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Evaluation of Cryoprotective Potential of *Jerusalem artichoke*' Inulin During Freeze-drying and Storage of *Lactobacillus paracasei* HII01

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The preservation of synbiotic preparations is the most important task than the formulation, due to the stability issues. Many advanced techniques are employed to preserve and stabilize the viability of synbiotic formulas. The current study deals with the development of freeze-dried (FD) *Lactobacillus paracasei* HII01 with selected commercial inulins (inulin GR, inulin SC, inulin FF), inulin extracted from Jerusalem artichoke (inulin JA), skim milk, maltodextrin #2, trehalose, and sorbitol as cryoprotectants and assessment of viability during intestinal transit and storage. The trehalose, inulin FF, and inulin JA retain the survivability of *L. paracasei* HII01 up to 46.1-79.5%, 19.0-51.0%, and 10.3-33.1%, respectively, whereas, the sorbitol showed least survival rate (1.4-12.7%) of *L. paracasei* HII01 and the FD-*L. paracasei* HII01 without any cryoprotectants had no viable cells. The FD-powders with the high survival of *L. paracasei* HII01 were selected to study the viability during gastrointestinal transit (Simulated gastric fluid, and simulated intestinal fluid of human). The FD-*L. paracasei* HII01 with inulins protects the survivability of *L. paracasei* HII01 during gastrointestinal transit more than other studied protectants. The results suggested that the storage of FD-*L. paracasei* HII01 with inulin JA at 4 °C can retain ~59.2% of the existence of live *L. paracasei* HII01 cells for 60 days than other tested commercially available inulins, while the samples stored at room temperature showed the drastic reduction in the viability. Collectively, the results suggested that the inulin JA can be used in freeze drying process of *L. paracasei* HII01 as a cryoprotectant, which effectively diminishes the degradation of probiotic cells during gastrointestinal transit and storage.

Keywords: Cryoprotectants, Freeze-drying, Inulin, *Lactobacillus paracasei* HII01, Synbiotic.

The cryoprotectants are any substance, either biological or chemical, which protects the microbial or eukaryotic cells/tissues/organ from degradation or inactivation during freeze-storage. The storage and formulation of bacterial cells for further pharmaceutical applications required potent cryoprotectants to enhance the survival during the process, storage and gastrointestinal transit, in the case of human consumption. Probiotics are live microbial cells which have the ability to offer some health benefits to the host system like inhibition of pathogenic growth by bacteriocins, enhancement of host immunity, lowering the colon cancer risk, and reducing blood cholesterol etc[1-3]. The efficiency and survivability of the probiotic bacteria can be supported by prebiotics, a

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fermented ingredient that will selectively adjust the composition and activity of gastrointestinal tract microbiota[4]. The supplementation of synbiotic preparation, both probiotic and prebiotic formula, are more efficient than separate interventions on enhanced survival and activity of probiotic and longer shelf-life of probiotic products[5, 6].

The formulation and accomplishment of an improved synbiotic preparation certainly depend on the delivery forms such as liquid, semi-solid, powder, etc. The current study employed the freeze-drying (FD) method, is a standard technique used to produce a probiotic powder. However, during the process, the viability of probiotics are affected due to the damage caused by freezing temperature and drying under high vacuum, FD is a relatively efficient method to preserve the microbial cells. The viability of the probiotic strain is also liable to storage conditions and gastrointestinal transit[7, 8]. Various cryoprotectants have been used as protective agents in FD of probiotics to improve the survival during the process, storage and gastrointestinal transit, such as glucose, sucrose, sorbitol, glycerol, skim milk, trehalose, maltodextrin and some prebiotics[7-10].

