. mesenteroides* DSA_O, scanning electron microscopy (SEM) and fluorescence microscopy were employed to observe the cell surface in control and MI-PEF-treated cells. The direct observation of the cell surface, therefore, offers invaluable insights into how MI-PEF physically interacts with this primary site of action, helping to explain observed changes in dextran yield or quality.

The cell wall is the main structural barrier against environmental factors and dictates bacterial shape (Pillet, Formosa-Dague, Baaziz, Dague, & Rols, 2016). Crucially, this structure, particularly the cell membrane, is the primary target for bacterial inactivation by various physical methods, including PEF. The electro permeabilization caused by PEF can influence cellular permeability and diffusion across membranes, potentially favouring the excretion of metabolites into the surrounding environment (Ewe, 2012).

In our observations, control cells of *Leuc. mesenteroides* DSA_O exhibited a smooth, integrated cell surface and formed typical chains of lenticular cells with narrowing junctions (Fig. 4A and B). In stark contrast, MI-PEF-treated cells underwent significant structural changes,

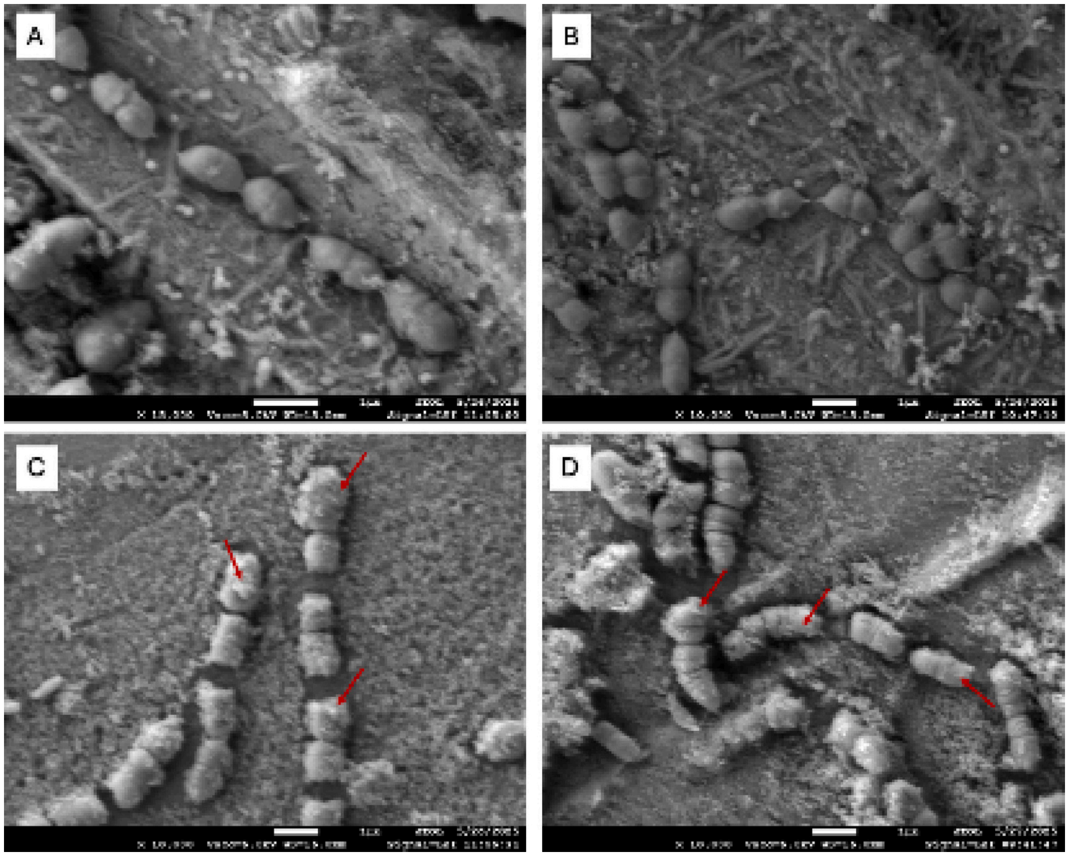

Fig. 4. Scanning electron microscope (SEM) images of *Leuc. mesenteroides* DSA_O. Control cells (A, B); MI-PEF (T6) treated cells (C, D). Red arrows indicate surface modification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

