EFFECT OF TWO LYOPROTECTANTS ON THE SURVIVAL RATE AND STORAGE STABILITY OF FREEZE-DRIED PROBIOTIC LACTIC ACID BACTERIAL STRAINS

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ABSTRACT

Lactic acid bacteria are very important for biotechnology, pharmaceutical and food industry, and are extensively used as probiotic products and starter cultures. Freeze-drying is a widely used and effective process for preservation of lactic acid bacteria in a dried form. To ensure stability and maintain biological activity of lactic acid bacteria, suitable protective media formulations should be used during the freeze-drying process. Twelve oral strains with functional and probiotic properties were subjected to freeze-drying and assessed for viability after the process and after long-term storage. Two lyoprotective media with different composition were used and the freeze-drying process was performed, where retained stability of the studied strains in both lyoprotectors was reported. Viability after four and eight months of storage was assessed, showing that the strains maintained high viability. The two lyoprotective media ensured the obtaining of freeze-dried samples with preserved viability for all studied strains of lactic acid bacteria. Four of the tested strains, L. fermentum N 2 and TC 3-11, L. delbrueckii subsp. lactis VG 2, and W. confusa NN 1 showed well preserved stability and viability after freeze-drying and long-term storage and, prepared in freeze-dried formulations, are suitable to be included in the composition of probiotic products for oral healthcare.

Keywords: lactic acid bacteria, freeze-drying, lyoprotectants, probiotics.

INTRODUCTION

Lactic acid bacteria (LAB) are a large group of microorganisms that are known to possess probiotic characteristics and are generally recognized as safe (GRAS). Their properties benefit to the human gastro-intestinal tract by exerting antimicrobial activity against pathogenic microorganisms, balancing the host microbiota, modulating the immune response, etc. [1, 2]. One of the important steps in the formulation and preparation of probiotic products is to ensure that beneficial bacteria remains viable during technological processes and storage for long periods of time.

Freeze-drying is a widely used process which improves the stability of thermally unstable biomaterials, LAB included, and their longevity when they are intended to be used as starter cultures, functional foods and probiotic supplements [3]. The freeze-drying process can be differentiated by three main phases: freezing to sub-zero temperatures, sublimation (first drying) and desorption (secondary drying). During the freezing step, ice crystals are formed from the water content in the extracellular medium from the gradual temperature decrease. Then, during the first drying step, sublimation of the frozen water in the bacterial suspension takes place, while in a vacuum environment, in order for the ice to transform directly from solid state to vapor without passing through liquid phase [4]. Finally, during the secondary drying, the bound water of the product is evaporated by desorption with slight raise of the temperature [5]. When the process is complete, the water content of the freeze-dried product is greatly reduced (< 5 % dry weight) [6].

Extreme conditions during freezing, drying, and post-process are critical for the viability of bacterial cells. Bacteria are subjected to mechanical and solute damage, damage to the cell wall and DNA structure, protein denaturation and inactivation, as well as chemical or osmotic impairments of the cells, which can significantly reduce their viability [7]. In order to minimize the stress and increase the stability during freeze-drying and subsequent storage, lyoprotective medium is added to the bacterial cultures. The formulation of lyoprotectants is comprised mainly from carbohydrates, with trehalose and lactose as the most widely studied [8]. The addition of either of these two sugars can mitigate mechanical damage by displacing water molecules or bond to the cell membrane in order to stabilize its structure and increase the glass transition temperature [7]. Other components, such as high protein materials (skim milk, etc.), can further strengthen the cell membrane. Different surfactants, including amino acids, can decrease the surface tension. Vitamins are used as agents with antioxidant properties that can scavenge for reactive oxygen species (ROS). Using different buffers can maintain stable pH values in the product. The addition of multiple components with varying modes of action can significantly increase stability of the product matrix and ensure increased survival rate of bacterial cultures [5]. The stability of the freeze-dried bacterial cultures largely depends on the storage temperature [9]. Storing freeze-dried cells at refrigerated temperatures shows better stability than at ambient temperatures during long-term storage [10]. At low temperature, kinetic energy is limited and bacterial metabolism is slowed down, which indicates lesser molecular movement and lower rates of detrimental reactions [11, 12].