Inulin isolated from Jerusalem artichoke (Inulin JA) is one of the proven prebiotic for *L. plantarum* [11]. Intervention of hydrolyzed inulin of JA suppress the azoxymethane influenced preneoplastic aberrant crypt foci formation in rat and also support the growth of *Lactobacillus* spp. and Bifidobacteria[12]. The synbiotic preparation consist of inulin JA and *L. plantarum* exhibiting greater protective effect in colorectal cancer induced rat system (unpublished data; manuscript under communication). In order to exert beneficial effect of probiotics, they need to survive during the manufacturing process, gastrointestinal transit and retain their viability during storage. It is important to consider these factors for synbiotic preparation. Thus, the objective of the current study was to prepare freeze dried *L. paracasei* HII01 cells with inulin JA and other selected cryoprotectants and to evaluate the cryoprotective potential of certain cryoprotectants, with respect to the viability of probiotic strain during gastrointestinal transit and storage at different temperatures.

MATERIALS AND METHODS

Bacterial strain and cryoprotectants

Lactobacillus paracasei HII01, isolated from Thai pickles of leeks and red shallots, and deposited at Thailand Institute of Scientific and Technological Research (TISTR)-microbial culture collection, was received from Health Innovation Institute, Chiang Mai and maintained in MRS (de Man, Rogosa and Sharpe, EMD Millipore, Germany). The LAB strain was further confirmed by sequencing of 16S rRNA coding gene using specific primers of 5'-GCCGCCTAA GGTGGGACAGAT-3' and, 5'-TTACCTAA CGGTAAATGCGA-3' and phylogenetic analysis as detailed in previous studies [13, 14]. Eight cryoprotectants were used including four different inulin such as inulin JA (hydrolyzed inulin from Jerusalem artichoke[11], Average Degree of Polymerization (DP_{av}) = 23), Inulin GR (Orafti, Germany, DP_{av} >10, from chicory), Inulin SC (Fuji Nihon, Japan, DP_{av} = 8, synthesized from sugar) and Inulin FF (Fuji Nihon, Japan, DP_{av} = 16, synthesized from sugar), and Skim milk, maltodextrin #2 (Matsutani Chemical, Japan), sorbitol and trehalose (Wako Pure Chemical, Japan). The probiotic was cultured in MRS broth at 37 °C for 20 h to reach the early stationary phase then the cells were harvested by centrifugation at 8000 *g* for 5 min at 4 °C (Beckman Allegra® 21R centrifuge, Germany). Then the pellets were washed and re-suspended in sterile saline. All cryoprotectants were used at the concentration of 2.5, 5.0, 7.5, and 10.0% (w/w). *L. paracasei* HII01 cells at the final concentration of 10^{10}-10^{12} CFU/g were mixed with different concentrations (2.5, 5.0, 7.5, and 10.0% (w/w)) of cryoprotectants. The suspensions (*L. paracasei* HII01 and cryoprotectants) were transferred to plates and were freeze stored at -40 °C for 1 h.

Freeze drying, survivability, and water content

The suspensions were placed in the freeze dryer (EYELA FD-100, Japan) and desiccated under vacuum for 24 h. Freeze dried powders were weighed, and stored in a tight container, until analysis. The survival of probiotic strain was determined by measuring the glucose-fermenting activity according to Lievense *et al* [15]. Briefly, the cell suspension was mixed with buffer (0.01 M

K_2HPO_4 (Wako), 0.01 M KH_2PO_4 (Wako) and 0.15 M NaCl (Wako)) and pre-incubated for 5 min in a water bath and constant stirring to mix the cells properly. Then 0.8 M glucose was added to start the fermentation, and the pH was registered between pH 4 and pH 7 (Horiba D54 pH Conductivity Meter, Japan). The activity of cell suspension was denoted as "pH.min^{-1} per gram of dry weight of the cells. The water content of the powders were measured by as follows. Water content = Weight of the cryopowder - Weight of the dry cryopowder (dried at 105 °C for 4 h). The freeze-dried *L. paracasei* HII01 with cryoprotectants, which had high survival rate were selected for viability and stability studies.

