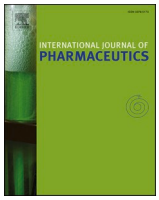
International Journal of Pharmaceutics 673 (2025) 125327

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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



# Viability of potential probiotics incorporated into nanofibers: Influence of genera, storage conditions, stabilizers and their solid-state

Nina Katarina Grilc [a,1], Spase Stojanov [b,1], Tomaž Rijavec [b], Aleš Lapanje [b], Aleš Berlec [a,b], Špela Zupančič [a,*]

[a] Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia
[b] Jožef Stefan Institute, Ljubljana, Slovenia

ARTICLE INFO

*Keywords:*
Probiotics
Delivery system
Nanofibers
Lyoprotectants
Storage
Viability
Solid-state

ABSTRACT

Electrospun nanofibers have emerged as a promising platform for probiotic delivery, with bacterial preservation posing a significant challenge in formulation design. This study examined the preservation of bacteria in various poly(ethylene oxide)-based nanofiber formulations and the solid-state behaviour of the excipients after electrospinning and during 24 weeks of storage under different conditions. Nanofiber formulations were loaded with bacteria from three different genera (oral cavity isolates *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S and vaginal *Lactobacillus jensenii*) and supplemented with 5 different stabilizers (sucrose, trehalose, glucose, mannitol or dextran), some of which also demonstrated nutrient characteristics. Efficacy of the tested stabilizers was species-dependent, with dextran as the most effective stabilizer for *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S and sucrose for *L. jensenii*. Low molecular weight stabilizers underwent complete (trehalose) or partial (sucrose, glucose, mannitol) amorphization during electrospinning in most formulations. Proportions of amorphous fractions of the semi-crystalline stabilizers were significantly influenced by the bacterial species, reaching up to 36% for sucrose and 28% for mannitol. Over 24 weeks, trehalose remained fully amorphous, while semi-crystalline stabilizers demonstrated instability of amorphous fractions, which underwent crystallisation. Notably, for oral isolate probiotics, amorphous stabilizers trehalose and dextran outperformed almost all semi-crystalline alternatives in preserving bacterial viability. In contrast, mannitol and glucose occasionally even reduced survival compared to PEO-only formulations, pointing out potential risks associated with physical instability of excipients. This study highlights the importance of selecting stabilizers tailored to specific bacterial species and understanding the solid-state properties of excipients to enhance probiotic survival in nanofiber-based formulations.

## 1. Introduction

Probiotics are live non-pathogenic microorganisms (i.e., bacteria and yeasts) with a variety of health benefits (Suez et al., 2019). Their mechanisms of action range from production of antimicrobial metabolites or competition for nutrients and adhesion with pathogens to immunomodulation and enhancement of epithelial barrier function (Barzegari et al., 2020; Kechagia et al., 2013; Stavropoulou and Bezirtzoglou, 2020). Probiotic use extends over a variety of diseases, most of which are related to gastrointestinal health (Rinninella et al., 2019) but also include other conditions, such as urogenital infections (mainly vaginal infections) (Ballini et al., 2018; Mei and Li, 2022; Verdenelli et al., 2016) and diseases of the oral cavity (mainly periodontal disease) (Rad et al., 2023; Saïz et al., 2021). Both vaginal infections and periodontal disease are dysbiosis-related diseases characterized by imbalance of the normal microbiota. While vaginal and oral microbiota are complex and heterogeneous in composition (France et al., 2022; Lebeer et al., 2023; Xian et al., 2018), multiple studies have shown that the dysbiosis of both can be ameliorated by supplementation with appropriate probiotic species. Autochthonous bacterial strains predominant in the healthy microbiota of the relevant mucosal sites have exhibited high efficacy in treating vaginal and oral dysbiosis (Lindo et al., 2024; Meng et al., 2024) and have thus been gaining attraction in the studies of the last decade compared to the previously highlighted gut-associated

* Corresponding author.
*E-mail address:* spela.zupancic@ffa.uni-lj.si (Š. Zupančič).
[1] The authors contributed equally with shared co-first authorship.

https://doi.org/10.1016/j.ijpharm.2025.125327
Received 7 October 2024; Received in revised form 10 January 2025; Accepted 6 February 2025
Available online 14 February 2025

strains (Jeng et al., 2020; Terai et al., 2015; Vicariotto et al., 2014; Vujic et al., 2013).

Optimal therapeutic effect of oral and vaginal probiotics requires local administration which involves the delivery of viable bacteria directly to specific mucosal sites. Thus, preservation of bacteria is a crucial step in producing probiotic formulations and is most often achieved through drying methods, with freeze-drying as the most common approach (Merivaara et al., 2021). However, alternative methods are gaining attention in probiotic formulation, including the production of nanofibers by electrospinning. Electrospun nanofibers incorporating various therapeutic compounds, including biological drugs (Stojanov and Berlec, 2020, 2024; Torres-Martínez et al., 2018) and various probiotic bacterial species (Zupančič et al., 2019) represent a promising approach that enables simultaneous drying, encapsulation and production of delivery system (Grilc et al., 2023; Hirscha et al., 2021). Nanofibers are also effective materials for delivering active compounds to the vaginal, oral and nasal mucosa (Sofi et al., 2020). Our and other groups have already demonstrated the incorporation of probiotics against oral diseases and vaginal infections (Grilc et al., 2023; Minooei et al., 2023; Silva et al., 2021; Stojanov et al., 2022; Stojanov et al., 2021a; Stojanov et al., 2024; Zupančič et al., 2018). Preservation of probiotics in nanofiber-based formulations is crucial and must be maintained during both electrospinning and storage to ensure therapeutic efficacy. Successful preservation, particularly long-term preservation of probiotics in nanofibers, can be improved by adding certain excipients with stabilizing activity (hereinafter referred as stabilizers) (Silva et al., 2021; Škrlec et al., 2019; Stojanov et al., 2022). Most of these stabilizers belong to the classes of saccharides and polyols and are postulated to act as lyoprotectants by thermodynamic or thermokinetic stabilization (Merivaara et al., 2021).

The preservational efficacy of stabilizers has been extensively studied in freeze-dried and spray-dried formulations (Kieps and Dembczynski, 2022; Martins et al., 2019; Tang et al., 2020; Zayed and Roos, 2004). Due to the dramatic differences of the processes and the structural differences (e.g., porosity, specific surface area) of the dry materials obtained by them, the stability of probiotics in nanofibers with different stabilizers cannot be addressed by extrapolating these data onto electrospun formulations. The existing studies on probiotic-loaded nanofibers with stabilizers have evaluated (i) the stabilizing effects of various stabilizers on the stability of a single probiotic species (Gensheimer et al., 2007; Hirscha et al., 2021; Salalha et al., 2006; Stojanov et al., 2022) or (ii) stabilizing effects of a nanofiber formulation on various probiotic species (Silva et al., 2021; Zupančič et al., 2019). However, there is a lack of information on how various stabilizers affect bacteria of different genera. Additionally, bacterial viability testing during storage has not been performed in all studies on nanofibers with stabilizers despite the known fact that storage conditions, such as temperature, relative humidity, exposure to light, moisture and oxygen content affect viability and potency of probiotics (Tripathi and Giri, 2014). Another important factor affecting the stabilizing efficacy of encapsulating matrices is their solid state. The solid state of stabilizers may affect their vitrifying efficacy during storage, while crystal formation can inflict potential cellular damage. Solid-state analysis is a well-established aspect of the characterisation of freeze-died or spray-dried probiotic formulations (Santivarangkna et al., 2011). Conversely, there is little data on the potential effects of the stabilizers' solid state in nanofibers, despite the fact that electrospinning is known to result in amorphization of many crystalline compounds (Kajdic et al., 2020) which could pose a problem in the use of crystallisation-prone stabilizers.

In the present study, we aimed to address these two research gaps by evaluating the stabilizing efficacy of various stabilizers for different potentially probiotic bacterial species during both electrospinning and nanofiber storage. Three species were selected on the basis of their differing genera, representation of both Gram-positive and Gram-negative bacteria and origin from two various microbiotas (oral and vaginal cavity) associated with potential dysbiosis. Thus, *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S were selected for treatment of periodontal disease, and *Lactobacillus jensenii* for treatment of vaginal infections. Each species was individually electrospun into nanofibers with poly(ethylene oxide) (PEO) as the carrier polymer alone or supplemented with one of the five stabilizers (sucrose, trehalose, glucose, mannitol and dextran), yielding a total of 18 probiotic-loaded nanofiber formulations. The excipients were also evaluated for their ability to serve as nutrient sources and facilitate bacterial growth.

Bacterial viability was tested at several time points during storage at different conditions and the stabilizers' solid state and interactions with the bacterial cells were evaluated by thermal and spectroscopic analysis. Our study design includes stabilizers with known stable glassy states and crystallisation-prone stabilizers in order to elucidate the potential effects of stabilizer solid-state after electrospinning and during storage on bacterial viability. This study highlights a promising approach for local delivery of probiotics targeting periodontal disease and vaginal infections, using nanofibers for effective delivery and long-term preservation of probiotic bacteria. Moreover, we evaluate the stabilizing efficacy of different stabilizers for a proper combination of bacterial species-stabilizer.

## 2. Materials and methods

### 2.1. Characterisation of bacterial strains

Three bacterial strains were used in the study as representatives of three different genera. All three strains were identified as potential probiotics – one for the treatment of vaginal infections and two for the treatment of periodontitis. *L. jensenii* ATCC 25258 is a type strain, isolated from the vaginal ecosystem (Gasser et al., 1970). Strains *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S were isolated as part of a previous study approved by the National Medical Ethics Committee of the Republic of Slovenia (ref. no. 78/04/14). The study was designed to establish a mini autochthonous buccal microbiota bank and screen the isolated strains for probiotic potential and use in the treatment of periodontal disease. The isolates originated from the oral cavity (tongue surface and saliva) of healthy individuals with dental records absent of periodontal disease, gingivitis, and caries. The two studied strains were chosen among those exhibiting antimicrobial activity against the following two Gram-negative bacteria: *Escherichia coli* TOP10 and *Aggregatibacter actinomycetemcomitans*, a known periodontopathogen. Strain identification was based on the sequencing of 16S rRNA gene (Supplementary data, Tables S1-S3). The strains 26.3.J and 27.3.S were assigned to the genera *Staphylococcus* and *Stenotrophomonas*, respectively, further on being named *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S throughout the continuation of the present study.

