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IMPACT OF TREHALOSE-BASED CRYOPROTECTANT ON IMMOBILIZATION AND RELEASE OF *LACTOBACILLUS PLANTARUM* OBTAINED FOR WOUND DRESSING

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ABSTRACT

Lactobacillus plantarum has been increasingly studied and applied in the context of wound healing and wound dressings, thanks to its antimicrobial, anti-inflammatory, and biofilm-disrupting properties. In some approaches, lyophilized (freeze-dried) *L. plantarum* is applied as a powder or mixed with a carrier to facilitate topical delivery.

L. plantarum was diluted in 10 % trehalose and a mixture of 5 % trehalose and skim milk. The mixtures were aseptically added on top of activated carbon (AC) pads, frozen at −80 °C, and freeze-dried for 5 h. Immobilized probiotics on AC pads were incubated in normal saline at 37 °C for 24 h. The immobilization efficiency after lyophilization and the release of probiotics from the pad after 10 minutes, 3 h, and 24 h were determined.

The combination of trehalose and skim milk showed significantly better results, maintaining almost 80 % viability after 24 h, Trehalose alone preserved almost half as many probiotics compared to trehalose with milk. Since the controlled release of probiotics is essential for their antimicrobial effectiveness in wound dressings, it can be concluded that the AC pad containing probiotics protected with both trehalose and skim milk is a more effective option than the probiotics protected with trehalose alone.

Keywords: *Lactobacillus plantarum*, freeze-dry, immobilization, AC pad, milk.

INTRODUCTION

Lactobacillus plantarum has been increasingly studied and applied in the context of wound healing and wound dressings, thanks to its antimicrobial, anti-inflamatory, and biofilm disruption properties. *L. plantarum* produces organic acids (like lactic acid), hydrogen peroxide, and bacteriocins that inhibit common wound pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*. In addition, it helps reduce inflammation by modulating cytokine production, which can accelerate the healing process.

One of the key challenges in developing efficient probiotic systems for various applications, such as wound dressing, is ensuring effective cell preservation (Krunic, et al., 2016; Krunic, et al., 2019). The primary goal of preserving cells is to maintain their viability without altering their biochemical, morphological, physiological, or genetic characteristics (Malik, 1988). Although several effective cell preservation strategies exist, lyophilization (or freeze-drying) is widely regarded as the gold standard in the biopharmaceutical industry, particularly when working with sensitive microbial cells. During freeze-drying, bacterial cells are subjected to low temperatures and the removal of water. The freezing phase can cause cellular damage due to ice crystal formation and osmotic stress.

To minimize such damage, various protectants are often added to the drying medium. These substances help stabilize the cells in the absence of water and form a supportive matrix that maintains the structure of the sample throughout the process. In doing so, protectants help preserve

cell viability, ensure stability, and facilitate easy rehydration. A wide range of materials have been tested for their protective capabilities, including skim milk, polyols, polysaccharides, disaccharides, amino acids, proteins, minerals, organic acid salts, and vitamins (Champagne, et al., 1991; Hubalek, 2003). However, even with effective protectants, sensitive microorganisms like lactic acid bacteria (LAB) may still experience a loss of viability and stability (Krunic, & Osmokrovic, 2025).

To address these limitations, researchers have explored new methodologies. One promising and straightforward approach is the immobilization of microorganisms on various carriers using physical or chemical methods (Bouabidi, et al., 2019).

Immobilization is a well-established technique widely used across various industries. When applied to probiotics, immobilization offers numerous advantages. By embedding probiotics in a carrier, they are protected from external environmental stressors, enhancing their ability to survive manufacturing processes, storage conditions, and extended shelf life. This technique significantly improves the viability of probiotic bacteria under adverse conditions during both processing and storage (Krunic, et al., 2016). Moreover, immobilized probiotics are better equipped to withstand the harsh environment of the human gastrointestinal tract, including low pH, digestive enzymes, and bile salts (Krunic, et al., 2016; Krunic, et al., 2019). A fundamental requirement of any immobilization technology is maintaining the viability of bacterial cells, which plays a key role in selecting the appropriate method.