Viability of L. paracasei HII01 during gastrointestinal transit

The viability of selected FD *L. paracasei* HII01 powders during gastrointestinal transit was investigated using a two-stage *in vitro* model which mimic the conditions of the human stomach and small intestine [7]. In Brief, samples were mixed with simulated gastric fluid (SGF) pH 1.2 containing 3 g/L of pepsin (Sigma-Aldrich, Japan), and incubated at 37 °C for 2 h then adjusted the sample pH to 6.8 and diluted to 10-fold in simulated intestinal fluid (SIF) pH 6.8 containing 10 g/L of pancreatin (Sigma-Aldrich, Japan) and 3 g/L of bile salt (Sigma-Aldrich, Japan) then incubated at 37 °C for 3 h. The viable bacteria were determined by plating on MRS agar. The CFU of untreated FD *L. paracasei* HII01 was compared with SGF, and SIF exposed samples.

Stability of the freeze dried powders during storage

The selected freeze dried powders were stored at 4 °C and room temperature (25°C) in separate air-tight containers for eight weeks. The survival of *L. paracasei* HII01 was determined during by glucose-fermenting activity method[15].

Statistics

All data were expressed as mean ± SD. One-way repeated analysis of variance and Duncan's least significant difference test had been used to compare the mean differences. Statistical significance was assigned at 0.05 interval level ($p < 0.05$).

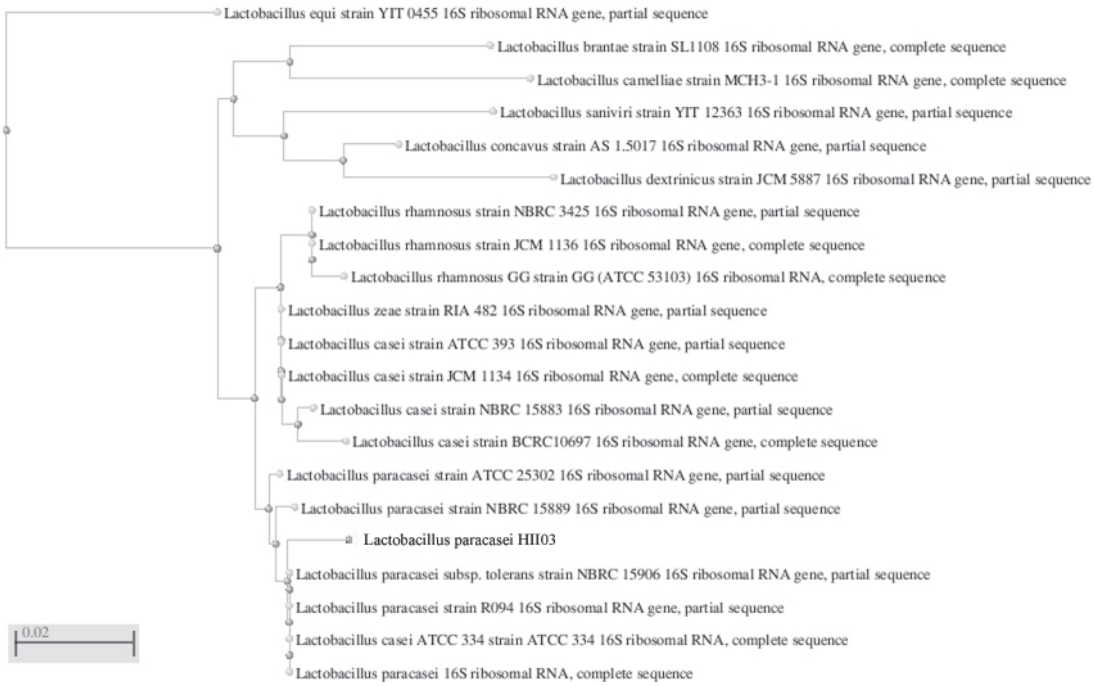

Fig. 1. The phylogenetic representation of *L. paracasei* HII01 strain acquired from HII, Chiang Mai showing sequence similarity with *L. paracasei* strains