### 2.2. Culturing of bacterial strains

Bacteria were cultured in different media that support the growth of the individual strains. *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S were grown in nutrient broth (NB) (Merck, Darmstadt, Germany), while *L. jensenii* ATCC 25258 was grown in De Man, Rogosa, and Sharpe (MRS) medium (Merck, Darmstadt, Germany) at 37 °C. Starter culture stocks of all three strains were kept frozen at −80 °C in the respective media supplemented with 20 % (v/v) glycerol. Bacteria were transferred from the frozen stock to solid media supplemented with agar. *L. jensenii* were grown anaerobically in anaerobic bags (GasPakTM EZ; Becton Dickinson, Franklin Lakes, NJ, USA) or jars (AnaeroGenTM 2.5 l; Thermo Scientific, Waltham, MA, USA) at 37 °C for 2–3 days. Several colonies were picked, transferred to 10 mL liquid MRS and grown for 1 day at 37 °C. Fresh inoculum (1:50) was transferred in 200 mL MRS and incubated at 37 °C for ~ 16 h. Both *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S were grown aerobically on nutrient agar at 37 °C for 30–36 h, followed by the picking of an individual colony which was

transferred into 15 mL of NB and grown overnight. This was then used as a fresh inoculum, transferred into 300 mL of NB and incubated at 37 °C with strain *Stenotrophomonas* 27.3.S grown for ~ 9h while *Staphylococcus* 26.3.J was grown for ~ 14 h (both while shaking at 150 rpm).

### 2.3. Evaluation of stabilizers as alternative nutrient sources for bacteria

To test whether the studied bacteria can use the carbohydrate stabilizers as nutrients, their growth kinetics were observed by culturing them in modified media supplemented with the studied stabilizers. Modified minimal M9 medium without the standard carbon source (glucose) was used to study the growth kinetics of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S, whereas growth of *L. jensenii* was studied in modified MRS medium without glucose. The media were supplemented with the carbohydrate stabilizers in amounts corresponding to the content of glucose in the commercial media compositions (NB, MRS). This resulted in 5 new MRS-based and 5 new M9-based media (supplemented with sucrose, trehalose, glucose, mannitol or dextran), while media without stabilizer served as a negative control. Compositions of the modified M9 and MRS media are described in supplementary materials. Overnight-cultures were diluted 50-fold and inoculated in the different fresh, sterile-filtered media with or without the individual added stabilizer. 200 μL of the resulting inoculated media were pipetted in 96-well sealed microplates. The growth characteristics were determined by incubating the bacteria at 37 °C for 42–50 h (depending on the bacterial strain) with absorbance measurements at 595 nm every 2 min in a microplate reader (Sunrise; Tecan, Salzburg, Austria) as previously described (Stojanov et al., 2021b). Two biological and three technical repeats were performed for the evaluation of the growth kinetics of each of the tested bacterial strains in all 6 media. Lag time and growth rate were analyzed with the DMFit 3.5 software according to the model of Baranyi and Roberts (Baranyi and Roberts, 1994).

### 2.4. Preparation of bacterial-polymer dispersions

After reaching stationary (for *L. jensenii* and *Staphylococcus* 26.3.J) or late-log phase (for *Stenotrophomonas* 27.3.S), the bacteria were harvested from cultures containing approximately $10^9$–$10^{14}$ CFU/mL, washed, concentrated and resuspended in water. The bacterial suspensions in water were mixed with solutions of PEO 900 kDa (Merck, Darmstadt, Germany) which was used as the carrier polymer. PEO was utilized alone or in combination with one of the following carbohydrate stabilizers screened for their potential stabilizing activity: sucrose (Merck, Darmstadt, Germany), trehalose (supplied in the form of D (+)-trehalose dihydrate by Sigma Aldrich, Darmstadt, Germany), D (+)-glucose (Merck, Darmstadt, Germany), mannitol (Parteck® M, Merck, Darmstadt, Germany) and dextran (dextran from *Leuconostoc* spp., Mr ~ 70 kDa, Sigma Aldrich, Darmstadt, Germany). The final concentration of excipients in the bacterial-polymer dispersion was 4 % (w/v) when PEO was used alone. When PEO was supplemented with stabilizers, the final concentration of both excipients was 8 % (w/v) with a 1:1 mass ratio of PEO to each stabilizer. A more detailed description of the washing and concentrating steps during the preparation of bacterial-polymer dispersions can be found in Supplementary materials (section 5).

### 2.5. Characterisation of polymeric solutions with stabilizers

Polymeric PEO-based solutions (with or without stabilizers) without bacteria and at concentrations used for preparation of probiotic-loaded nanofibers were analysed by the following properties relevant to the electrospinning process: surface tension, conductivity and rheological properties.

Surface tension was determined in triplicates using the Krüss K-12 tensiometer (Krüss Scientific, Hamburg, Germany), with the Wilhelmy plate method. Conductivity was determined in triplicates using the Mettler FiveEasy conductivity meter (Mettler, Greifensee, Germany). Dynamic viscosity was analysed using the Physica MCR 301 rheometer (Anton Paar, Graz, Austria), with a cone-plate measuring system CP50-2 (cone angle 2°, cone diameter 50 mm, sample thickness 0.209 mm). The rotational test was performed at 20 °C with the shear rate varying from 1 $s^{-1}$ to 100 $s^{-1}$.

### 2.6. Electrospinning of bacterial-polymer dispersions

The bacterial-polymer dispersion was filled into a 5 mL syringe that was fixed to an electrospinning machine. Two electrospinning machines were used to fabricate the nanofibers, both equipped with grounded aluminium foil-covered plate collectors. Fluidnatek LE100 (BioInicia SL, Valencia, Spain) was used for electrospinning of formulations with *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S, while Spinbox (BioInicia SL, Valencia, Spain) was used for the incorporation of *L. jensenii* as the latter are more temperature-sensitive and the Spinbox enabled the incorporation of cooling elements.

#### 2.6.1. Electrospinning of polymer dispersions with Staphylococcus 26.3.J and Stenotrophomonas 27.3.S

Electrospinning was conducted on a vertically oriented setup with a needle-to-collector distance of 15 cm. The dispersions were fed through the needle at a flow rate of 475–500 μL/h and the voltage of 13–15 kV. The electrospinning process was carried out at room temperature and ~ 30 % relative humidity (RH).

#### 2.6.2. Electrospinning of polymer dispersions with L. jensenii

Electrospinning was conducted on a horizontally oriented setup with a needle-to-collector distance of 15 cm. During electrospinning, the pump along with the syringe and the plastic tube connecting it to the needle were placed in a cooling bag containing cold elements to maintain a low temperature for the temperature-sensitive *L. jensenii*. The dispersions were fed through the needle at a flow rate of 250–400 μL/h and the voltage of 10–12 kV. The electrospinning process was carried out at room temperature and ~ 30 % relative humidity (RH).

### 2.7. Freeze-drying of bacterial dispersions

Known volumes of bacterial dispersions in water were freeze-dried in order to determine the dry mass of the bacteria added into the dispersions used for electrospinning and thus determine the mass proportions of dry constituents of nanofibers (PEO, stabilizer and loaded bacteria). 3 mL of bacterial dispersion in vials was frozen at −80 °C and freeze-dried using the Beta 1–8 K Manual (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Primary drying was performed at shelf temperature of −5 °C and 0.65 mbar for 24 h and secondary drying at 20 °C for 1 h.

### 2.8. Scanning electron microscopy

Air-dried bacteria and freshly prepared bacteria-loaded nanofibers were visualized under scanning electron microscope (Supra 35 VP; Carl Zeiss, Oberkochen, Jena, Germany). Individual species were concentrated, washed and redispersed in highly purified water, and 5 μL of the resulting suspension was pipetted onto the metal sample-holder pin and air-dried. Meanwhile, nanofiber mats loaded with bacteria were fixed onto the metal pins using double-sided conductive tape. The imaging process was conducted at a 1 kV accelerating voltage with a secondary electron detector. Bacteria length and width and nanofiber diameters were measured using ImageJ 1.44p software (National Institutes of Health, Bethesda, MD, USA), by randomly selecting 50 bacteria or nanofibers.

### 2.9. Bacterial viability in polymer solutions, nanofibers after electrospinning and after long- term storage

Bacterial viability of the tested strains was evaluated in the polymer solution and nanofibers immediately after electrospinning as well as after storage of the nanofibers at different conditions for varying lengths of time. Viability was evaluated using the drop-plate method (Herigstad et al., 2001) performed in triplicate using three separate nanofiber mat pieces. The theoretical viability of loaded bacteria in a piece of the nanofiber mat was calculated based on the known dry mass of the bacterial-polymer dispersion.

Bacteria-loaded nanofiber mats were stored at refrigerated conditions (4–8 °C) in a desiccator with silica that maintained the RH at ~ 8–10 %. Refrigerated samples were analysed for bacterial viability after 1, 2, 4, 8, and 24 weeks. Additionally, nanofiber mats stored at room temperature (~ 22 °C) and ~ 45 % RH and −20 °C were tested for bacterial viability after 2 weeks and 24 weeks, respectively. Bacterial viability in both polymer solutions and nanofibers was normalized to the mass of nanofiber sample (CFU/g).

Each evaluation of bacterial viability at a specific time point consisted of diluting three aliquots of the homogenous bacterial-polymer dispersion (collected from the left-over dispersion in the syringe after electrospinning) or dissolving three nanofiber samples (approximately 5 mg) in different dilution media in Eppendorf tubes, followed by serial ten-fold dilution of the content of each tube. Sterile 0.9 % NaCl was used to dissolve and dilute samples of strains *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S, whereas *L. jensenii*-loaded samples were dissolved and diluted in 4 % (w/v) sucrose solution. Five 10 μL drops of each dilution were pipetted on agar plates and incubated at 37 °C, followed by colony enumeration. Nutri-agar plates were used to culture *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S and were incubated for 30–36 h in aerobic conditions. MRS agar plates were used to culture *L. jensenii* and were incubated anaerobically in bags or jars for 48–72 h. The survival of the bacteria was evaluated on the basis of the reduction of logarithmic viability (log CFU/g) of bacteria as calculated by the difference between the log viability per dry mass in polymer dispersion before electrospinning (theoretical viability) and the log viability per dry mass of nanofibers immediately after electrospinning. Additionally, for stored samples, the survival was determined by comparing the log CFU/g in the initial nanofiber sample (at time 0) to the log CFU/g in the final nanofiber sample (2 weeks for samples stored at room temperature and 24 weeks for samples stored at 4 °C and − 20 °C).