The group of LAB strains present in the current study were selected according to their assessed probiotic properties in a previous study [13, 14]. The obtained data for these strains showed probiotic potential regarding their survival ability and growth in the environment of the oral cavity and lower parts of the GIT, and expressed adhesive properties, as well. Antimicrobial activity against common Gram– and Gram+ pathogens, including *Bacillus subtilis, Bacillus cereus* and *Escherichia coli* was reported [13]. Antagonistic activity against oral pathogens was reported as well, where four strains, *Limosilactobacillus fermentum* N 2, TC 3-11, and NA 2-2, and *Weissella confusa* NN 1 showed significant inhibition of *Streptococcus mutans* and all studied strains inhibited *Candida albicans* by up to 2 logs. Evaluation of antibiofilm activity of the studied strains showed antibiofilm properties by most of them above 79 % against *S. mutans* and above 50 % against *C. albicans* [14].

In this context, the aim of the study was to evaluate the stability of a group of oral LAB strains after a freeze-drying process. Two lyoprotective media with different composition were prepared and the survival rate and long-term storage stability of the freeze-dried LAB strains was evaluated.

EXPERIMENTAL

Microorganism strains

The LAB strains included in the current study were previously isolated from the microbiome of the human oral cavity and identified as representatives from the *Lactobacillaceae* family: *Limosilactobacillus fermentum* N 2, N 4-5, TC 3-11 and NA 2-2, *Latilactobacillus curvatus* KG 12-1, *Lactobacillus delbrueckii* subsp. *sunkii* VG 1, *Lactobacillus delbrueckii* subsp. *lactis* VG 2 and MK 13-1*, Lacticaseibacillus rhamnosus* NA 1-8, *Lacticaseibacillus paracasei* AV 2-1, and *Weisella confusa* AG 2-6 and NN 1 [13].

Lyoprotectants

Two lyoprotective media were formulated on the basis of previously unpublished data of performed freeze-drying processes in the Department of Biotechnology, Faculty of Biology, Sofia University and other scientific studies [11, 15 - 17]. 10 % skim milk was used as a base for both lyoprotectants, with the addition of 5 % lactose (SML) or 5 % trehalose (SMT) and 2 % ascorbic acid. The prepared lyoprotectants were then sterilized before usage by autoclaving at 121°C for 5 min.

Sample preparation

The studied LAB strains were cultivated overnight at 37°C in de Man, Rogosa, Sharpe (MRS) broth (HiMedia, Mumbai, India) and centrifuged at 6000×g for 10 min at 4°C. The collected biomass was then resuspended in the lyoprotectants, and 1.5 mL aliquots were added to sterile 6 mL glass vials with legged rubber stoppers for freeze-drying.

Freeze-drying

The prepared vials were loaded in Christ Epsilon 2 - 4 LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) freeze-dryer and the work program was run for 48 h. The process was optimized, according to the targeted samples and lyoprotective media, in order to achieve a minimum water content in the dry product: lower sub-zero temperature (-40°C) to ensure optimal freezing of the samples; longer sublimation for maximum removal of the free water; desorption at higher temperature (24°C - 25°C) for removal of most of the bound water [17, 18]. After completion, the vials were closed under vacuum and stored at 4°C, for ensuring better stability, for eight months [11, 12].

Viable cell counting Survival ability assessment

Sample vials were analyzed before and after freeze-drying, and after four and eight months of storage by the pour plate method [19 - 21]. The samples were rehydrated with the same volume of sterile dH_2O and ten-fold serial dilutions were made. The strains were pour plated in MRS agar and cultivated at 37°C for 48 h. The results were reported as CFU mL^{-1} from the viable cell count.

Data analysis

The experiments were performed in triplicate. The obtained data was analyzed by Microsoft Excel built-in functions and the results were expressed as mean ± standard deviation. One-way ANOVA was performed as a statistical analysis, followed by a post hoc Tukey test, to detect differences between controls before freeze-drying and samples after freeze-drying and storage. p-value of $p < 0.05$ was considered as statistically significant difference.