RESULTS AND DISCUSSION

Survival and water content of FD-*L. paracasei* HII01

The strain of *L. paracasei* HII01 has been confirmed by 16S rRNA sequencing and phylogenetic analysis (Fig. 1). The survival rate (%) of FD-*L. paracasei* HII01 with studied cryoprotectants were assessed and represented as glucose-fermenting activity (Fig. 2). FD-*L. paracasei* HII01 without cryoprotectants had no survival whereas addition of trehalose, as cryoprotectants, retain the survivability of *L. paracasei* HII01 up to 46.1-79.5% compared to another tested protectant. Inulin FF protects *L. paracasei* HII01 up to 19.0-51.0%, and sorbitol showed only 1.4-12.7% of *L. paracasei* HII01 survival. Moreover, FD-*L .paracasei* HII01 with inulin JA showed 10.3-33.1% of the cryoprotective effect on the survivability of *L. paracasei* HII01. The water content of FD-*L. paracasei* HII01 with various cryoprotectants are shown in Fig. 3. The maximum (7.2-11.5%) and minimal (2.4-6.2%) level of water content have been recorded in the FD-*L. paracasei* HII01 with inulin SC and FD-*L. paracasei* HII01 with skim milk samples, respectively. Obviously, the concentration of cryoprotectants is directly proportional to a water content of the FD powers.

The trehalose has been shown to increase the viability of lyophilized *L. paracasei* subsp. *tolerance* and *L. delbrueckii* subsp. *Bulgaricus* [9]

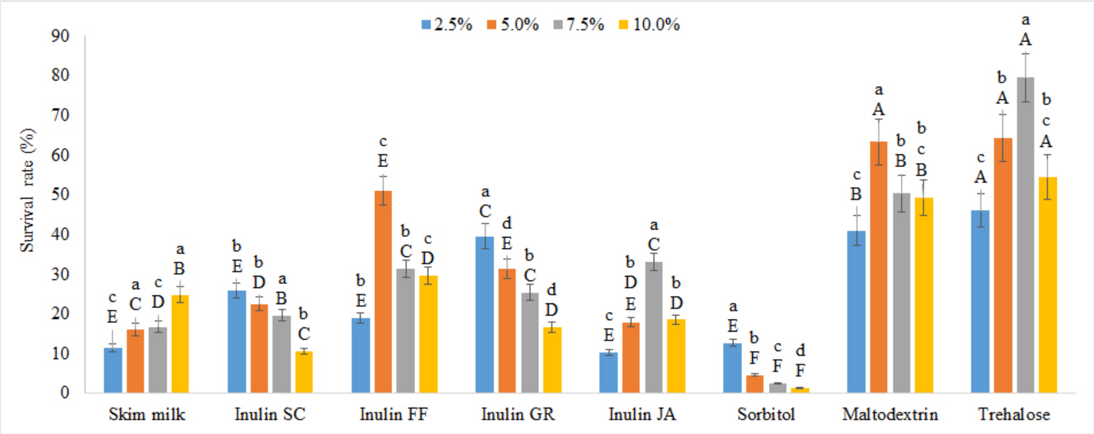

Fig. 2. Survival rate (%) of freeze-dried *L. paracasei* HII01 with different concentration (2.5, 5.0, 7.5 and 10.0%) of various cryoprotectants. Values are denoted as mean ± SD. [a-d] superscripts indicates the significant difference of concentrations of cryoprotectants, [A-F] superscripts indicates the significant difference of cryoprotectants at same concentration, $p < 0.05$.