### 2.10. Thermal analysis

Differential scanning calorimetry (DSC 1, STARe,Mettler Toledo, Greifensee, Switzerland) was used to evaluate the solid-state characteristics of the excipient powders, lyophilized bacteria, placebo nanofiber mats and bacteria-loaded nanofiber mats. The samples (~ 5 mg) were weighed into aluminium pans with a pinhole heated at the rate of 10 K/min within a temperature range of 30 to 300 °C under a nitrogen flow of 50 mL/min. Immediately after electrospinning, placebo nanofiber samples were recorded in quadruplicates and bacteria-loaded nanofiber samples in duplicates. In order to study the changes in the solid state of the nanofibers during storage, stored samples were re-analysed after 24 weeks of refrigerated storage at ~ 8–10 % RH. The proportions of crystalline fractions of individual stabilizers were evaluated by determining the melting enthalpy ($\Delta H_m$) (where possible in case of present melting endotherms) in various samples (supplied pure stabilizers, placebo nanofibers, bacteria-loaded nanofibers). To enable comparison of crystalline fractions of stabilizers between samples of pure excipient powders, placebo nanofibers and bacteria-loaded nanofibers, $\Delta H_m$ were normalized to the mass ratio of the stabilizer in the nanofiber mat. The fraction of crystalline stabilizer ($x_c$) was calculated by Equation (1) with $\Delta H_m$ of the pure stabilizer powder representing the basis for 100 % crystallinity. The calculation included mass ratio normalization, taking the mass proportion of the stabilizer in nanofibers into account.

$$x_c(\%) = \frac{\Delta H_{mstab-nf}}{\Delta H_{mstab-p} \cdot w_{stab.}} \cdot 100\% \quad (1)$$

$\Delta H_{m\ stab\text{-}nf}$ presents the melting enthalpy determined from the stabilizer melting-associated peak in the nanofiber samples, $\Delta H_{m\ stab\text{-}p}$ the melting enthalpy determined from the stabilizer melting-associated peak of pure stabilizer powder and $w_{stab}$ is the mass proportion of the stabilizer in nanofibers, determined from the known dry masses of each component in the sample (PEO, stabilizer and dry bacteria). $\Delta H_m$ were quantified for PEO, sucrose, mannitol and trehalose dihydrate. While the reduction of glucose melting peak could be observed on the thermograms, the overlap of glucose melting and decomposition hinders the quantification of $\Delta H_m$ (Verma et al., 2020).

### 2.11. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to evaluate the interactions between bacteria, PEO and stabilizers. The spectra were obtained (Nexus, Thermo Nicolet, Madison, USA) in the spectral range of 4000 $cm^{-1}$ to 600 $cm^{-1}$ with 64 scans recorded at a resolution of 2 $cm^{-1}$. The samples were analysed immediately after electrospinning and after 24 weeks of refrigerated storage.

### 2.12. Statistical analysis

One-way analysis of variance (ANOVA) with post-hoc Tukey's test was carried out for comparison of mean nanofiber diameters of nanofibers of different compositions loaded with the same bacterial species, comparison of log CFU/g reductions during electrospinning of various formulations containing a specific bacterial species, and for and comparison of log CFU/g reductions in various formulations containing a specific bacterial species after storage for 24 weeks. Two-sample t-tests were employed for comparison of bacterial viability in dispersions and after electrospinning and bacterial viability in nanofibers immediately after electrospinning. Two sample t tests were also employed for comparison of melting enthalpies of crystalline and semi-crystalline stabilizers in the supplied powder form vs the corresponding nanofiber formulations, and the growth kinetics parameters (growth rate, lag time) of bacteria in non-supplemented and supplemented modified media. The results were visually presented as means ± standard deviation (SD). Statistical analyses were conducted with OriginPro 2018 software for t tests, while one-way ANOVA was conducted in Python (v3.12) with Pandas (v2.2.3), StatsModels (v0.14.4) and SciPy (v1.14.1) libraries.

## 3. Results and discussion

### 3.1. Stabilizers as alternative nutrients for bacteria

In order to study the potential of the stabilizers to act as carbohydrate nutrients for the bacterial strains, the latter were cultured in modified media that were either completely or partially depleted of the standard carbon sources, and in modified media where the depleted carbon sources were replaced with the tested stabilizers. The minimal media without any carbon source were used in the case of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S, and the modified depleted media with limited carbon source (MRS without glucose) in the case of *L. jensenii*.

Media without added stabilizers enabled limited bacterial growth which was significantly weaker than that in media supplemented with stabilizers (Fig. 1). The presence of most stabilizers notably enhanced the growth of all species, suggesting their utility as an energy source for *Staphylococcus* 26.3.J, *Stenotrophomonas* 27.3.S and *L. jensenii*. Notably, dextran was not utilized as an energy source by any of the three species as evidenced by their comparable growth in non-supplemented media

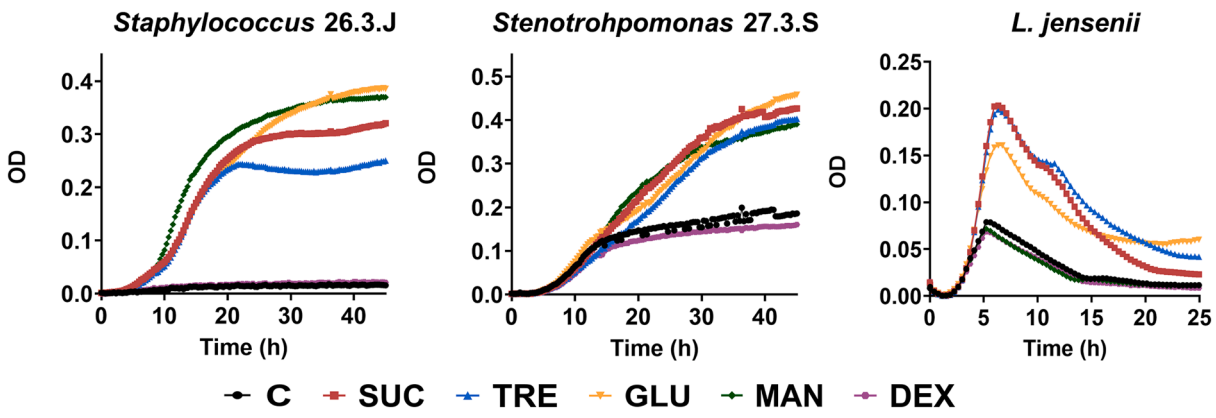



**Fig. 1.** Growth curves of *Staphylococcus* 26.3.J, *Stenotrophomonas* 27.3.S and *L. jensenii* in modified growth media without carbon source as a control (C) or supplemented with with 1% (w/v) (in the case of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S) or with 2% (w/v) (in the case of *L. jensenii*) sucrose (SUC), trehalose (TRE), glucose (GLU), mannitol (MAN) or dextran (DEX).

and media supplemented with dextran. Dextran can be utilized as a nutrient by bacteria harbouring dextran-hydrolyzing enzymes which break the polymer down and use different oligomers as carbon source (Khalikova et al., 2005), indicating that the three bacterial strains tested in our study did not express these enzymes.

Meanwhile, other tested stabilizers (trehalose, sucrose, glucose and mannitol) served as energy sources to all three tested bacterial species with the exception of mannitol that could not be utilized by *L. jensenii*. They increased the bacterial biomass as seen by the increase in the maximal OD ($OD_{max}$). Variations in growth characteristics (lag time, growth rate and $OD_{max}$) were observed among individual stabilizers and bacterial species, indicating the ability of certain bacteria to utilize specific stabilizers (Figs. S1 and S2). Mannitol and glucose were the best carbon sources for *Staphylococcus* 26.3.J, with the highest $OD_{max}$ and the shortest lag time. Despite no statistically significant changes in lag time and growth rate, glucose also emerged as the preferred energy source of *Stenotrophomonas* 27.3.S, stimulating bacterial growth to $OD_{max}$ of 0.37. Sucrose and trehalose were the best energy sources for *L. jensenii*, with sucrose enabling bacterial growth with the highest mean $OD_{max}$ of 0.20. These results correlate with our previous findings where we observed utilization of sucrose as a carbohydrate nutrient of three different species of lactobacilli grown in supplemented MRS, including *L. jensenii* (Stojanov et al., 2022). Similar results were observed with trehalose, where the lyoprotectant enhanced the bacterial growth to an $OD_{max}$ of 0.20. In contrast, glucose, the default energy source in commercial MRS media, yielded a mean $OD_{max}$ of 0.16.

These findings indicate that most of the tested stabilizers act as nutrients for the three studied bacterial strains. This could be useful for the promotion of their growth and colonization of the target mucosa as the dissolution of these stabilizers could act as an additional nutrient for the locally delivered bacteria immediately upon their release (Ji et al., 2023).

### 3.2. Effects of stabilizers on the properties of polymeric solutions for electrospinning

Polymeric PEO-based solutions (with or without stabilizers) without bacteria and at concentrations used for preparation of probiotic-loaded nanofibers were analyzed for surface tension, conductivity and rheological properties. Addition of stabilizers to PEO resulted in minor differences in these properties (Supplementary material section 2, Fig. S3 and Table S4), however, it did not prevent nanofiber formation during electrospinning experiments. Thus, the addition of stabilizers on these three studied properties was disregarded as minor in terms of effects on spinnability.

### 3.3. Effect of stabilizers on morphology of nanofibers with incorporated bacteria

Bacterial morphology and nanofiber formulations were observed under SEM (Fig. 2). The length and width of bacteria differed between the species, with *Staphylococcus* 26.3.J being the only cocci characterized with spherical-shape, while *Stenotrophomonas* 27.3.S and *L. jensenii* are bacilli with rod-shaped morphology. The average width of *Staphylococcus* 26.3.J was 1076 ± 162 nm, while the average width of *Stenotrophomonas* 27.3.S and *L. jensenii* were 699 ± 82 nm and 381 ± 40 nm respectively. Additionally, the lengths of these rod-shaped bacteria differed, with *Stenotrophomonas* 27.3.S being significantly longer (1664 ± 310 nm) compared to *L. jensenii* (960 ± 224 nm). To the best of our knowledge, we are the first to isolate and measure the size of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S. While *L. jensenii* ATCC 25258 is a widely used type strain, published data detailing its physical dimensions are scarce. An exception is our previous study, which examined genetically engineered *L. jensenii* expressing fluorescent proteins and reported similar findings (Stojanov et al., 2021).