appearing notably rougher and covered by blister-like structures (Fig. 4C and D). Similar surface alterations have been reported in other studies on pressure- and PEF-treated bacteria (Kaletunç et al., 2004; Lye, Karim, Rusul, & Liong, 2011; Pillet et al., 2016). The observed surface roughness in treated cells could be a visible manifestation of alterations at the nanometric or micrometric level of the membrane, consistent with permeabilization induced by MI-PEF treatments. This permeabilization, if reversible and controlled, could facilitate the transport of substrates (such as sucrose, a dextran precursor) from the external environment to either intracellular sites or to extracellular enzymes. Given that dextransucrase, the key enzyme for dextran synthesis, is often associated with the cell surface or secreted externally (Robyt, Yoon, & Mukerjea, 2008), a rougher surface might indicate a change in membrane conformation that either exposes the enzyme more, enhances its accessibility to the substrate, or increases its catalytic activity. Alternatively, this could also signify increased secretion of the enzyme into the extracellular space, where it then deposits on the cell surface or in the culture medium. Another hypothesis is that the "roughness" itself could be an accumulation of dextran on the cell surface, produced in a denser or more structured way as a direct result of MI-PEF treatments.

Further confirmation of these hypotheses came from confocal microscopy observations of *Leuc. mesenteroides* DSA_O treated with ConA, a lectin known for its specific binding to carbohydrates, particularly α-D-glucose and α-D-mannose residues (Leriche et al., 2000). Control cells showed a weak fluorescence signal upon staining with Alexa Fluor 488-conjugated ConA (Fig. 5A and B), indicating minimal exposed carbohydrate on their surfaces under normal conditions. Conversely, the MI-PEF-treated cells exhibited a markedly increased fluorescence following ConA staining (Fig. 5C and D). This enhanced fluorescence in treated cells may reflect two possibilities: either an increased secretion of EPS by the bacterial cells, or structural modifications in the cell envelope that expose previously inaccessible glycoconjugates.

Indeed, PEF is known to induce reversible or irreversible electroporation with subsequent membrane permeabilization, which can facilitate the release of intracellular components, including polysaccharides (Kotnik, Rems, Tarek, & Miklavčič, 2019; Lye et al., 2011). On the other hand, the application of a sub-lethal MI-PEF treatment could also stimulate the bacteria's stress responses, directly leading to enhanced EPS synthesis (Ohba et al., 2016). We postulate that a possible reason for the enhancement of EPS production during fermentation could be that MI-PEF may have caused the permeabilization of the bacterial envelope, allowing the release of dextransucrase, a key enzyme involved in dextran production (Robyt et al., 2008). Collectively, these findings suggest that MI-PEF treatment significantly influences the extracellular matrix composition of *Leuc. mesenteroides* DSA_O, potentially affecting its functional or technological properties in fermentation processes.

4. Conclusion

This pioneering study successfully demonstrated that MI-PEF pretreatment of *Leuc. mesenteroides* DSA_O inoculum profoundly increased EPS yields, achieving a more than seven-fold increase compared to the control. A linear relationship was observed between treatment intensity and EPS yield at both 250 and 700 Hz, indicating that increasing intensity proportionally enhances production. Investigations of cellular behavior in liquid and solid media, combined with SEM and CLSM observations, strongly support reversible cell damage as a key mechanism, likely facilitating substrate transport or enzyme release. Importantly, preliminary NMR characterization showed that the dextran's macromolecular architecture remained unchanged, thereby preserving its established functional and technological properties. These findings highlight the significant potential of MI-PEF for scaling up EPS