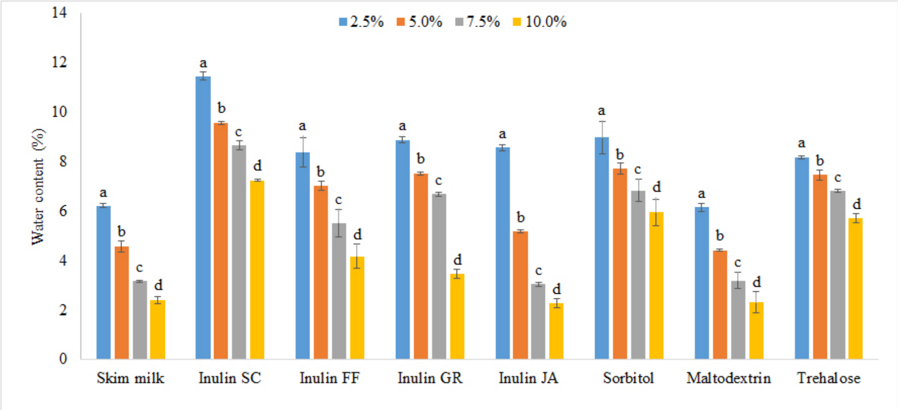

Fig. 3. Water content (%) of freeze-dried *L. paracasei* HII01 with different concentration (2.5, 5.0, 7.5 and 10.0%) of various cryoprotectants. Values are denoted as mean ± SD. [a-d] superscripts indicates the significant difference between concentrations of each cryoprotectant, $p < 0.05$.

and also enhanced the survival of *L. salivarius* after freeze-drying and storage[16], and protects the various strains of lactic acid bacteria during freeze drying[17]. The prebiotics was used as cryoprotectants during freeze drying process of probiotic strains[18, 10]. The freeze dried *Bifidobacterium bifidum* cells with fructooligosaccharide, xylo-oligosaccharide, isomaltooligosaccharide and inulin at the concentration of 4-20% (v/v) showed the viability of 20, 16, 12 and 4%, respectively[18].

Viability of *L. paracasei* HII01 during gastrointestinal transit

The survival of probiotic bacteria during human gastrointestinal transit is influenced by pH, gastric enzymes, bile salt and residence time[19]. It has been suggested that the addition of non-digestible carbohydrates called as prebiotics may increase the viability of probiotics passing through the gastrointestinal tract and thus exert a beneficial effect to host health[20, 21]. The protective ability of selected cryoprotectants in FD- *L. paracasei* HII01 powder has been determined. The survival rate (% of Log CFU/g of sample) of FD-*L. paracasei* HII01 was calculated. The survival rate of FD-*L. paracasei* HII01 without protectant (control) was significantly ($p < 0.05$) decreased to 54.7% after simulated gastric conditioning at pH 1.2 for 2 h and there was no viable cells after simulated intestinal conditioning at pH 6.8 for 3 h. While the addition of cryoprotectants significantly ($p < 0.05$) protected FD- *L. paracasei* HII01 during gastrointestinal transit (Fig. 4). The FD- *L. paracasei* HII01 with all test inulin showed the protective effect on the survival of *L. paracasei* HII01 during gastrointestinal transit. The FD- *L. paracasei* HII01 with 5.0% of inulin FF exhibited about 79.4% of survival followed by the maltodextrin and trehalose protects 40.8 and 30.4% of FD- *L. paracasei* HII01 survival, respectively. Whereas, inulin JA showed 75.5 % of the cryoprotective effect.

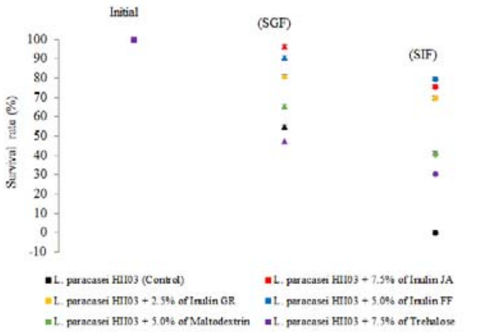

Fig. 4. Survival rate (%) of freeze-dried *L. paracasei* HII01 with selected cryoprotectants during *in vitro* simulated gastrointestinal transit. The viability of *L. paracasei* at initial point (%), simulated gastric fluid (SGF ;2%), and simulated intestinal fluid (SIF;·) were denoted.