Nanofiber formulations with bacteria were successfully developed, with incorporation evidenced by specific thickenings in the nanofibers (Fig. 2). Eighteen formulations were developed using PEO alone or supplemented with sucrose, trehalose, glucose, mannitol or dextran to embed *Staphylococcus* 26.3.J, *Stenotrohpomonas* 27.3.S and *L. jensenii*. Potential stabilizers were incorporated at high proportions (polymer: stabilizer ratio 1:1), also acting as bulking agents in the final product. This affected the nanofiber diameter as in most cases, stabilizer addition significantly increased the nanofiber diameter compared to PEO nanofibers (Fig. 3). This is to be expected as electrospinning of the dispersions with the added stabilizers resulted in a 2-fold higher amount of cumulative excipient mass supplied to the needle at the same solution flow rate. The stabilizer which resulted in the highest average nanofiber diameter in case of all three species is dextran, likely due to its high molecular weight which enables additional polymer chain entanglements. The diameter was also influenced by the presence of incorporated bacteria. Moreover, when utilizing the Fluidnatek LE100 system, nanofibers tended to be thicker in comparison to those produced with the Spinbox. This can also be attributed to several other factors other than the machine used, such as increased conductivity and lower flowrate. Spinbox was employed to produce nanofibers with lactobacilli that release extracellular matrix and other compounds which can increase conductivity of the electrospinning dispersion. Additionally, this effect of lactobacilli on dispersion conductivity was accounted for by electrospinning at lower flowrates. Notably, electrospinning of *Staphylococcus* 26.3.J with dextran as a stabilizer resulted in the largest average diameter (434 ± 42 nm). Conversely, electrospinning of *L. jensenii* in

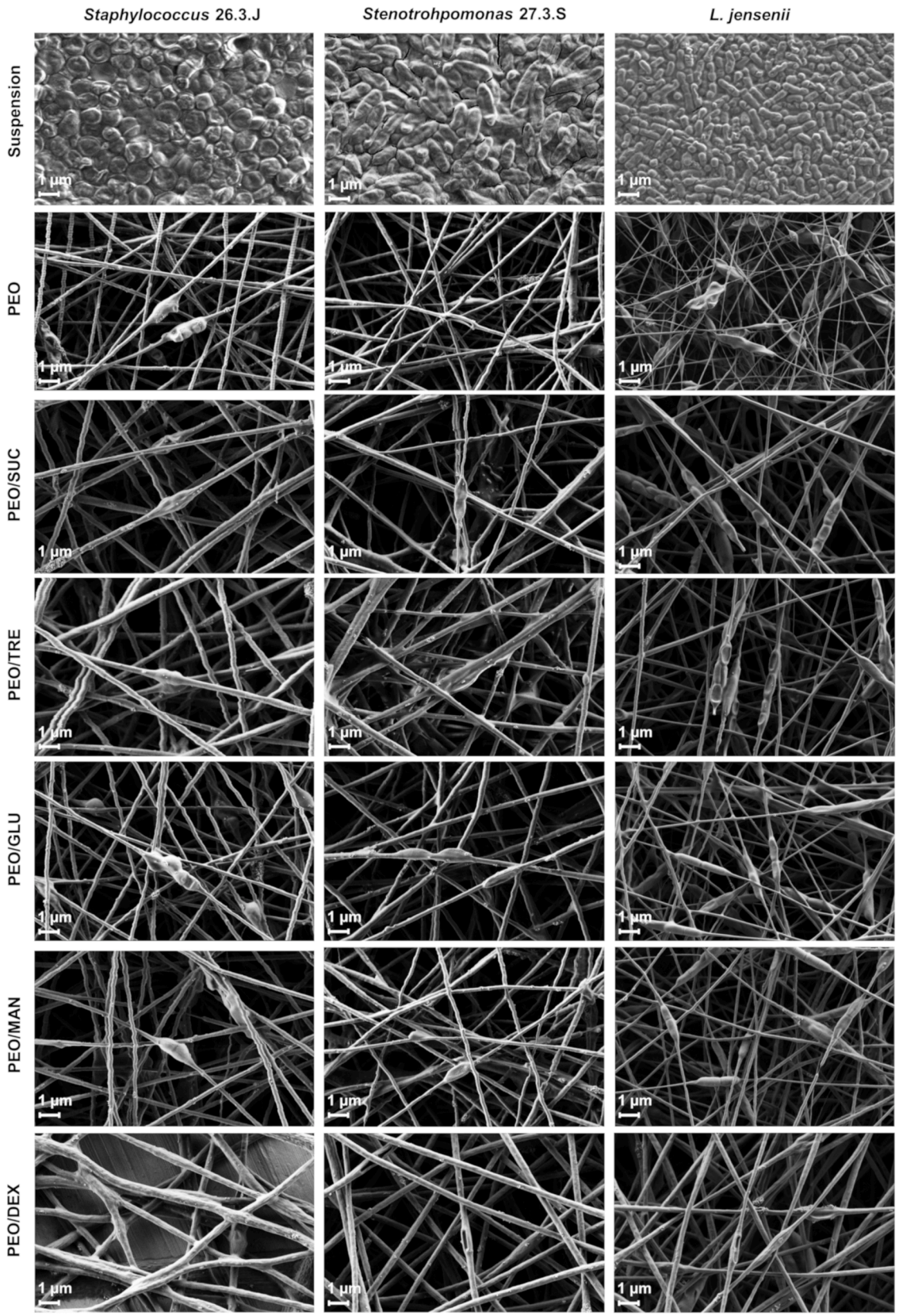



**Fig. 2.** SEM images of individual air-dried bacteria and bacteria incorporated into different nanofiber formulations, with polyethylene oxide (PEO) as the carrier polymer, alone or supplemented with sucrose (SUC), trehalose (TRE), glucose (GLU), mannitol (MAN) or dextran (DEX).

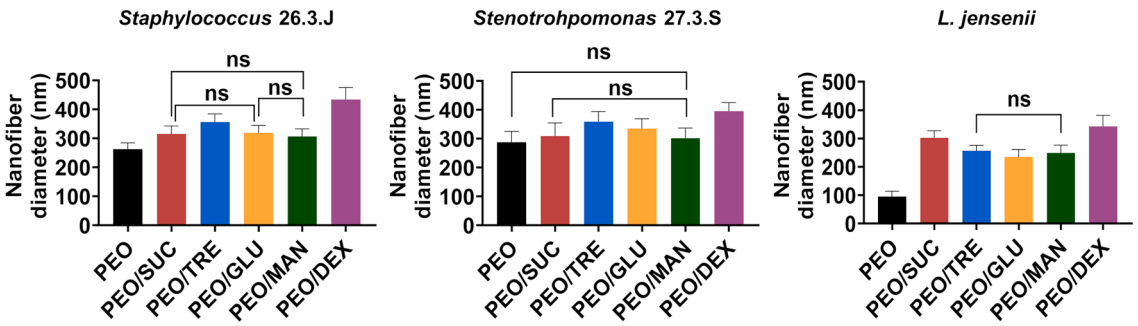



**Fig. 3.** The average diameter of the nanofiber formulations loaded with specific bacteria with PEO as the carrier polymer, alone or supplemented with sucrose (SUC), trehalose (TRE), glucose (GLU), mannitol (MAN) and dextran (DEX). Unless specified as statistically non-significant (ns) at the $p < 0.05$ level (ANOVA, post-hoc Tukey), mean diameters of nanofibers loaded with the same bacterial species but with different excipient compositions differed ($p < 0.05$).

PEO without stabilizers led to the formation of the thinnest nanofibers, with an average diameter of 96 ± 19 nm.

### 3.4. Effects of stabilizers on preservation of bacterial viability during electrospinning

Electrospinning of the three species *Staphylococcus* 26.3.J, *Stenotrophomonas* 27.3.S and *L. jensenii* resulted in significant viability reduction ($p < 0.05$) in all formulations, except for *L. jensenii* in PEO supplemented with sucrose and glucose (Fig. S4). This can be attributed to dehydration along with the osmotic stresses accompanying the latter (Merivaara et al., 2021), as well as other electrospinning-associated stresses, such as mechanical stress (Salalha et al., 2006) or high voltage (Szumski et al., 2011). Despite this, all species partially survived the electrospinning process and maintained viability in all nanofiber formulations following dissolution. The survival varied among the bacterial species ($p < 0.05$), with *Staphylococcus* 26.3.J showing highest survivability (average log CFU/g reduction 0.9 in PEO-only formulation), followed by *L. jensenii* (average log CFU/g reduction 4.3 in PEO-only formulation) and *Stenotrophomonas* 27.3.S (average log CFU/g reduction 5.4 in PEO-only formulation) (Fig. 4). This alone demonstrates the inherent differences in the sensitivity of the three studied bacterial species to the electrospinning process. The lowest survival of the Gram-negative *Stenotrophomonas* 27.3.S is in line with the previously reported higher sensitivities to the stresses during electrospinning compared to Gram-positive bacteria, represented here by *L. jensenii* and the *Staphylococcus* 26.3.J. The lower sensitivity of Gram-positive bacteria to electrospinning (as well as other drying methods) has been attributed to the thicker peptidoglycan layer in the cell wall offering better support during the dehydration of the cell and the accompanying structural changes (Gensheimer et al., 2007; Miyamoto-Shinohara et al., 2008; Salalha et al., 2006).

The protective effects of the stabilizers on the individual species during electrospinning were evaluated by comparison of the log CFU/g reduction occurring during electrospinning of formulations with different stabilizers with the control PEO formulation (Fig. 4). Dextran improved the survival of all three tested bacterial species ($p < 0.05$). Meanwhile, *L. jensenii* was the only tested bacterial species stabilized by sucrose, trehalose and glucose ($p < 0.05$). Stabilizers likely acted as lyoprotectants and thus played a crucial role in improving the viability during electrospinning. Lyoprotectants interact with the membrane bacterial proteins, phospholipid bilayers and other structurally important (macro)molecules of the bacterial cell during the removal of water (water replacement hypothesis) and entrap the cell in an immobile (glassy) matrix (Tripathi and Giri, 2014; Zupančič et al., 2018). The other tested excipients did not improve survival ($p > 0.05$) or, in rare cases, even exhibited negative effects on survival during electrospinning ($p < 0.05$). For example, sucrose exhibited negative effects on the survival of *Staphylococcus* 26.3.J, increasing the log CFU/g reduction during electrospinning ($p < 0.05$). Interestingly, this was the opposite to the protective effects of sucrose on *L. jensenii*. Meanwhile, mannitol negatively affected the survival of *Stenotrophomonas* 27.3.S.