Another crucial factor for using immobilized material is the controlled release of it into the surrounding environment. Depending on the application, it may be necessary to release a large number of cells rapidly, or in some cases, a sustained and gradual release is more desirable. These release dynamics must be carefully considered when choosing both the immobilization technique and the carrier material (Krunic, et al., 2016).

In this study, we investigated the impact of trehalose alone and in combination with milk on the viability of probiotic cells during the immobilization process, as well as their effect on the subsequent release of cells from the carrier. Activated carbon (AC) pads were used as the immobilization carrier, while freeze-drying was an immobilization technique.

MATERIALS AND METHODS

Materials

AC pads were prepared in a circular shape by punching from activated carbon fabric (ACF, ConvaTec, USA). Other materials were trehalose dehydrate (Carl Roth, Germany), skim milk (Imlek, Serbia), NaCl (Sigma, USA), De Man, Rogosa and Sharpe (MRS) broth, and Agar (Torlak, Serbia). Other analytical grade reagents were purchased from Sigma-Aldrich Chemie Gmbh, USA. *Lactobacillus plantarum* (Lp299v) was obtained from Abela Pharm, Croatia.

Preparation of probiotic for freeze-drying

L. plantarum from the frozen stock was subsequently incubated under anaerobic conditions in MRS broth at 37 °C for 18 hours, twice in succession. After incubation, the bacterial culture was washed three times with 10 mL of NS (normal saline solution, 0.9 % w/v NaCl). The resulting culture was then centrifuged at 4000 rpm for 5 minutes, and the bacterial pellets were resuspended in 1 mL of various freeze-drying media. Two protective media were used: 10% trehalose and a mixture of 5 % trehalose with 5 % skim milk. All freeze-drying solutions were sterilized using a 0.2 µm filter prior to mixing with the bacterial cells. Control was a culture without a cryoprotectant.

Freeze-drying immobilization of *L. plantarum* on AC pads

AC pads were put in a glass Petri dish, sterilized with hot air at 160°C for 2h, and then used for bacterial cell immobilization. Concretely, 100 µL of freeze-drying media mixed with cells was aseptically added on top of each pad and left to settle for 15 min at room temperature. The samples were then placed in plastic Petri dishes, frozen at −80 °C, and subsequently freeze-dried for 5 hours at −40 °C under a pressure of 0.12 mbar using a Beta 2-8 LD plus freeze dryer (Christ, Germany).

After freeze-drying, AC pads with immobilized cells were gold-coated and examined using a MIRA 3 XMU Field Emission Scanning Electron Microscope (Tescan USA Inc., Cranberry Township, PA).

Probiotic viability and immobilization efficiency (IE) during freeze-drying

Viable cell numbers were determined before and after immobilization by the pure plate method. The cell number was expressed as CFU/pad and calculated percentage of viability after freeze-drying, which is the immobilization efficiency (IE).

$$IE\ \% = \frac{Ni}{N} x100$$

Ni is the total number of bacteria immobilized on the carrier (CFU/g) calculated relative to the weight of the carrier; N is the total number of bacteria in the suspension before the immobilization procedure (CFU/g).

The obtained values for the number of cells are recalculated in relation to the logarithmic value of the obtained number of cells.

Probiotic viability and release capacity of AC pads after freeze-drying process during 24 h

The pads containing immobilized probiotics were incubated in 2 mL of normal saline at 37 °C for 10 minutes, 3, and 24 h. At each time point, the release of viable cells into the solution was assessed. The number of live cells was determined using the pour plate method and expressed as $\log_{10}$ CFU/pad

Statistical analysis

Experiments were performed in triplicate. All values are expressed as mean ± standard deviation. Mean values were analyzed using one-way ANOVA. The Tukey post hoc test was performed for means comparison (Origin Pro 8 (1991-2007) computer package, Origin Lab Co., Northampton, USA). Data analysis was conducted using Microsoft Excel (Microsoft Office 2013 Edition, USA) and OriginPro 8 (Origin Lab Co., Northampton, USA). Data was considered significantly different when $P < 0.05$.