The cryoprotectants significantly ($p < 0.05$) protected the FD- *L. paracasei* HII01 during SGF and SIF exposure. Moreover, FD- *L. paracasei* HII01 with inulin showed the greater survival of *L. paracasei* HII01 than maltodextrin and trehalose

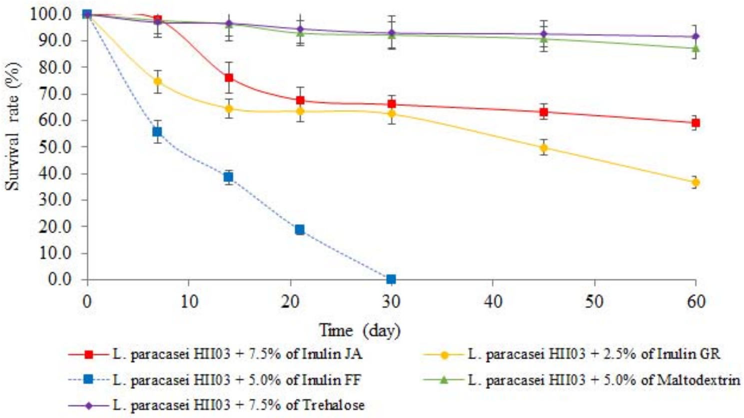

Fig. 5. Survival rate (%) of freeze-dried *L. paracasei* HII01 with selected cryoprotectants at room temperature (25°C) for 60 days.

(Fig. 4). The artichoke, an edible plant enriched with inulin, can retain and improved the survival of probiotic strain, *L. paracasei* in gastrointestinal digestion[22]. The inulin considerably enhances the survival of *L. rhamnosus* during gastrointestinal tract passage[23].

Stability of the freeze dried powders during storage

The FD- *L. paracasei* HII01 samples were stored at 4 °C and 25 °C (Room Temperature; RT) for 60 days and samples were collected regular interval for the assessment of stability regarding viability. About 7.5% of trehalose and 5.0% maltodextrin protects the cells up to the survival rate of 91.6% and 87.2% at 4 °C, respectively, but the cells stored at RT showed the gradual decrease in the viability. Among tested inulins, 7.5% of inulin FF hold the survivability *L. paracasei* HII01 up to 59.2% stored at 4 °C for 60 days, While at RT, all inulins could maintain the survival of *L. paracasei* HII01 for 21 days (Fig. 5).

It has been recommended that probiotic dairy product should contain at least 7 log CFU/g of viable probiotic bacteria at the time of consumption. Thus, it is the core to developing products to retain the viable probiotic cells during storage. The evidence suggested that the combination of prebiotic with probiotic improves the survival of probiotic during storage[10, 24-26]. Some of the prebiotics like Raftilose (FOS), Hi-maize and Raftiline (inulin) were evaluated for their ability to retain the viability of probiotic and proved that the selected prebiotics reduces the rate of cell death during storage[27]. The results of the present study suggested that the viability of FD- *L. paracasei* HII01 was reduced with storage time especially at RT, whereas, the samples stored at 4 °C retain the viability of *L. paracasei* HII01 up to 91.6 ± 4.3 % in the presence of trehalose for 60 days. The inulin JA maintain 59.2±2.8 % of survivability of FD- *L. paracasei* HII01 (Fig. 6). Nagpal and Kaur evaluated the effect of inulin, oligofructose, lactulose, raftilose and honey on the viability of *Lactobacillus* strains during storage at 4 °C for five weeks and found that the viability of lactobacilli was stabilized by inulin up to 60-78%[24]. The addition of inulin can maintain the survival of *L. paracasei* during 30 days of storage with only 0.47-0.71of log CFU reduction[26]. The overall results suggested that the effective preservation of probiotic strains along with prebiotics as cryoprotectants by freeze drying method is influenced by many factors like percentage of cryoprotectants used, storage temperature, and period of storage.

CONCLUSION

Synbiotic (*L. paracasei* HII01 + cryoprotectants) powders were prepared using freeze drying method and FD- *L. paracasei* HII01 with trehalose showed 46.1–75.5% of survival, moreover, it retains the viability of *L. paracasei* HII01 up to 96.1% and 75.5% during SGF and SIF transit, respectively. Our prepared inulin from JA displayed only 33.1% of *L. paracasei* HII01