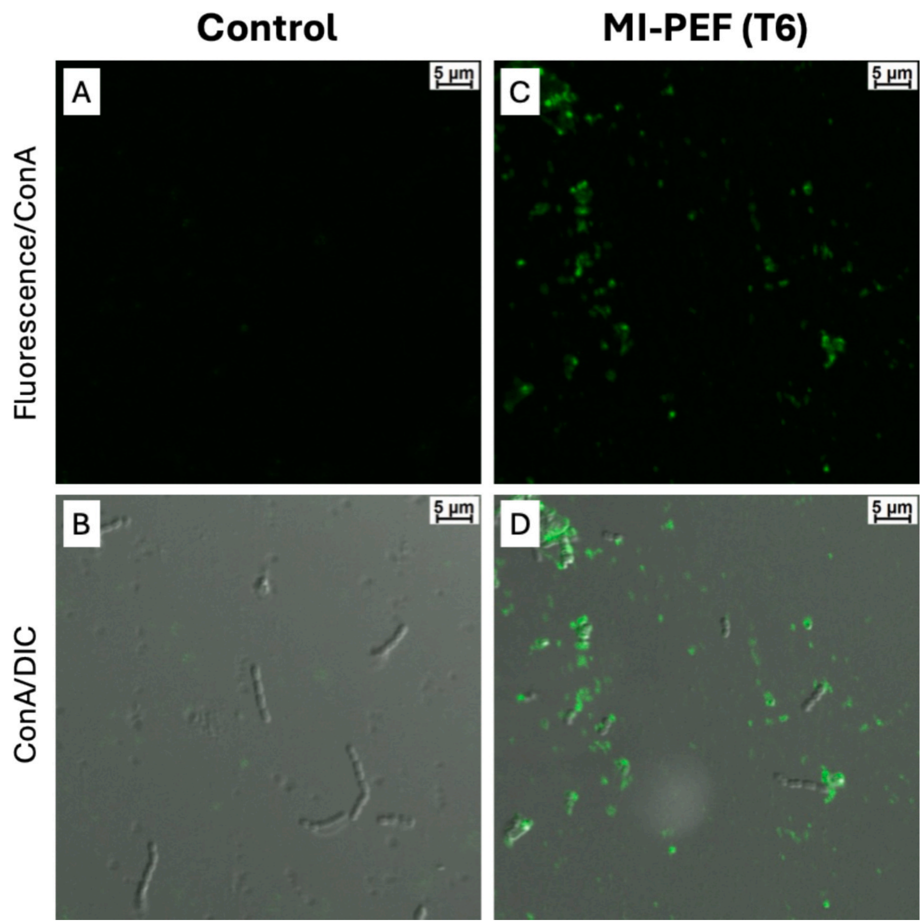

Fig. 5. Confocal laser scanning microscopy of non-treated (Control) and treated /MI-PEF T6) *Leuc. mesenteroides* DSA_O stained with Alexa Fluor 488-conjugated ConA. DIC, Differential Interference Contrast.

production while maintaining product quality, addressing current yield limitations in desirable LAB strains.

Nevertheless, further research is needed to evaluate the effects of MI-PEF on other *Leuc. mesenteroides* strains, to elucidate possible strain-specific responses to environmental stresses. Moreover, since the experiments were performed under controlled laboratory-scale fermentation conditions, additional work is needed to adapt and validate the MI-PEF process parameters for larger-scale implementations. Future research should further elucidate the underlying mechanisms and assess the applicability of this approach to other LAB species to expand its industrial relevance in sustainable EPS manufacturing.

CRediT authorship contribution statement

Giulia Bisson: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Sofia Melchior:** Formal analysis, Investigation, Writing – review & editing. **Anna Rossi:** Formal analysis, Investigation, Writing – review & editing. **Clara Comuzzi:** Formal analysis, Investigation, Writing – review & editing. **Matteo Zanocco:** Investigation, Writing – review & editing. **Francesca D'Este:** Investigation, Writing – review & editing. **Francesco Andreatta:** Investigation, Writing – review & editing. **Maria Cristina Nicoli:** Resources, Writing – review & editing. **Sonia Calligaris:** Resources, Supervision, Writing – review & editing. **Marilena Marino:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2025.104320.

Data availability

Data will be made available upon request.

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