RESULTS AND DISCUSSION

Figure 1 illustrates the experimental process employed in this study to create an innovative probiotic–activated carbon-based wound pad.

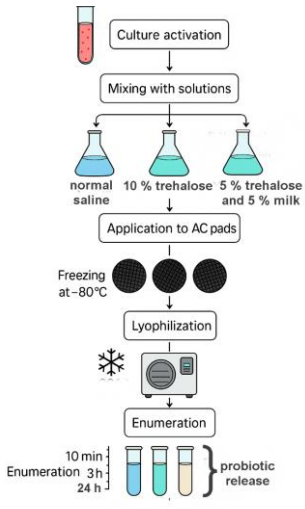

Figure 1. Scheme of experimental process.

AC pads fabricated via a punching process had an average diameter of approximately 12 mm (Fig. 2) and a mean weight of 0.017 ± 0.001 g. Bacterial immobilization was performed by directly applying 100 µL of a bacterial cell suspension, with or without protective agents, onto each AC pad. Following freeze-drying, the morphology of the carrier and the immobilized cells was examined using optical microscopy and field-emission scanning electron microscopy (FE-SEM) (Figure 2).

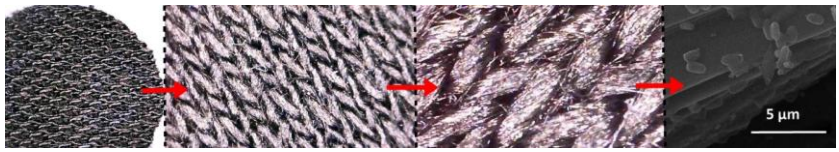

Figure 2. Optical and field emission scanning electron microscopy.

In this study, ACF was selected as a carrier for the freeze-drying of immobilized probiotics due to its excellent thermal conductivity. Carbon-based materials are known to facilitate efficient heat transfer throughout the sample matrix, thereby minimizing the risk of cellular damage during the lyophilization process (Malik, 1990).

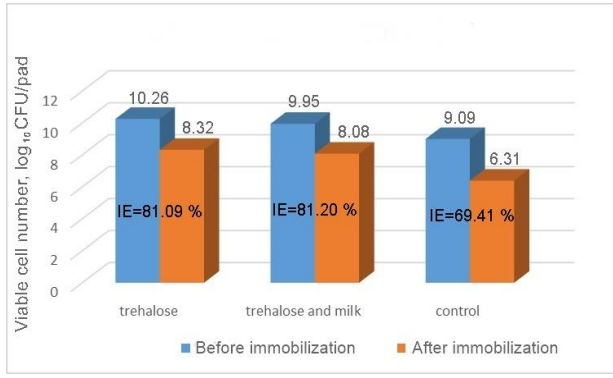

Figure 3. Probiotic viability before and after 5 h of the freeze-dry process and immobilization efficiency (IE) for samples with 10 % trehalose, with 5 % trehalose and 5 % milk, and control (without cryoprotectant).

As shown in Figure 3, trehalose and the combination of trehalose and milk exhibit very similar protective capacities, which significantly increased the number of viable probiotics during the immobilization process compared to the control sample without cryoprotectants. This is consistent with the literature, which has demonstrated that trehalose, as well as sucrose, provides a high degree of protection against bacterial freezing, similar to milk (Krunic, & Osmokrovic, 2025; Oluwatosin, et al., 2022). Also, Jia-Peng, et al. (2008) showed that a mixture of 3.34 % skim milk and 1.85 % trehalose resulted in a 96.8% survival rate of *L. plantarum* after lyophilization based on response surface methodology (RSM). Another RSM-based study found that 24.06 % skim milk and 5.63 % trehalose significantly improved *L. plantarum* viability, and highlighted the synergistic effect between milk and trehalose (Gisela, et al., 2014). Trehalose is a well-known and widely utilized protectant across the food, medical, pharmaceutical, and cosmetic industries. Although recent advancements in enzymatic production technologies have substantially reduced the cost of trehalose, it remains more expensive than commonly used sugars such as glucose and sucrose. However, a disadvantage associated with sucrose and other sugars is the increased stiffness of the sample following the application of these cryoprotectants. In such cases, milk is preferred, as is the sample containing both milk and trehalose in our study, as it results in a considerably less stiff structure, as well as being less expensive. These cryoprotectants not only influence the viability and survival of the probiotic culture during the immobilization process, but also affect the release of probiotics from the carrier. As shown in Figure 4, trehalose and the combination of trehalose