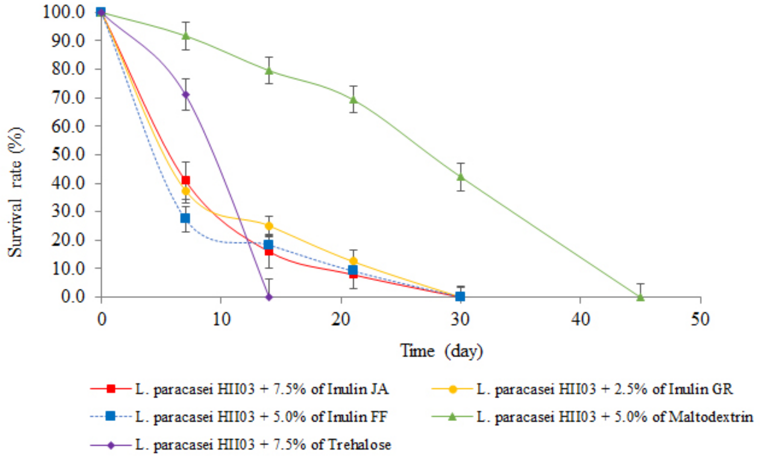

Fig. 6. Survival rate (%) of freeze-dried *L. paracasei* HII01 with selected cryoprotectants at 4°C for 60 days.

survivability. The storage of FD- *L. paracasei* HII01 at 4 °C with inulin JA protects the probiotic cells (59.2%), which was significantly higher than the protective ability of tested commercially available inulins. Thus, the current study has endorses the use of inulin JA as a protectant of *L. paracasei* HII01 during freeze-drying process and storage at 4 °C for the improved recovery of viable cells.

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REFERENCES

1. Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L., Poli, A., Pregliasco, F., Salvini, F., Zuccotti, G. V. Probiotics and health: an evidence-based review. *Pharmacol Res.,* 2011; **63**: 366-376.
2. Nagpal, R., Kumar, A., Kumar, M., Behare, P. V., Jain, S., Yadav, H. Probiotics, their health benefits and applications for developing healthier foods: a review. *FEMS Microbiol. Lett.*, 2012; **334**: 1-15.
3. Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., Fakiri, E. M. 2013. Health Benefits of Probiotics: A Review. *ISRN Nutrition.*, 2013; **2013**: 1-7. Article ID 481651.
4. Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E. F., Saulnier, D., Loh, G., Macfarlane , S., Delzenne, N., Ringel, Y., Kozianowski, G., Dickmann, R., Lenoir-Wijnkook, I., Walker, C., Buddington, R. Dietary prebiotics: current status and new definition. *Food Sci. Tech. Bull. Funct. Foods.,* 2010; **7**: 1-19.
5. Gibson, G. R., Roberfroid, M.B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.*, 1995; **125**: 1401-1412.
6. Chaluvadi, S., Hotchkiss, A. T., Jr., Call, J. E., Luchansky, J. B., Phillips, J. G., Liu, L., Yam, K. L Protection of probiotic bacteria in a synbiotic matrix following aerobic storage at 4 degrees C. *Benef. microbes.,* 2012; **3**: 175-187.
7. Crittenden, R., Weerakkody, R., Sanguansri, L., Augustin, M. Synbiotic Microcapsules That Enhance Microbial Viability during Non-refrigerated Storage and Gastrointestinal Transit. *Appl. Environ. Microbiol.,* 2006; **72**: 2280-2282.
8. Meng, X. C., Stanton, C., Fitzgerald, G. F., Daly, C., Ross, R. P. Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chem.,* 2008; **106**: 1406–1416.
9. Jalali, M., Abedi, D., Varshosaz, J., Najjarzadeh, M., Mirlohi, M., Tavakoli, N. Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. tolerance and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules. *Res. Pharm. Sci.,* 2012; **7**: 31-36.
10. Dhewa, T., Pant, S., Mishra, V. Development of freeze dried synbiotic formulation using a probiotic strain of Lactobacillus plantarum. *J. Food Sci. Technol.,* 2014; **51**: 83-89.
11. Pattananandecha, T., Sirilun, S., Sivamaruthi, B. S., Suwannalert, P., Peerajan, S., Chaiyasut, C. Hydrolyzed Inulin with Different Degree of Polymerization as Prebiotic for *Lactobacillus plantarum. J. Pure Appl. Microbio.,* 2015; **9**: 973-979.
12. Pattananandecha, T., Sirilun, S., Duangjitcharoen, Y., Sivamaruthi, B. S., Suwannalert, P., Peerajan, S., Chaiyasut, C. Hydrolyzed inulin alleviates the azoxymethane-induced preneoplastic aberrant crypt foci by altering selected intestinal microbiota in Sprague-Dawley rats. *Pharm. biol.,* 2016; Doi: 10.3109/13880209.2015.1110597.
13. Woraharn, S., Lailerd, N., Sivamaruthi, B.S., Wangcharoen, W., Sirisattha, S., Chaiyasut, C. Screening and kinetics of glutaminase and glutamate decarboxylase producing lactic acid bacteria from fermented Thai foods. Food Sci. Technol. (Campinas)., 2014; **34** (4): 793-799
14. Woraharn, S., Lailerd, N., Sivamaruthi, B.S., Wangcharoen, W., Peerajan, S., Sirisattha, S., Chaiyasut, C. Development of fermented Hericium erinaceus juice with high content of L-glutamine and L-glutamic acid. *Int. J Food Sci. Tech.*, 2015; **50**: 2104-2112.
15. Lievense, C., van't Riet, K., Noomen, A. Measuring and modelling the glucose-fermenting activity of *Lactobacillus paracasei*. *Appl. Microbiol. Biotechnol.,* 1990; **32**: 669-673
16. Zayed, G., Roos, Y. H. Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. *Process Biochem.,* 2004; **39**: 1081-1086.