RESULTS AND DISCUSSION

Assessment of technological stability of LAB strains after freeze-drying process

The determination of the technological application of LAB strains with probiotic potential also includes evaluation of their stability and viability in different technological processes [6]. Two lyoprotective media have been used during freeze-drying in order to reduce adverse changes on the freeze-dried cells, including minimizing the inactivation of membrane lipids or reducing the structural changes of sensitive proteins of LAB [22]. The freeze-drying was performed by aseptically loading the vials in the freeze-dryer, monitoring the parameters of the process and closing of the vials under vacuum at the end (Fig. 1). After freeze-drying, the dried samples were analyzed for stability by determining the CFU. The results show that the used lyoprotective media retain stable viability of all LAB strains during the freeze-drying process (Fig. 2). In the SML lyoprotectant, *L. fermentum* N 2, *L. curvatus* KG 12-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1, retain their cell density at 10^9 CFU mL^{-1} and *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1 at 10^7 CFU mL^{-1} after the end of the freeze-drying process. No statistically significant difference in CFU mL^{-1} is observed for *L. fermentum* NA 2-2. For *L. fermentum* N 4-5 and TC 3-11, cell density decreases to 10^9 CFU mL^{-1}, and for *W. confusa* AG 2-6 and *L. delbrueckii* subsp. *sunkii* VG 1 to 10^7 CFU mL^{-1} (Fig. 2(a)). In the SMT lyoprotectant, *L. fermentum* N 2, N 4-5, TC 3-11 and NA 2-2, *L. curvatus* KG 12-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, and *L. paracasei* AV 2-1 retain their cell density at 10^9 CFU mL^{-1}, *L. delbrueckii*

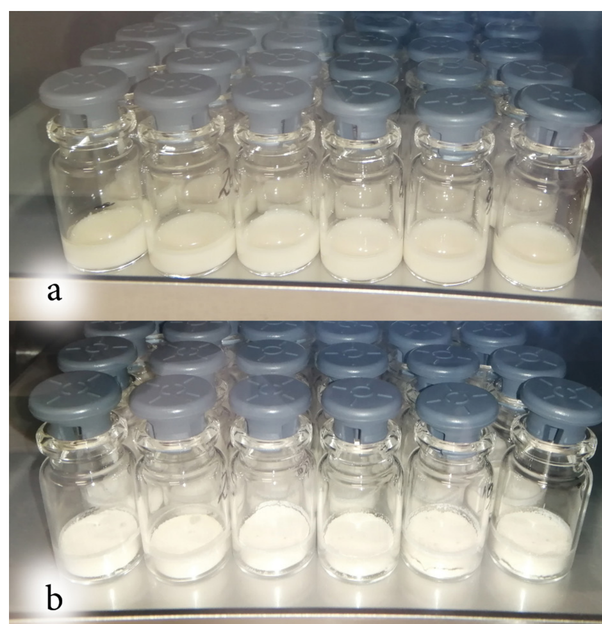

Fig. 1. Prepared samples of the studied LAB strains before freeze-drying (a) and after freeze-drying (b).