and milk result in a high release rate within the first 10 minutes as well as within the first 3 hours in normal saline solution. The control sample exhibits a statistically significantly lower number of released viable bacteria in the first 10 min, which is a consequence of the considerably lower survival rate during the immobilization process compared to the other two samples. After 3 hours, the number of viable probiotics in the control decreases by 17 %, while in the samples with cryoprotectants, the reduction is 3 % for trehalose and 1 % for the trehalose-milk combination. The greatest difference among the samples is observed after 24 hours: the number of viable probiotics in the control sample drops by 100 %, whereas the decrease is 44 % in the trehalose sample and 3.7 % in the sample with the trehalose-milk combination.

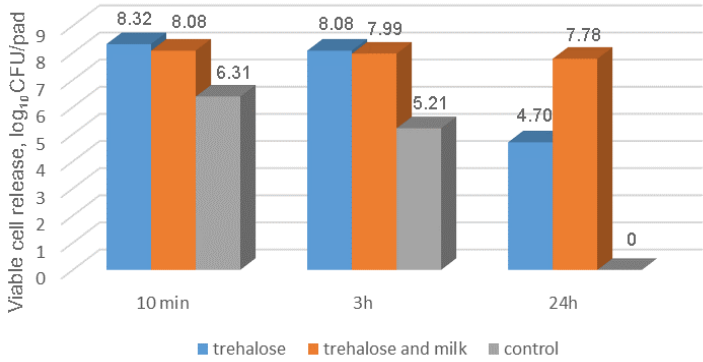

Figure 4. Viable cell number of release *L. plantarum* from AC pads in NS after 24h.

The release of bacteria from the carrier matrix is a critical factor when applying immobilized probiotics in wound dressing applications. For probiotics to exert their beneficial effects—such as modulating the local microbiota, producing antimicrobial compounds, reducing inflammation, and promoting tissue regeneration—they must be able to detach from the carrier and colonize the wound environment. An efficient and controlled release ensures that a sufficient number of viable cells reach the wound site and remain metabolically active, which is essential for achieving the desired therapeutic outcomes (Osmokrovic, et al., 2025). Furthermore, sustained release can prolong the probiotic action, enhancing the overall efficacy of the wound treatment.

Based on the obtained results, it can be concluded that the combination of trehalose and milk significantly enhances the survival of the probiotic culture during the immobilization process compared to the control sample, as well as during the release process when compared to both the control and the trehalose-only sample. Overall, these findings suggest that the combination of milk and trehalose is the most effective choice for the immobilization of *L. plantarum* by lyophilization onto an AC pad intended for wound dressing applications.

CONCLUSIONS

The results of this study demonstrate that the use of cryoprotectants, particularly the combination of trehalose and milk, plays a critical role in enhancing the viability of *L. plantarum* during the immobilization process onto AC pads and in promoting its subsequent release in a simulated wound environment. Compared to the control sample and the sample containing trehalose alone, the trehalose-milk combination significantly improved bacterial survival rates post-lyophilization and ensured a more efficient and sustained release of viable probiotic cells over 24 hours. These findings are consistent with previous reports highlighting the protective effects of trehalose and milk during freeze-drying. Moreover, the ability of probiotics to be released from the carrier is essential for their functional performance in wound care, including microbiota modulation, antimicrobial activity, and tissue regeneration. Therefore, the combination of trehalose and milk appears to be the most effective and practical choice for the immobilization of *L.*

plantarum on AC wound dressing pads, offering both enhanced protection during processing and optimal functionality upon application.

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DECLARATIONS OF INTEREST STATEMENT

The authors affirm that there are no conflicts of interest to declare in relation to the research presented in this paper.

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