Impact of protectants on the storage stability of freeze-dried probiotic *Lactobacillus plantarum*

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Abstract The ability of rice protein supplemented with various prebiotics to protect probiotic *Lactobacillus plantarum* TISTR 2075 upon freeze-drying and subsequent storage was determined. A combination of rice protein-fructooligosaccharide (RF) provided the best storage stability with the lowest specific rate of cell death (k) of 1.20×10^{-2} and 5.79×10^{-2} 1/day during subsequent storage at 4 °C for 180 days and 30 °C for 90 days, respectively. Glass transition temperatures (T_g) of freeze-dried probiotic in various protectants were 14.2–25.4 and 42.9–50.1 °C after storage at 4 and 30 °C, respectively. The functional properties of freeze-dried probiotic with protectants remained stable. The presence of RF could effectively protect and enhance the probiotic functionality during exposure to gastrointestinal tract conditions. The pathogenic inhibition of freeze-dried probiotic against foodborne pathogens was not different from the active cells. Protective agents were able to maintain high degrees of cell surface hydrophobicity.

Keywords Freeze-drying · Probiotic · Probiotic property · Protective agents · Storage stability

Introduction

The development of functional foods containing probiotics and prebiotics has been gaining interest in global market due to their potential in promoting human health. The stability and viability of probiotic are both a marketing and technological challenge for industrial producers (Jofré et al., 2015). In this regard, the dried form of probiotic has been purposed due to the advantages in term of transportation, storage cost and industrial application. The survival in sufficient numbers and functionality expression of probiotics are influenced by preservation methods (Santivarangkna, 2015). Freeze-drying is considered as the most mild dehydration technique for long-term preservation of probiotic cultures because of the capability to maintain a high degree of cells viability (Halim et al., 2017). Stability of probiotic cells during freeze-drying and storage could be enhanced by the addition of protective agents such as proteins, amino acids, sugars, and carbohydrates in order to minimize the cellular damage (Gwak et al., 2015). In addition, the application of prebiotics as freeze-drying media was carried out. According to Santos et al. (2014), there was no significant loss of viable cell number of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after freeze-drying and 21 days storage when GOS cup Oligo H70®, GOS Biotempo, and lactulose were used as freeze-drying media. Furthermore, inulin and acacia gum provided a great protective effect on *L. plantarum* with no significant reduction in viability after storage under refrigerated condition. Unfortunately, the viable cell count decreased by 5–6 log cycles after storage at room temperature for

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90 days (Dhewa et al., 2014). A combination of protein and sugar can have synergistic effect on cell survival rather than acting individually. A mixture of plant protein and trehalose significantly ($p < 0.05$) improved the viability of probiotic *L. plantarum* TISTR 2075 with the lowest specific rate of cell death of 7.45×10^{-4} and 1.79×10^{-2} 1/day after storage at 4 °C for 168 days and 30 °C for 90 days (Savedboworn et al., 2017). Different protective agents variably affect not only the viability of probiotics, but also their probiotic functionalities (Santivarangkna, 2015). Freeze-dried *L. salivarius* CFR-2158 with the combination of skim milk and fructooligosaccharide were capable to survive during exposure to simulated gastric juice pH 2.5 with the survival rate of more than 95% which was higher than that of free cell (Reddy et al., 2009). Moreover, encapsulated *Pediococcus acidilactici* ATCC 8042 in alginate coated bead and freeze-dried with skim milk could be able to survive more than 5 log CFU/g after exposure to simulated bile juice pH 8.25 for 30 min. Montel Mendoza et al. (2014) reported that the degree of cell surface hydrophobicity of freeze-dried *L. plantarum* CRL 1606 in skim milk mixed with sucrose and inhibitory activity of this freeze-dried strains against *Listeria monocytogenes* Scott A and *Pseudomonas aeruginosa* were not different from the original cells before freeze-drying. Of the protective agents, skim milk powder is generally a suitable carrier medium to improve bacterial survival (Romano et al., 2016). However, the increasing demands of non-dairy protective agents incorporated in probiotic products have been proposed. This was probably because lactose intolerance and the allergy to milk protein might cause adverse effect to consumers (Gwak et al., 2015). Soy powder and plant protein extract have been studied for their protective effect on probiotic cells during freeze-drying and storage (Gwak et al., 2015; Savedboworn et al., 2017). Brown rice is a great source of protein contains complete amino acid profiles (Kalman, 2014) which could be considered as an alternative carrier medium in freeze-drying process. Moreover, there is no information regarding an impact of rice protein as protectants for probiotic. Therefore, this work focuses mainly on the protective capability of rice protein supplemented with prebiotics as protectants on the viability of freeze-dried probiotic *L. plantarum* TISTR 2075 after freeze-drying and subsequent storage. The comparison was made with the addition of various protective agents including rice protein and prebiotics. The physical property of matrices after storage in term of the glass transition temperature (T_g) was also evaluated. The impacts of freeze-drying process with emphasis on the retention of probiotic functionality including gastrointestinal tract tolerance, pathogenic inhibition, and cell surface hydrophobicity after freeze-drying were carried out.

Materials and methods

Bacterial strains and preparation of cultures

Probiotic strain: *Lactobacillus plantarum* TISTR 2075 obtained from Microbiological Resource Center, Thailand Institute of Scientific and Technological Research (TISTR), Thailand was used in this study. Stock culture was preserved in Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) mixed with 20% (v/v) glycerol at − 20 °C. The strain was subcultured twice in MRS broth at 37 °C for 24 h before use.