17. Giulio, B. D., Orlando, P., Barba, G., Coppola, R., De Rosa, M., Sada, A., De Prisco, P. P., Nazzaro, F. Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. *World J. Microbiol. Biotechnol.*, 2005; **21**: 739-746.
18. Shu, G.W., Hu, M., Qin, T., Chen, H., Ma, Q. Effect of fructo-oligosaccharide, isomalto-oligosaccharide, inulin and xylo-oligosaccharide on survival of *B. bifidum* during freeze-drying. *Adv. Mater. Res.*, 2011; **382**: 454-457.
19. Conway, P. L., Gorbach, S. L., Goldin, B. R. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci.*, 1987; **70**: 1-12.
20. Roberfroid, M., Slavin, J. Nondigestible oligosaccharides. Crit Rev Food Sci Nutr., 2000; **40**: 461-480.
21. Iyer, C., Kailasapathy, K. Effect of Co-encapsulation of Probiotics with Prebiotics on Increasing the Viability of Encapsulated Bacteria under In Vitro Acidic and Bile Salt Conditions and in Yogurt. *J. Food Sci.*, 2005; **70**: M18-M23.
22. Valerio, F., De Bellis, P., Lonigro, S. L., Morelli, L., Visconti, A., Lavermicocca, P. *In vitro* and *in vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastrointestinal tract. *Appl. Environ. Microbiol.*, 2006; **72**: 3042-3045.
23. Khalf, M., Dabour, N., Kheadr, E. and Fliss, I. Viability of probiotic bacteria in maple sap products under storage and gastrointestinal conditions. *Bioresour. Technol.*, 2010; **101**: 7966-7972.
24. Nagpal, R., Kaur, A. Synbiotic effect of various prebiotics on in vitro activities of probiotic lactobacilli. *Ecol. Food Nutr.*, 2011; **50**: 63-68.
25. Savini, M., Cecchini, C., Verdenelli, M. C., Silvi, S., Orpianesi, C., Cresci, A. Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. *Nutrients.*, 2010; **2**: 330-339.
26. Valero-Cases, E., Frutos, M. Effect of different types of encapsulation on the survival of Lactobacillus plantarum during storage with inulin and *in vitro* digestion. LWT - *Food Sci. Technol.*, 2015; **64**, 824-828.
27. Capela, P., Hay, T. K. C., Shah, N. P. Effect of cryoprotectants, prebiotics and microencapsulation on survival of probiotic organisms in yoghurt and freeze-dried yoghurt. *Food Res. Int.*, 2006; **39**: 203-211.