Our data show that different excipients exhibit different stabilizing activity on bacterial species during electrospinning. Thus, these results imply the existence of species-specific mechanisms in the stabilizing activity of stabilizers, affecting bacterial viability during electrospinning.

### 3.5. Effects of stabilizers on preservation of bacterial viability during long-term storage

Nanofibers loaded with probiotic bacteria were stored under different conditions to evaluate the influence of temperature on their viability. At 4 °C, the tested bacterial species exhibited differing stability in terms of their survival which depended on the species and nanofiber formulation (Fig. 5 and Fig. S5). Overall, the Gram-positive *L. jensenii* and *Staphylococcus* 26.3.J showed similar survival during storage, while the most sensitive species was again the Gram-negative *Stenotrophomonas* 27.3.S, which exhibited complete loss of viability after only a week of storage in formulations with glucose and mannitol.

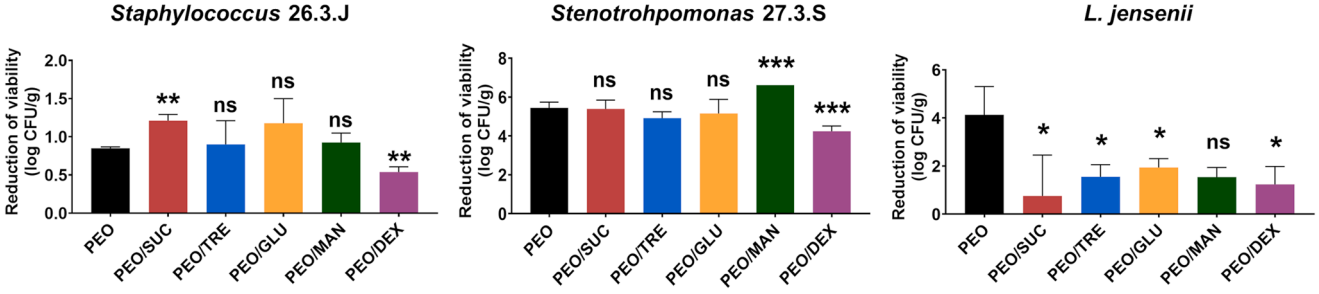



**Fig. 4.** Reduction of bacterial viability during electrospinning in different formulations. PEO, polyethylene oxide; SUC, sucrose; TRE, trehalose; GLU, glucose; MAN, mannitol; DEX, dextran. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (relative to PEO without stabilizers).

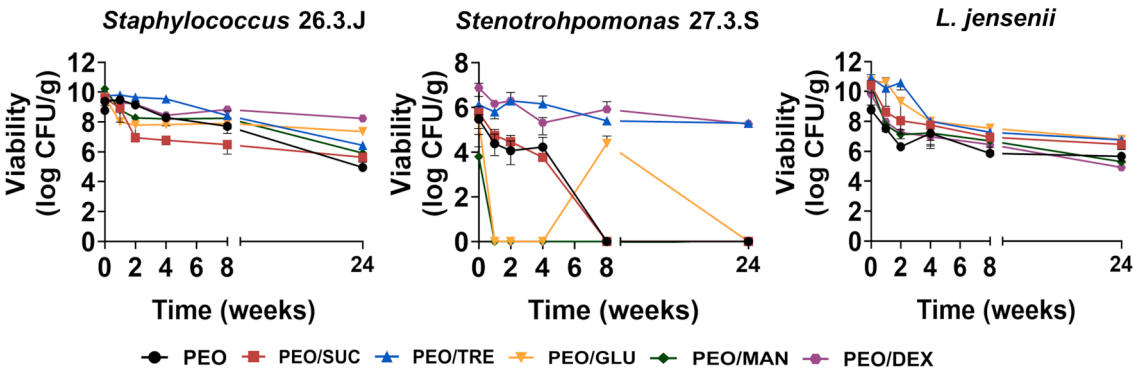



**Fig. 5.** Long-term viability of *Staphylococcus* 26.3.J, *Stenotrophomonas* 27.3.S and *L. jensenii* incorporated into different nanofiber formulations at different time points, stored at 4 °C and relative humidity of 8–10 %. PEO, polyethylene oxide; SUC, sucrose; TRE, trehalose; GLU, glucose; MAN, mannitol; DEX, dextran. The appearance of detectable viability of *Stenotrophomonas* 27.3.S at 8 weeks is attributed to an experimental artefact.

Reduction of bacterial viability in each formulation (in log CFU/g, calculated from the viabilities at the 0 and 24 weeks time points) was determined in order to analyze the effects of stabilizer type on long-term survival. Formulation composition was found to affect the total reduction of bacterial viability during 24 weeks of refrigerated storage in the case of *Stenotrophomonas* 27.3.S and *Staphylococcus* 26.3.J ($p < 0.05$) while it did not affect *L. jensenii* ($p > 0.05$) (Fig. S5). More specifically, dextran, glucose and trehalose emerged as stabilizers of *Staphylococcus* 26.3.J and resulted in 1.3, 2.2 and 3.3 log CFU/g reduction of viability, respectively. Meanwhile, mannitol and sucrose did not affect survival during 24 weeks of storage. For *Stenotrophomonas* 27.3.S, the effective stabilizers during storage were trehalose and dextran, resulting in 0.8 and 1.6 log CFU/g reduction of viability respectively. All other formulations resulted in complete loss of viability of *Stenotrophomonas* 27.3.S even before the 6-month mark. Interestingly, mannitol and glucose decreased the stability of *Stenotrophomonas* 27.3.S, as its viability was completely lost already after a week of refrigerated storage, while in the PEO and PEO/SUC formulation, complete loss of viability was only observed after 8 weeks of refrigerated storage.

To further test the protective effects of the stabilizers, we stored nanofiber formulations at room temperature (23 ± 2 °C) for 2 weeks and at −20 °C for 24 weeks. Bacterial survival notably differed when stored under these conditions (Fig. 6). In general, the survival at room temperature was significantly lower compared to −20 °C, particularly for *L. jensenii*, which did not survive in any formulation. These results are in line with the literature where probiotics stored at lower temperatures are reported to maintain a higher level of viability compared to probiotics stored at higher temperatures (Bruno and Shah, 2003). During 2 weeks of storage at room temperature, the most stable species was *Staphylococcus* 26.3.J, followed by *Stenotrophomonas* 27.3.S and *L. jensenii*. The stabilizers showed negative effects on the survival of *Staphylococcus* 26.3.J when stored at room temperature, since PEO without stabilizers showed the best survival for *Staphylococcus* 26.3.J (0.7 log CFU/g reduction), followed by nanofibers supplemented with dextran (1.9 log CFU/g reduction), mannitol (2.9 log CFU/g reduction), trehalose (3.2 log CFU/g reduction), glucose (4.4 log CFU/g reduction) and sucrose (4.7 log CFU/g reduction). On the other hand, *Stenotrophomonas* 27.3.S was the most stable in PEO supplemented with trehalose (1.2 log CFU/g reduction) which is in line with its superior survival in PEO/TRE nanofibers during 24 weeks of refrigerated storage. This was followed by PEO without stabilizers (1.5 log CFU/g reduction) and PEO supplemented with dextran (2.4 log CFU/g reduction), while it did not survive in the other formulations.

The survival of the three species after 24 weeks of storage at −20 °C was improved compared to room temperature and to refrigerated conditions. The viability of *L. jensenii* was the highest, among the three species, with maximum viability loss of only 0.6 log CFU/g. Contrary to the previous results, dextran emerged as the most effective stabilizer for *L. jensenii* when stored at −20 °C, resulting in a 0.2 higher log CFU/g after 24 weeks of storage. The maximum survival reduction of *Staphylococcus* 26.3.J was 0.6 log CFU/g with PEO without stabilizers proving to be the most efficacious, resulting in a 0.6 higher log CFU/g after 24 weeks of storage. *Stenotrophomonas* 27.3.S exhibited the lowest survival, with only three formulations showing viable bacteria after 24 weeks of storage. Specifically, PEO alone or PEO supplemented with glucose and mannitol failed to retain viable bacteria after storage. Optimal survival was observed in PEO supplemented with trehalose, where the survival reduction reached 0.5 log CFU/g.

Based on bacterial viability testing during nanofiber storage, long-term stabilization efficacy of the tested stabilizers was revealed to be species-dependent, same as the species-dependent stabilization during electrospinning. Additionally, the stabilizing efficacy of the stabilizers differed between electrospinning and storage, as some stabilizers conferred protection to a certain bacterial species during the electrospinning process, while others were more effective during storage (Figs. 4, 5 and Fig. S5). We postulated that this might be a result of instabilities of the stabilizers themselves. Moreover, short-term storage at room temperature and higher RH revealed complete viability loss of *L. jensenii* in all formulations and of strain *Stenotrophomonas* 27.3.S in nanofibers with sucrose, mannitol and glucose. At such storage conditions, solid-state changes of stabilizers might occur at faster rates than at refrigerated conditions used to evaluate long-term stability which indicated that, especially for those stabilizers, there is a need for further solid-state characterisation by DSC analysis (discussed in section 3.6).