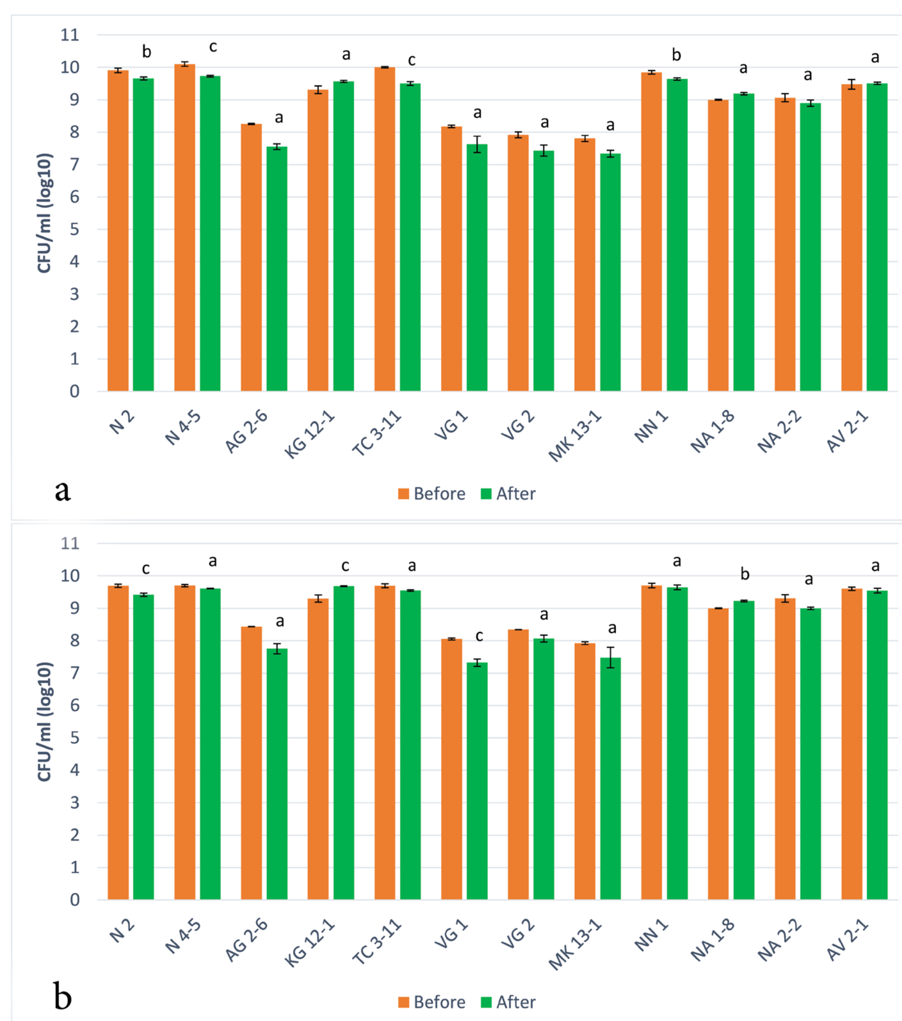

Fig. 2. Viability of the studied LAB strain after freeze-drying process in SML (a) and SMT (b) lyoprotectants.
Values are expressed as mean ± standard deviation. Statistical analysis was performed by ANOVA and post hoc Tukey test: a - nonsignificant ($p > 0.05$); b and c - significant ($p < 0.05$ and $p < 0.01$, respectively). Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L.s delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2 and *L. paracasei* AV 2-1.

subsp. *lactis* VG 2 at 10^8 CFU mL^{-1} and *L. delbrueckii* subsp. *lactis* MK 13-1 at 10^7 CFU mL^{-1} after the end of the freeze-drying process. For *W. confusa* AG 2-6 and *L. delbrueckii* subsp. *sunkii* VG 1 cell density decreases to 10^7 CFU mL^{-1} (Fig. 2(b)). The viability decrease of the tested LAB strains after freeze-drying is less than 1 log in both lyoprotectors. Although the small differences in survival ability, the two constructed lyoprotective media ensured excellent stability of LAB during the freeze-drying process.

For *L. fermentum* N 2, *L. curvatus* KG 12-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 no difference in viability can be observed in the two used lyoprotectors. This suggests that both protective media are suitable for freeze-drying these five strains. For *L. fermentum* N 4-5, TC 3-11 and NA 2-2, *L. delbrueckii* subsp. *lactis* VG 2, and *W. confusa* AG 2-6, freeze-drying in the SMT lyoprotectant showed better stability than in the SML lyoprotectant. In a study by Mendoza et al., the authors evaluated *Lactococcus* strains and *Lactiplantibacillus plantarum* CRL 1606 in nine lyoprotective media, where two of them, comprised of only lactose and skim milk + lactose showed excellent viability preservation after freeze-drying [23]. Jalali et al.

evaluated ten lyoprotectors with different ratio of skim milk, trehalose and sodium ascorbate. Freeze-drying of *Lacticaseibacillus paracasei* subsp. *tolerans* DSM 20258 and *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 showed that the studied strains were most stable in a protector with 6 % skim milk, 8 % trehalose and 4 % sodium ascorbate [24]. Studies by Gul et al. and Ren et al. used different protective media, comprised of skim milk, lactose and sucrose for freeze-drying of *Ligilactobacillus salivarius* and *Ligilactobacillus agilis*, and *L. curvatus* N 19, respectively. The results indicated that increasing the skim milk concentration resulted in an increase of cell viability and lactose and sucrose retained cell stability after freeze-drying [17, 25].

Assessment of viability of freeze-dried LAB strains after long-term storage

The long-term storage stability of LAB is as important as the stability during the freeze-drying process itself. While in storage, probiotic bacteria must maintain high viable cell density in order to be successfully included in the composition of different probiotic products or used as starter cultures [26, 27]. Closed under vacuum, the freeze-dried LAB were stored for four and eight months and analyzed for viability by determining the CFU. The results from the long-term storage show that all of the tested LAB strains retain high viability after four and eight months of storage at 4°C (Fig. 3). In the SML lyoprotectant, *L. fermentum*

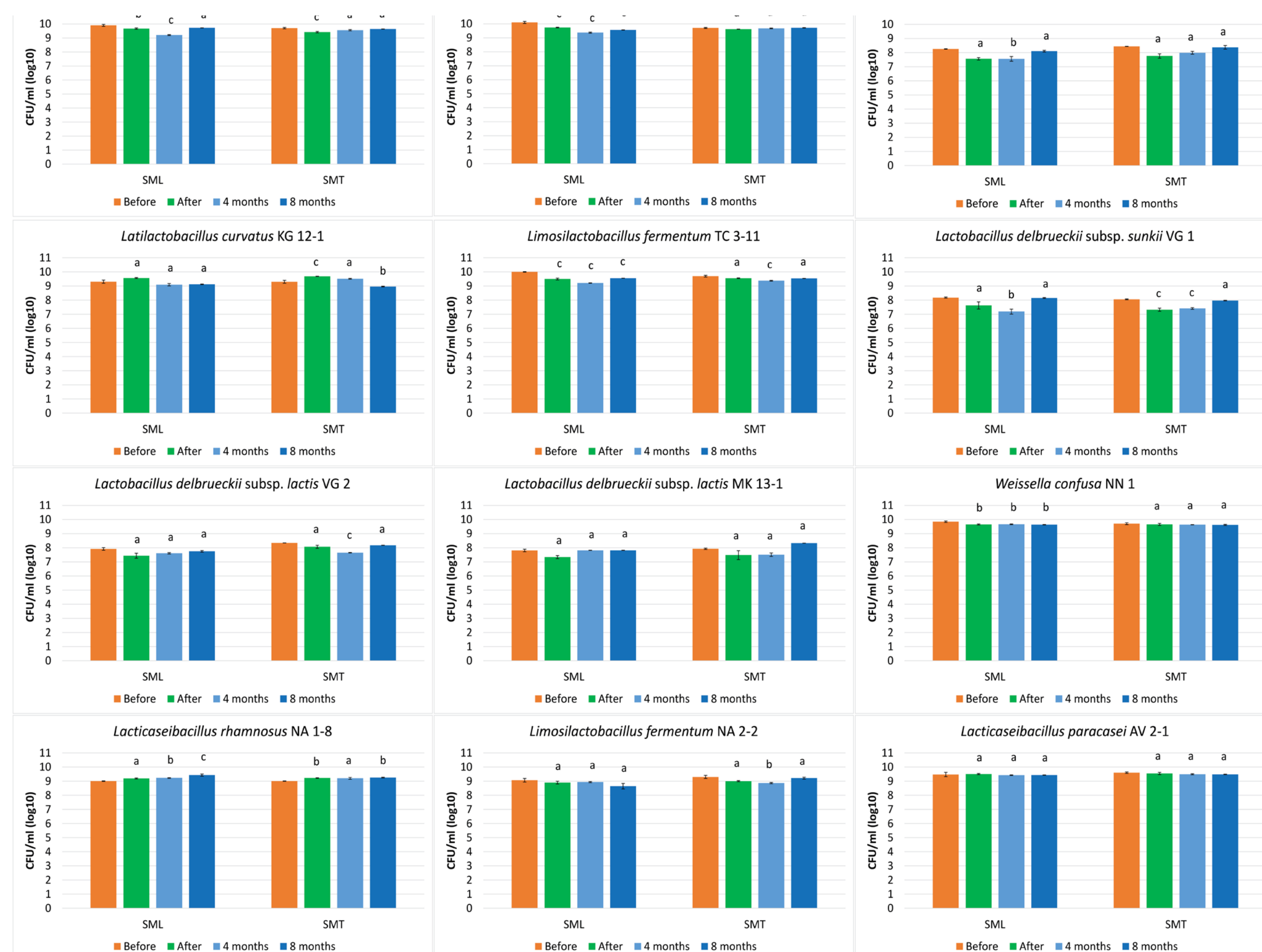

Fig. 3. Viability of the studied LAB strains after 4 and 8 months of storage at 4°C in SML and SMT lyoprotectants. Values are expressed as mean ± standard deviation. Statistical analysis was performed by ANOVA and post hoc Tukey test: a – nonsignificant ($p > 0.05$); b and c – significant ($p < 0.05$ and $p < 0.01$, respectively).