### 3.6. Thermal solid-state analysis of nanofiber formulations

DSC analysis was performed for solid-state characterisation of the bacteria-encapsulating matrix in nanofibers as well as to uncover potential interactions between the excipients and bacteria. Thermograms of individual excipient powders, placebo nanofibers and bacteria-loaded nanofibers with *Staphylococcus* 26.3.J are shown in Fig. 7 and in Figs. S6 and S7 for the other two bacterial species tested. For analysis of the proportions of the excipient crystalline fraction in a specific sample, we analyzed endotherms which we attributed to the following thermal events: melting of the crystalline fraction of PEO (a semi-crystalline polymer) at approximately 70 °C (Wen et al., 2021), dehydration of trehalose dihydrate to the α-anhydrate form at 98 °C, followed by α-anhydrate melting at 127 °C and β-anhydrate melting at 192 °C (Amis et al., 2020), melting of sucrose at approximately 190 °C (Jawad et al., 2018), and melting of mannitol at approximately 170 °C (Gil et al., 2013), whereas dextran was amorphous without any melting-related thermal events. Melting of glucose was attributed to the endothermic events at approximately 150 °C, however, the $T_m$ of glucose decrease to 145 °C in PEO/GLU physical mixtures and even lower to approximately 125 °C in placebo nanofibers, which we attribute to formation of

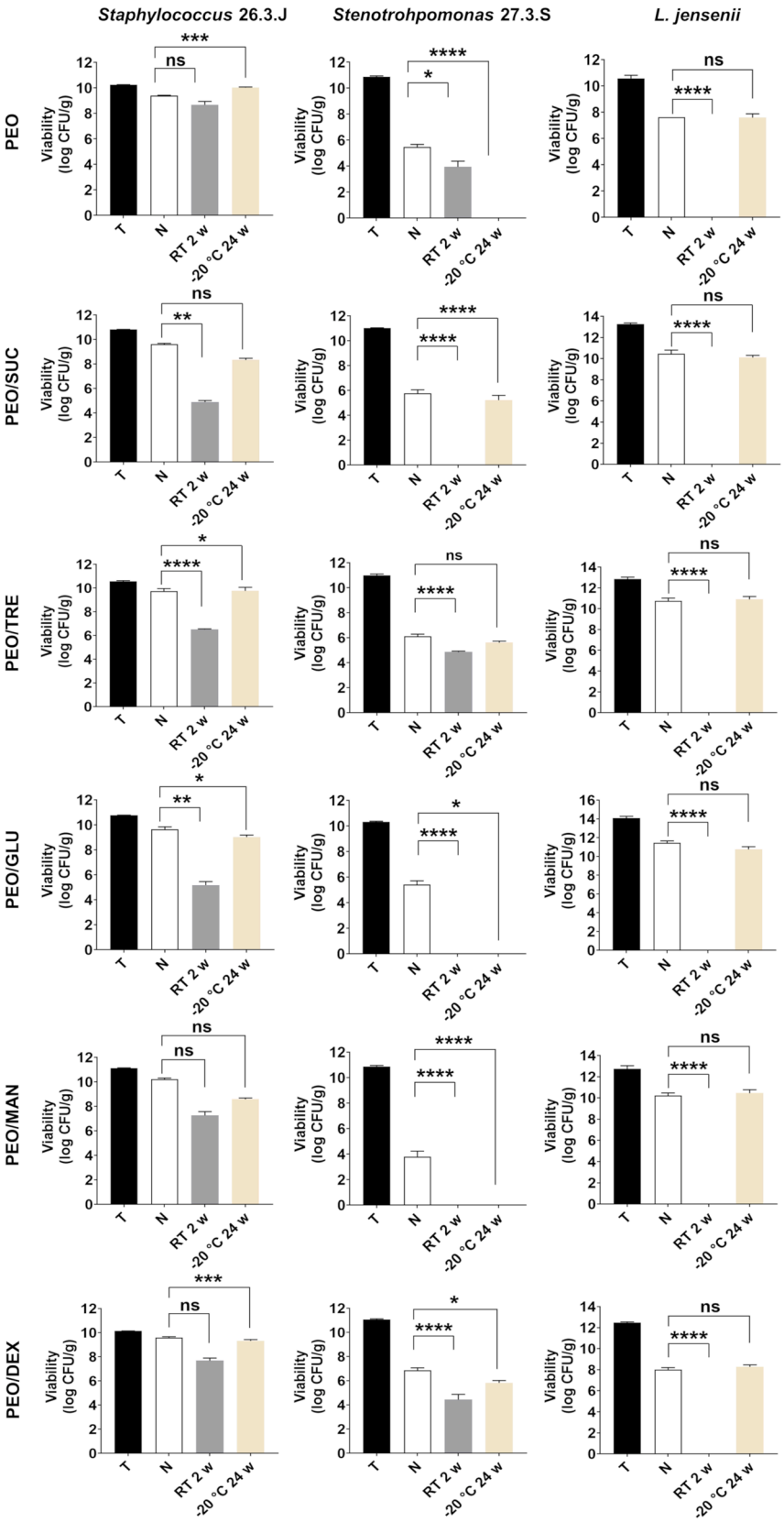



**Fig. 6.** Viability of *Staphylococcus* 26.3.J, *Stenotrophomonas* 27.3.S and *L. jensenii* in nanofibers, as theoretically determined from the polymer dispersions (T), nanofibers immediately after electrospinning (N), after 2-week storage at room temperature (RT 2 w) and after 24-week storage at −20 °C (−20 °C 24 w). PEO, polyethylene oxide; SUC, sucrose; TRE, trehalose; GLU, glucose; MAN, mannitol; DEX, dextran. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (two-sample *t* test), relative to nanofibers immediately after electrospinning.

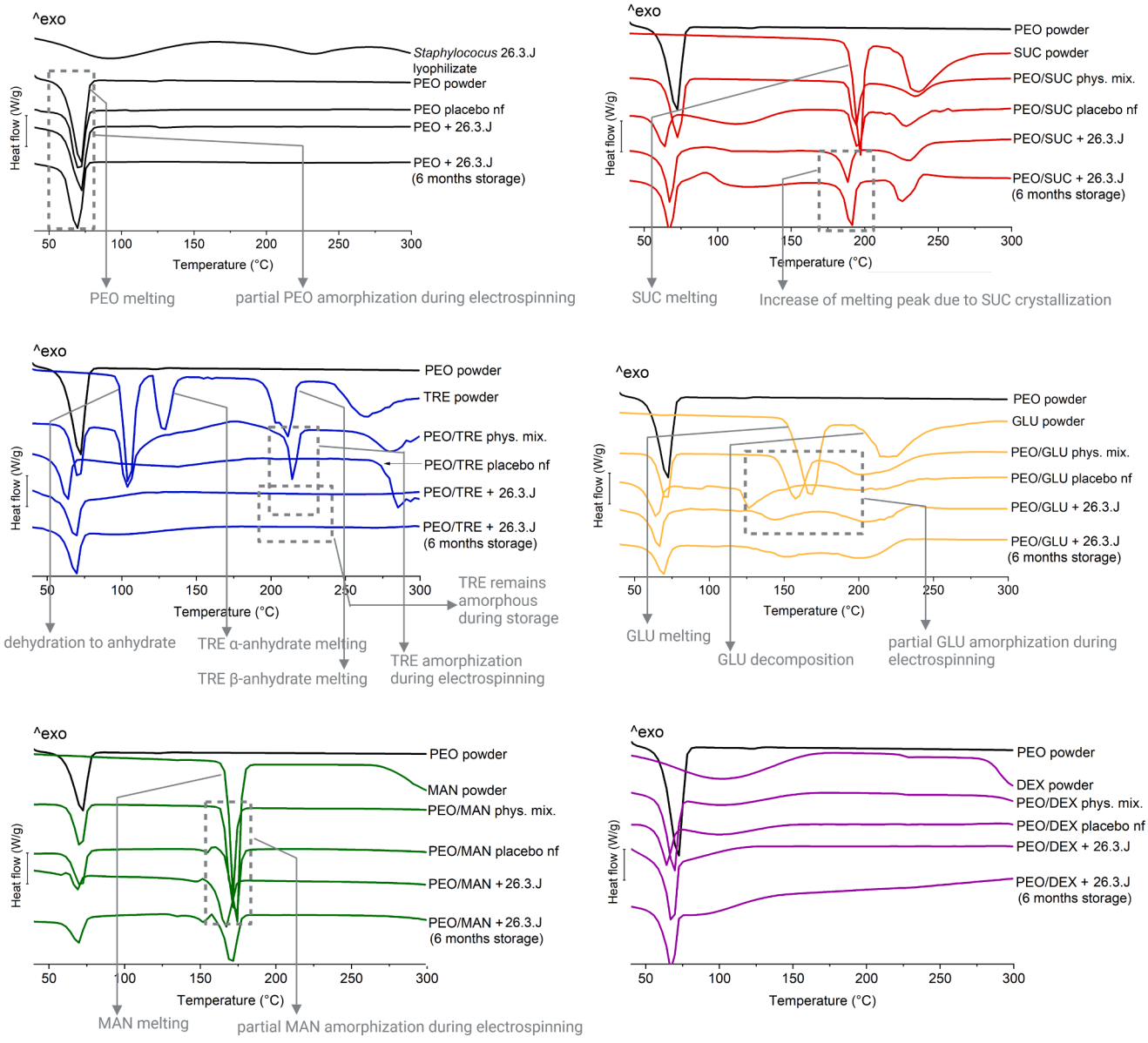



**Fig. 7.** Thermograms obtained for DSC analysis of nanofibers with *Staphylococcus* 26.3.J. Each smaller figure contains stacked thermograms of the following samples (in order from top to bottom): bacterial lyophilizate (only for the upper left figure showing PEO-only formulations without stabilizers), pure PEO powder, pure stabilizer powder, PEO/stabilizer powder physical mixtures, freshly prepared PEO/stabilizer placebo nanofibers, freshly prepared PEO/stabilizer + *Staphylococcus* 26.3.J nanofibers, and PEO/stabilizer + Stenotrophomonas 26.3.J nanofibers after 24 weeks of refrigerated storage. PEO, polyethylene oxide; SUC, sucrose; TRE, trehalose; GLU, glucose; MAN, mannitol; DEX, dextran.

interactions between glucose and PEO. It is possible, that the decrease of glucose $T_m$ is a result of the existence of its interactions with PEO (Schultheiss and Newman, 2009). This decrease of $T_m$ was also noticeable in nanofibers with incorporated bacteria, albeit less prominent, which is likely the result of the interference of the bacterial cells or residues of solutes from the growth media in the formation of PEO-GLU interactions.

The degree of amorphization of the studied stabilizers in placebo nanofibers during electrospinning varied. Partial amorphization of PEO during electrospinning is a known phenomenon (Song et al., 2018) and was also confirmed in this study (Table S5). Total or partial amorphization of certain stabilizers can be expected due to rapid solvent evaporation which causes a swift increase in viscosity of the dispersion jet, reduced mobility of both small molecules (i.e., sugars) and polymers (i.e., PEO) and their quick solidification (Yu et al., 2018). In our study, trehalose and dextran were examples of fully amorphous stabilizers in placebo nanofibers, evidenced by thermograms absent of any stabilizer-associated melting endotherms (Fig. 7, Figs. S6 and S7). Dextran as a polymeric stabilizer was already amorphous in its supplied powder form, whereas trehalose was fully amorphized during electrospinning. Glucose, mannitol and sucrose are crystallisation-prone stabilizers (Crowe et al., 1998; Leinen and Labuza, 2006). Thus, only partial amorphization was observed for glucose and mannitol in placebo nanofibers, while sucrose recrystallized fully.