N 2, *L. curvatus* KG 12-1, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 maintain their cell density unchanged for the whole period of storage. In the SMT lyoprotectant, *L. fermentum* N 2, N 4-5 and TC 3-11, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 maintain their cell density unchanged for the 8-month storage.

No statistically significant difference on the viability of *L. curvatus* KG 12-1, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1 can be observed for the whole period of storage in the SML lyoprotectant. While in the SMT lyoprotectant, no statistically significant difference can be observed on the viability of *L. fermentum* N 4-5, *W. confusa* AG 2-6 and NN 1, *L. delbrueckii* subsp. *lactis* MK 13-1, and *L. paracasei* AV 2-1 for the eight months of storage. *L. fermentum* N 2, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 show better survival ability after freeze-drying and maintain their viability better after long-term storage in both lyoprotective media. In the SML lyoprotectant, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 are observed to have better stability than the other strains and in the SMT lyoprotectant, *L. fermentum* N 2, N 4-5 and TC 3-11, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 were more stable. These results point that the stability of the studied LAB is better retained when trehalose is added to the lyoprotectant formulation. Different authors reported that better survival rates were observed after long-term storage at refrigeration temperature [28 - 30]. In the study by Jalali et al., the studied *L. paracasei* subsp. *tolerans* DSM 20258 and *L. delbrueckii* subsp. *bulgaricus* DSM 20081 were stored at 4°C for three months and the results showed that the strains were most stable in the same protector, comprised of 6 % skim milk, 8 % trehalose and 4 % sodium ascorbate [21]. In two studies by Gul et al., *L. curvatus* N 19 and *Levilactobacillus brevis* ED25 were subjected to six months of storage at refrigeration temperature in optimized protectant composition (20 % skim milk, 3.57 % lactose and 10 % sucrose, and 17.28 % skim milk, 2.12 % lactose, and 10 % sucrose, respectively) and showed excellent stability with viability loss of less than 1 log [17, 30]. In a study by Sun et al., the authors reported that adding trehalose in the composition of freeze-drying solutions significantly increased the survival ability of *L. plantarum* LP105 after 240 days of storage at 4°C with viability decrease by less than 1 log [11].

CONCLUSIONS

The obtained results from this work include substantial data regarding the technological application of previously isolated oral LAB strains in freeze-drying process. All studied LAB strains showed excellent stability after freeze-drying and viability after storage for four and eight months. Among them, *L. fermentum* N 2 and TC 3-11, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 showed to be the most stable and retain their viability unchanged for the whole period of storage. Utilizing different compositions of lyoprotectants, comprised of skim milk, lactose or trehalose, and ascorbic acid showed to have significant impact on maintaining cell viability of the studied LAB strains. Both lyoprotectant formulations had similar viability preservation, but the lyoroptector with added trehalose showed to better maintain the viability of most of the studied strains. Four strains, *L. fermentum* N 2 and TC 3-11, *L. delbrueckii* subsp. *lactis* VG 2, and *W. confusa* NN 1 who possess observed probiotic properties and significant antagonistic activity against oral pathogens [13, 14], also show well preserved stability and viability after freeze-drying and long-term storage. These four strains, prepared in freeze-dried formulations, are suitable to be included in the composition of probiotic products for oral healthcare.

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REFERENCES

1. J. Ji, H. Yang, Using Probiotics as Supplementation for *Helicobacter pylori* Antibiotic Therapy, Int. J. Mol. Sci., 21, 3, 2020, 1136, doi: 10.3390/ijms21031136.