Incorporation of bacteria in nanofibers affected the degree of amorphization of the tested stabilizers during electrospinning with the exception of trehalose and dextran which remained fully amorphous in both placebo and bacteria-loaded nanofibers. The presence of all three bacterial species in nanofibers reduced the degree of PEO amorphization during electrospinning compared to placebo nanofibers ($p < 0.05$) (Table S5), whereas their incorporation increased the amorphization of glucose in the nanofibers (Fig. 7, Figs. S6 and S7). The degree of partial

amorphization of sucrose and mannitol was also affected by the presence of bacteria, evident by the difference in the proportion of the stabilizers' crystalline fraction in bacteria-loaded and placebo nanofibers ($p < 0.05$). Moreover, this effect was also species-specific. For example, loading of *L. jensenii* and *Staphylococcus* 26.3.J into nanofibers resulted in more pronounced amorphization of sucrose and mannitol ($p < 0.05$), whereas this effect was not observed in the case of *Stenotrophomonas* 27.3.S which even inhibited amorphization of mannitol ($p < 0.05$), resulting in its complete recrystallisation during electrospinning (Table 1). These species-dependent effects of bacteria on amorphization of crystallisation-prone low molecular weight stabilizers during electrospinning could be either a result of the presence of ions, other lower molecular weight solutes or exopolysaccharides released from the bacterial cells, remaining from incomplete removal of the culturing medium (Rajoka et al., 2020; Škrlec et al., 2019) or formation of specific species-dependent interactions between the excipients and the bacterial cells (Škrlec et al., 2019).

There was no trend indicating that a significant effect of a specific bacterial species on amorphization results in better preservation of bacterial viability during electrospinning. For example, *Staphylococcus* 26.3.J exhibited a lower survival in PEO/SUC nanofibers after electrospinning compared to PEO/MAN nanofibers despite having a higher effect on amorphization of sucrose than mannitol. Therefore, such solid-state analysis alone cannot predict relevant stabilizing efficacy of an excipient based on the formation of specific interactions between the bacteria and the excipients during electrospinning.

Bacteria-loaded nanofiber mats were re-analyzed after 24 weeks of refrigerated storage. The amorphous states of trehalose and dextran in nanofibers were preserved (Fig. 7, Figs. S6 and S7), pointing to the known high vitrifying potential of trehalose due to the stability of its glassy state during storage (Cao et al., 2022; Crowe et al., 1998). Thus, trehalose seems to be the most promising additive choice for long-term storage of probiotic-loaded nanofibers compared to the other low molecular weight stabilizers tested in this study. These results are also in line with the well-performing stabilizing activity of trehalose on bacterial viability as seen by the highest viability preservation of *Stenotrophomonas* 27.3.S and *L. jensenii* during storage. Dextran was also a top-performing stabilizer of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S during storage which could be a result of its stable solid state. Interestingly, it did not exhibit the same efficacy for *L. jensenii*, which could be a result of species-specific interactions of the bacterial cells with the glassy matrix of the nanofibers.

Nanofibers with semi-crystalline stabilizers (sucrose, mannitol and glucose) varied in the stability of the stabilizers' amorphous fractions. The stability of the amorphous fraction of sucrose and mannitol depended on the loaded bacterial species. We also observed a trend of improved long-term bacterial preservation in formulations where the amorphous fraction was more stable. For example, PEO/SUC + *L. jensenii*, exhibited a stable amorphous sucrose fraction and was also among the top-performing formulations for preservation of this bacterial species. Meanwhile, crystallinity of sucrose increased during storage of PEO/SUC + *Staphylococcus* 26.3.J nanofibers and this formulation also performed poorly for long-term stabilization of this bacterial species compared to those with other stabilizers, particularly those characterized by stable amorphous states (i.e., trehalose and dextran). Crystallinity was also increased during storage for mannitol in formulations PEO/MAN + *L. jensenii* and PEO/MAN + *Staphylococcus* 26.3.J nanofibers (Fig. 7) and accordingly, these formulations also performed poorly in terms of bacterial preservation compared to those with stable amorphous trehalose and dextran. As previously mentioned, the extent of glucose crystallisation during storage could not be quantified due to the hindered determination of its $\Delta H_m$, however, glucose crystallisation during storage was observed based on the changes of nanofiber morphology which exhibited crystal growth observed by SEM (Fig. S11).

Interestingly, despite this, glucose emerged as an effective stabilizer of both strain *Staphylococcus* 26.3.J and *L. jensenii* during storage and was only ineffective for stabilization of strain *Stenotrophomonas* 27.3.S, indicating that the extend of its crystallisation during storage might not have been as significant or did not cause damage to the bacterial cells of the former two species. Based on these results, we propose that solid-state stability of semi-crystalline stabilizers is related to their long-term stabilizing efficacy, since crystallisation during storage can decrease bacterial viability due to potential devitrification or crystallisation-related damage to bacterial cells. Crystallisation of the amorphous fraction changes the physical structure of the matrix surrounding the bacteria, thus lowering its ability for thermokinetic stabilization by immobilization of the bacterial cells. Additionally, it is widely accepted that thermodynamic stabilization of proteins and membranes of the bacterial cell by the water-replacement theory is possible due to the amorphous behavior of the stabilizer which enables hydrogen bonding in place of water molecules. This means that the decrease of the amorphous fraction of the stabilizer might diminish the stabilizing hydrogen bonds that were formed during drying between the stabilizer and the bacterial cell (Cao et al., 2022; Crowe et al., 1998; Grilc et al., 2025). Moreover, there seems to be a species-dependent effect of bacterial loading in nanofibers on the kinetics of solid-state changes of crystallisation-prone stabilizers during storage which in

**Table 1**
Effects of bacterial loading into nanofibers on amorphization of crystallisation-prone stabilizers during electrospinning. A – $\Delta H_m$ of excipient is normalized to its mass proportion in sample to enable comparison. B, C – % of amorphous fraction in nanofiber samples is based on the crystalline fraction determined by $\Delta H_m$ normalized to the mass proportion in nanofiber sample. Effects of specific bacterial species on stabilizer amorphization during electrospinning are reported for all statistically significant changes ($p < 0.05$). n.s. – not significant at $p = 0.05$ level. PEO, polyethylene oxide; SUC, sucrose; TRE, trehalose; GLU, glucose; MAN, mannitol; DEX, dextran.

| | $\Delta H_m$ of stabilizer (J/g)[A] | Fraction of amorphized stabilizer (%)[B] | Effect of bacteria of stabilizer amorphization[C] |
|---|---|---|---|
| SUC powder | −124.3 ± 10.4 | / | / |
| PEO/SUC placebo | −121.1 ± 3.9 | n.s. | / |
| PEO/SUC + *Staphylococcus* 26.3.J | −78.7 ± 7.1 | 36.7 ± 5.7 | 35 % increase |
| PEO/SUC + *Stenotrophomonas* 27.3.S | −116.4 ± 3.9 | n.s. | n.s. |
| PEO/SUC + *L. jensenii* | −102.8 ± 5.5 | 17.3 ± 4.4 | 15 % increase |
| MAN powder | −287.7 ± 9.9 | / | / |
| PEO/MAN placebo | −255.0 ± 2.8 | 11.4 ± 1.0 | / |
| PEO/MAN + *Staphylococcus* 26.3.J | −236.0 ± 4.7 | 18.0 ± 1.6 | 7 % increase |
| PEO/MAN + *Stenhotrophomonas* 27.3.S | −290.8 ± 5.7 | n.s. | 14 % decrease |
| PEO/MAN + *L. jensenii* | −207.8 ± 7.5 | 27.8 ± 2.6 | 19 % increase |
| TRE powder | −108.7 ± 5.2 | / | / |
| PEO/TRE (placebo, loaded) | no melting endotherms | 100 % (no melting endotherms) | fully amorphous in both loaded and placebo |
| DEX powder | no melting endotherms | / | / |
| PEO/DEX (placebo, loaded) | | remains fully amorphous (no melting endotherms) | fully amorphous in both loaded and placebo |
| GLU powder | not accurately quantifiable (decomposition overlap with melting) | | |
| PEO/GLU (placebo, loaded) | | | |

turn affect the rates of bacterial death.

Based on the DSC analysis and viability testing of bacteria, the stabilizing efficacy of stabilizers with high crystallizing tendency in nanofibers could prove questionable for long-term storage, as seen particularly well in the case of PEO/SUC and PEO/MAN nanofibers. Moreover, the proportion of unstable amorphous fractions of these semi-crystalline stabilizers and kinetics of their crystallisation are seemingly bacterial species-specific. Thus, we show here the importance of basing stabilizer selection on (i) solid-state characterisation and (ii) studies of bacterial preservation during long-term storage. Both these aspects of formulation design for probiotic-loaded nanofibers are often omitted and should be evaluated for each individual combination of bacterial strain and stabilizer. Additionally, it must be noted that solid-state characterisation in this study was performed on samples stored under refrigerated conditions. Crystallisation of amorphous fractions of semi-solid crystallisation-prone stabilizers during storage may be even faster and more pronounced when stored at higher temperatures (i.e., room temperature) or high relative humidity (Leinen and Labuza, 2006). Thus, we suggest that future studies include solid-state characterisation of formulations stored at relevant conditions expected for practical use of the final product.

### 3.7. Interactions between bacteria and nanofibers

FTIR analysis was performed to gain further insight into the stabilizing mechanism of the potential stabilizers studied. Shifts of FTIR spectral band positions, intensity and width occurring during drying can provide insight into structural changes induced by dehydration or other stresses exerted on the bacterial cell during processing (i.e., osmotic stress, mechanical stress) (Al-Azzam et al., 2002; Girardeau et al., 2022). Based on the water-replacement theory, lyoprotective activity of stabilizers originates from thermodynamic stabilization of the bacterial cell components (i.e., structurally important (macro)molecules). This theory suggests that, during removal of water, the stabilizers take on the role of hydrogen bonding with bacterial cell components, such as proteins and phospholipids. Stabilizing excipients can thus mitigate dehydration-induced protein unfolding and phospholipid bilayer phase transitions, thereby supporting the maintenance of the bacterial cell's structural and functional integrity (Crowe et al., 1998; Merivaara et al., 2021). Particularly amide bands (amide I, amide II and amide A) can serve as relevant indicators of protein conformation and shifts of their positions are also associated with dehydration-related conformational changes (Al-Azzam et al., 2002; Liltorp and Maréchal, 2005).

Both lyophilized and hydrated bacteria exhibited comparable FTIR spectra with typical bands seen in Fig. 8 and presented in Table S6 along with their literature-based assignation (Garip et al., 2007). Comparison of FTIR spectra of hydrated bacteria and lyophilizates without added excipients revealed a shift of amide I band from 1651 to 1638 $cm^{-1}$, respectively, in *Staphylococcus* 26.3.J, and from 1651 to 1649 $cm^{-1}$ in *Stenotrophomonas* 27.3.S. Due to the broad amide I band of the hydrated bacteria, the shift between the hydrated and lyophilized *L. jensenii* could not be established (Fig. 8). We attribute the shift of amide I band to lower wavenumbers to dehydration-related protein conformational changes in these two strains. Thus, if position of the amide I band in bacteria in nanofibers remains the same as in the hydrated form or if the shift of amide I band is smaller than in the case of lyophilization, we can assume that less protein conformation-related damage occurred during electrospinning than during lyophilization. This can be attributed to protective action of excipients (PEO and/or added stabilizers) and therefore, amide I position was examined for all electrospun samples. Amide II band position of the hydrated bacteria was not examined due to the fact that its broad shape overlapped with the tailing of the amide I band, hindering the exact determination of its position.

FTIR analysis revealed stabilization of bacterial protein conformation in *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S by PEO and *L. jensenii* by mannitol and dextran. Stabilization of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S by PEO was implied by the amide I band peak position in PEO + *Staphylococcus* 26.3.J and PEO + *Stenotrophomonas* 27.3.S nanofibers remaining the same as in the hydrated form (at 1651 $cm^{-1}$), indicating that the bacterial protein conformation was better preserved than in lyophilizates. Additionally, the position of the amide I band was also preserved in PEO/MAN + *Stenotrophomonas* 27.3.S nanofibers; however, we cannot deduce whether this was a result of stabilization by PEO alone or by both PEO and MAN. The latter, however, is unlikely due to the fact that a large fraction of mannitol crystallized during electrospinning and was also not shown to act as a stabilizer of strain *Stenotrophomonas* 27.3.S based on viability testing. FTIR analysis did not indicate *L. jensenii* protein conformation stabilization by nanofiber excipients based on amide I position.

We also analyzed the position of amide II band as it was prominent in the spectra of *L. jensenii*-loaded nanofibers as opposed to it not being visible in the spectra of *Staphylococcus* 26.3.J and *Stenotrophomonas* 27.3.S-loaded nanofibers. Protein conformation in *L. jensenii* was stabilized by dextran and mannitol, evidenced by the shift of amide II band from 1538 to 1549 $cm^{-1}$ in PEO/DEX + *L. jensenii* nanofibers and from 1538 to 1544 $cm^{-1}$ in PEO/MAN + *L. jensenii* nanofibers compared to the lyophilized bacteria. As such a shift of amide II band was not observed in PEO nanofibers without stabilizers, the stabilizing action on bacterial protein conformation of *L. jensenii* can be attributed to mannitol and dextran. Such stabilizing properties are also in line with the improved survival of *L. jensenii* during electrospinning as seen from the lower log-reduction of viability in freshly electrospun PEO/MAN and PEO/DEX nanofibers compared to PEO nanofibers.

Unfortunately, stabilization of bacterial cell structure via interactions with other stabilizers (trehalose, sucrose, glucose) could not be elucidated due to the overlap of most relevant bands of the bacteria with those typical of the excipients observed in spectra of physical mixtures and placebo nanofibers. Moreover, due to the relatively low mass fraction of the bacteria in some of the dry nanofiber mats, some characteristic peaks from spectra of lyophilized bacteria could no longer be detected in the nanofiber samples (e.g., amide II bands of *Stenotrophomonas* 27.3.S and *Staphylococcus* 26.3.J).

Comparison of FTIR spectra of bacteria-loaded nanofibers after electrospinning and after 24 weeks of storage failed to reveal any shifts of relevant amide bands that would indicate a change, e.g. reversion back to band positions associated with dry bacteria not stabilized by the potential stabilizers (Figs. S8-S10). It is unclear whether the lack of such spectral changes after 24 weeks of storage arises from the difficult interpretation of stabilizing mechanisms due to spectral overlaps discussed above or due to the amide band positions of bacteria remaining the same despite dramatic reductions of their viability. Thus, a deeper understanding is needed and a more detailed FTIR analysis by inclusion of different proportions of stabilizers could aid in the elucidation of their stabilizing mechanisms and the comparison of those across formulations with different bacterial strains.

## 4. Conclusion

This study highlights the species-dependent efficacy of carbohydrate excipients as stabilizers for probiotic bacteria in nanofibers during electrospinning and storage. We confirm previous findings of significant bacterial species-dependent sensitivity to the electrospinning process. Additionally, we show that no universal stabilizer exists; instead, stabilizer efficacy for improved bacterial survival varies by bacterial species and the stage of bringing the final product to the patient as the same stabilizers may not exhibit optimal stabilizing activity during both electrospinning as the production process and storage. Trehalose emerged as the most promising choice for initial formulation due to its stable glassy state; conversely, crystallisation-prone excipients like sucrose and mannitol require caution due to the potential storage-induced crystallisation of their amorphous fraction. These findings are critical for designing delivery systems and underscore the need for tailored

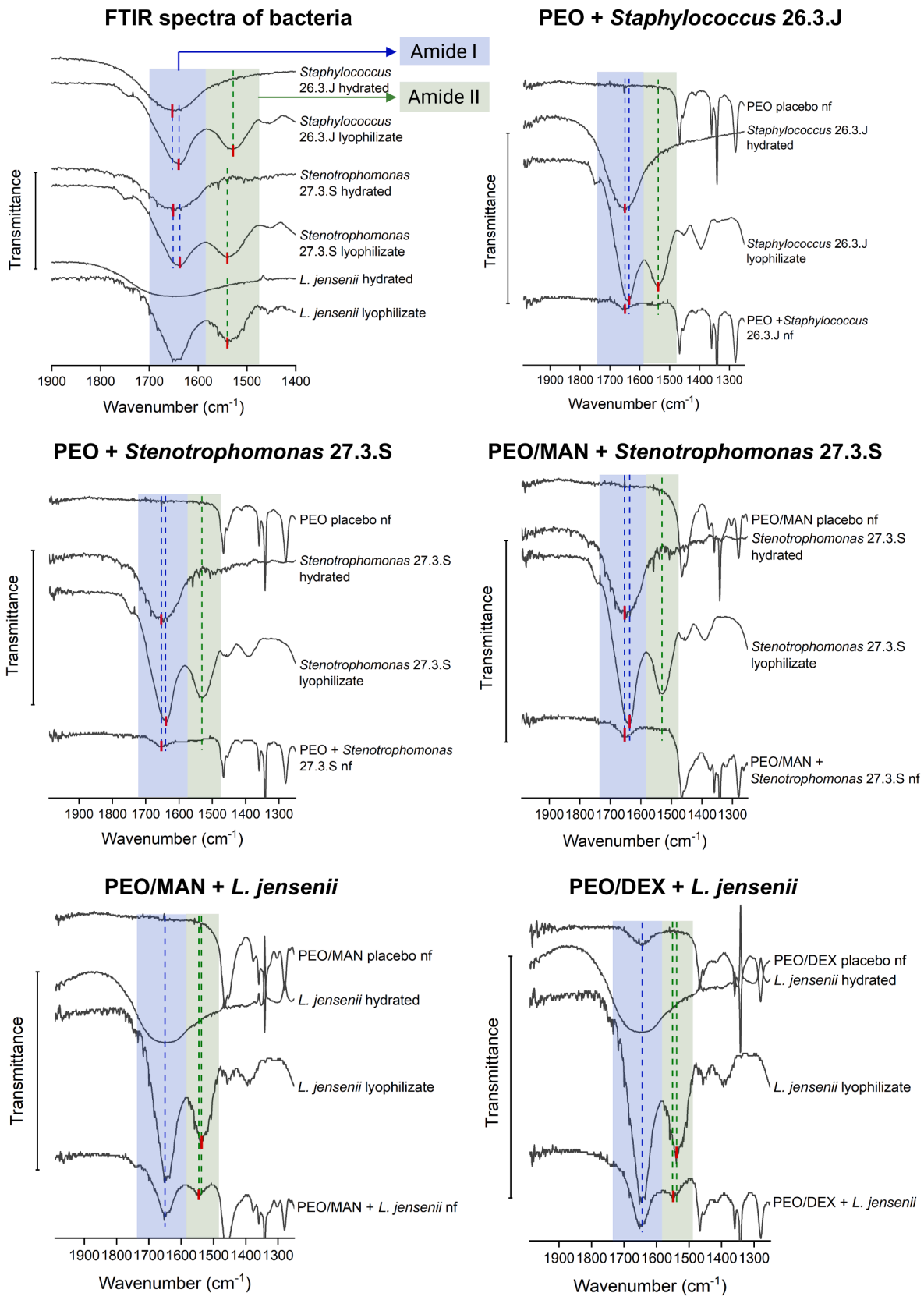



**Fig. 8.** FTIR spectra of samples where amide I (blue shading) and/or amide II (green shading) band shifts were observed, pointing to potential stabilization of bacterial protein structure by excipients. Spectra of hydrated bacteria, dehydrated (lyophilized) bacteria without excipients and nanofibers with the incorporated bacteria are shown. PEO, polyethylene oxide; MAN, mannitol; DEX, dextran. Red markings emphasize individual peak positions of amide bands and are overlaid on dotted lines, where two parallel dotted lines of the same colour indicate the two differing peak positions that underwent a shift and a singular dotted line indicates a peak position that did not undergo any shifts during electrospinning.

formulation design, particularly for the delivery of multi-species consortia, wherein the presence of various bacterial species requires targeted stabilizer selection for improved bacterial preservation.

## Funding sources

This work was supported by the Slovenian Research Agency [Research Core Funding No. P1-0189 and P4-0127 and Projects J7-4418 and J4-4556] and University of Ljubljana Start-up Research Programme (SN-ZRD/22-27/0510). Data availability statement.

## CRediT authorship contribution statement

**Nina Katarina Grilc:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Spase Stojanov:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Tomaž Rijavec:** Writing – review & editing, Resources, Funding acquisition, Formal analysis. **Aleš Lapanje:** Resources, Formal analysis. **Aleš Berlec:** Supervision, Writing – review & editing, Conceptualization. **Špela Zupančič:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## 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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2025.125327.

## Data availability

Data will be made available on request.

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