2. W.-K. Lai, Y.-C. Lu, C.-R. Hsieh, C.-K. Wei, Y.-H. Tsai, F.-R. Chang, Y. Chan, Developing Lactic Acid Bacteria as an Oral Healthy Food, Life, 11, 2021, 268, doi: 10.3390/life11040268.
3. G. Broeckx, D. Vandenheuvel, I.J.J. Claes, S. Lebeer, F. Kiekens, Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics, Int. J. Pharm., 505, 2016, 303-318, doi: 10.1016/j.ijpharm.2016.04.002.
4. P. Yeboah, N. Wijemanna, A. Eddin, L. Williams, S. Ibrahim, Lactic Acid Bacteria: Review on the Potential Delivery System as an Effective Probiotic, Dairy Processing - From Basics to Advances, Intech Open, 2023, doi: 10.5772/intechopen.111776
5. A. Bodzen, A. Jossier, S. Dupont, P.Y. Mousset, L. Beney, S. Lafay, P. Gervais, Design of a new lyoprotectant increasing freeze-dried *Lactobacillus* strain survival to long-term storage, BMC Biotechnol., 21, 1, 2021, 66, doi: 10.1186/s12896-021-00726-2.
6. F. Fonseca, S. Cenard, S. Passot, Freeze-drying of lactic acid bacteria, Methods Mol. Biol., 1257, 2015, 477-488, doi: 10.1007/978-1-4939-2193-5_24.
7. C. Santivarangkna, M. Wenning, P. Foerst, U. Kulozik, Damage of cell envelope of *Lactobacillus helveticus* during vacuum drying, J. Appl. Microbiol., 102, 3, 2007, 748-756, doi: 10.1111/j.1365-2672.2006.03123.x.
8. S. Cui, M. Hu, Y. Sun, B. Mao, Q. Zhang, J. Zhao, X. Tang, H. Zhang, Effect of Trehalose and Lactose Treatments on the Freeze-Drying Resistance of Lactic Acid Bacteria in High-Density Culture, Microorganisms, 11, 2023, 48, doi: 10.3390/microorganisms11010048.
9. A. Yao, F. Bera, C. Franz, W. Holzapfel, P. Thonart, Survival rate analysis of freeze-dried lactic acid bacteria using the arrhenius and z-value models, J. Food Prot., 71, 2, 2008, 431-434, doi: 10.4315/0362-028x-71.2.431.
10. I. Ibrahim, J.A. Ayariga, J. Xu, R.K. Boakai, O.S. Ajayi, J. Owusu-Kwarteng, A Comparative Study of Skimmed Milk and Cassava Flour on the Viability of Freeze-Dried Lactic Acid Bacteria as Starter Cultures for Yogurt Fermentation, Foods, 12, 2023, 1207, doi: 10.3390/foods12061207.
11. H. Sun, M. Zhang, Y. Liu, Y. Wang, Y. Chen, W. Guan, X. Li, Y. Wang, Improved viability of *Lactobacillus plantarum* embedded in whey protein concentrate/pullulan/trehalose hydrogel during freeze drying, Carb. Pol., 260, 2021, 117843, doi: 10.1016/j.carbpol.2021.117843.
12. Y. Lu, L. Huang, T. Yang, F. Lv, Z. Lu, Optimization of a cryoprotective medium to increase the viability of freeze-dried *Streptococcus thermophilus* by response surface methodology, LWT, 80, 2017, 92-97, doi: 10.1016/j.lwt.2017.01.044.
13. N. Atanasov, Y. Evstatieva, D. Nikolova, Probiotic Potential of Lactic Acid Bacterial Strains Isolated from Human Oral Microbiome, Microbiol. Res., 14, 1, 2023, 262-278, doi: 10.3390/microbiolres14010021.
14. N. Atanasov, Y. Evstatieva, D. Nikolova, Antagonistic Interactions of Lactic Acid Bacteria from Human Oral Microbiome against *Streptococcus mutans* and *Candida albicans*, Microorganisms, 11, 6, 2023, 1604, https://doi.org/10.3390/microorganisms11061604.
15. D. Teng, K. Kawai, S. Mikajiri, Y. Hagura, Stabilization of freeze-dried *Lactobacillus paracasei* subsp. *paracasei* JCM 8130T with the addition of disaccharides, polymers, and their mixtures, Biosci. Biotechnol. Biochem., 81, 4, 2017, 768-773, doi: 10.1080/09168451.2017.1279852.
16. R.F. Stefanello, E.H. Nabeshima, B.T. Iamanaka, A. Ludwig, L.L.M. Fries, A.O. Bernardi, M.V. Copetti, Survival and stability of *Lactobacillus fermentum* and *Wickerhamomyces anomalus* strains upon lyophilisation with different cryoprotectant agents, Food Res. Int., 115, 2019, 90-94, doi: 10.1016/j.foodres.2018.07.044.
17. L.B. Gul, A.H. Con, O. Gul, Storage stability and sourdough acidification kinetic of freeze-dried *Lactobacillus curvatus* N19 under optimized cryoprotectant formulation, Cryobiology, 96, 2020, 122-129, doi: 10.1016/j.cryobiol.2020.07.007.
18. T. Heidebach, P. Först, U. Kulozik, Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells, J. Food Eng., 98, 2010, 309-316, doi: 10.1016/j.jfoodeng.2010.01.003.
19. L. Maturin, J.T. Peeler, Aerobic plate count, In Bacteriological analytical manual online, 8th ed, FDA, Silver Spring, MD, 2001.
20. E.R. Sanders, Aseptic laboratory techniques: plating methods, J. Vis. Exp., 63, 2012, e3064, doi: 10.3791/3064.
21. M. Nemati, A. Hamidi, S. Maleki Dizaj, V. Javaherzadeh, F. Lotfipour, An Overview on Novel

Microbial Determination Methods in Pharmaceutical and Food Quality Control, Adv. Pharm. Bull., 6, 3, 2016, 301-308, doi: 10.15171/apb.2016.042.

22. D. Nowak, E. Jakubczyk, The Freeze-Drying of Foods-The Characteristic of the Process Course and the Effect of Its Parameters on the Physical Properties of Food Materials. Foods, 9, 10, 2020, 1488, doi: 10.3390/foods9101488.
23. G. Montel Mendoza, S.E. Pasteris, M.C. Otero, M.E. Fatima Nader-Macías, Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage, J. Appl. Microbiol., 116, 1, 2014, 157-166, doi: 10.1111/jam.12359.
24. M. Jalali, D. Abedi, J. Varshosaz, M. Najjarzadeh, M. Mirlohi, N. Tavakoli, Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules, Res. Pharm. Sci., 7, 1, 2012, 31-36.
25. H. Ren, J. Zentek, W. Vahjen, Optimization of Production Parameters for Probiotic *Lactobacillus* Strains as Feed Additive, Molecules, 24, 2019, 3286, doi: 10.3390/molecules24183286.
26. K. Fenster, B. Freeburg, C. Hollard, C. Wong, R. Rønhave Laursen, A.C. Ouwehand, The Production and Delivery of Probiotics: A Review of a Practical Approach, Microorganisms, 7, 3, 2019, 83, doi: 10.3390/microorganisms7030083.
27. A. Terpou, A. Papadaki, I.K. Lappa, V. Kachrimanidou, L.A. Bosnea, N. Kopsahelis, Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value, Nutrients, 11, 7, 2019, 1591, doi: 10.3390/nu11071591.
28. G. Shu, Z. Wang, L. Chen, H. Wan, H. Chen, Characterization of freeze-dried *Lactobacillus acidophilus* in goat milk powder and tablet: optimization of the composite cryoprotectants and evaluation of storage stability at different temperature, LWT, 90, 2018, 70-76, doi: 10.1016/j.lwt.2017.12.013.
29. W. Savedboworn, K. Teawsomboonkit, S. Surichay, W. Riansa-Ngawong, S. Rittisak, R. Charoen, K. Phattayakorn, Impact of protectants on the storage stability of freeze-dried probiotic *Lactobacillus plantarum*, Food Sci. Biotechnol., 28, 3, 2019, 795-805, doi: 10.1007/s10068-018-0523-x.
30. L.B. Gul, O. Gul, M.T. Yilmaz, E. Dertli, A.H. Con, Optimization of cryoprotectant formulation to enhance the viability of *Lactobacillus brevis* ED25: Determination of storage stability and acidification kinetics in sourdough, J. Food Process. Preserv., 44, 2, 2020, 14400, doi: 10.1111/jfpp.14400.