Indicator strains: *Escherichia coli* O157:H7 DMST 12743 and *Salmonella typhimurium* ATCC 13311 derived from the Department of Medical Science, Ministry of Public Health, Thailand were used as indicator microorganisms for antimicrobial activity. Indicator cultures were cultured in tryptic soy broth (TSB) (Difco, Detroit, MI, USA) supplemented with 0.6% yeast extract, combined with 20% (v/v) glycerol and then stored at − 20 °C. The strains were subcultured twice and incubated at 37 °C for 24 h.

Preparation of rice extracts fermentation

In the present study, Plai Ngahm Prachin Buri rice was obtained from Suan Dusit Rice Mill Company Limited, Suan Dusit Rajabhat University, Prachinburi province, Thailand. The rice extract fermentation procedure was performed following the method of Savedboworn et al. (2017) with a little modification. The rice was first washed and soaked overnight in distilled water. The soaked rice was mixed with distilled water (rice:water = 1:10 w/v) and then comminuted in a blender for 5 min. The resultant slurry was filtered through double-layered cheesecloth 2 times to yield rice extracts. Rice extract was dispensed into containers and sterilized by heating at 121 °C for 15 min. Sterilized rice extract was inoculated with overnight culture of 2% (v/v) *L. plantarum* TISTR 2075. The fermentations were carried out under no pH control in Duran screwcapped glass bottles at 37 °C for 24 h. The viable cell counts were determined by the standard plate count method with MRS medium supplemented with 0.5% CaCO_3 (Scharlau Chemie, Barcelona, Spain) at 37 °C for 24 h.

Freeze-drying of probiotic

Freeze-drying procedure was performed following the method of Savedboworn et al. (2017). Briefly, the 24-h incubated culture of probiotic *L. plantarum* TISTR 2075 grown in Plai Ngahm Prachin Buri rice extract was combined with 15% (w/v) protectants (Rice protein or Rice

protein mixed with various prebiotics) listed as follows: 15% Rice protein (R; Brown rice protein, Growing naturals, Los Angeles, CA, USA), 10% Rice protein + 5% Fructooligosaccharides (RF; Orafit[®]P95, Beneo, Tienen, Belgium), 10% Rice protein + 5% Gum arabic (RG; Gum Arabic Powder KB-120, Chemipan Corporation Co., Ltd., Bangkok, Thailand), 10% Rice protein + 5% Inulin (RI; Orafit[®]GR, Beneo, Tienen, Belgium), 10% Rice protein + 5% Polydextrose (RP; Litesse[®] powder, Danisco, Terre Haute, Indiana, USA), and 10% Rice protein + 5% Xylitol (RX; Futaste Pharmaceutical Co., Ltd., Shandong, China). The suspensions were mixed by a magnetic stirrer for 30 min and transferred into lyophilized flask under aseptic condition and frozen at -18°C for 17 h. A bench-scale freeze dryer (Alpha LSC1-4, Christ, Osterode am Harz, Germany) was performed at 0.110 mbar and -50°C for 18 h. Control of this experiment was *L. plantarum* TISTR 2075 cells freeze-dried without protective agent. All of freeze-dried probiotic samples were determined immediately for the viable cell number.

Storage stability of freeze-dried probiotic

Freeze-dried probiotic powders were kept in sealed aluminum foil laminated bags (3.25×6 cm) under atmosphere and then stored at 4 and 30°C . The viable cell count was determined every 15 days for 4°C and every 10 days for 30°C . The specific rate of cell death (k , 1/day) of freeze-dried probiotic in different protectants was calculated as a first-order reaction from $k = \ln(N_0/N)/t$, where N refers to the bacterial cell count at a particular storage period (CFU/g), N_0 represents the bacterial cell count at the beginning of the storage (CFU/g) and t is the storage time (day) (Sayedboworn et al., 2017).

Determination of simulated gastrointestinal tract tolerance of freeze-dried probiotic

Simulated gastric juice at pH 2.0 was prepared by suspending pepsin from porcine gastric mucosa (P-7000, Sigma-Aldrich, St. Louis, MO, USA) in sterile 0.5% NaCl to a final concentration of 3 g/L and adjusting to pH 2.0 with concentrated HCl (Michida et al., 2006). Simulated small intestinal juice at pH 8.0 was prepared by suspending pancreatin USP (P-1500, Sigma-Aldrich, St. Louis, MO, USA) in sterile 0.5% NaCl to a final concentration of 1 g/L, adding with 0.45% bile salt (Oxoid, Basingstoke, England) and adjusting to pH 8.0 with sterile 0.1 M NaOH. The pH value was measured with a pH meter (Inolabph level 1, WTW, Weilheim, Germany).

For the determination of tolerance to simulated gastric juice and small intestinal juices, freeze-dried powder (1 g) was suspended in 0.85% NaCl (9 mL). An aliquot of

0.2 mL of the suspension was transferred to a sterile tube, mixed with sterile 0.5% NaCl (0.3 mL) and blended with 1.0 mL of the simulated gastric juice or small intestinal juice. Viable cell counts were measured after 30, 60, 90, and 180 min for the simulated gastric juice and after 60 and 240 min for small intestinal juice tolerance determination (Sayedboworn and Wanchaitanawong, 2015). Active cells refer to fresh probiotic cells which have never been in freeze-drying process.

Antimicrobial activity of freeze-dried probiotic

Antimicrobial activity of freeze-dried probiotic was performed according to the method of Sayedboworn and Wanchaitanawong (2015) with a slight modification. Briefly, freeze-dried probiotic sample with various protective agents (1% w/v) was inoculated into MRS broth and cultured at 37°C for 24 h. The inhibitory activity against indicator microorganisms, *E. coli* O157:H7 DMST 12743 and *S. typhimurium* ATCC 13311, was evaluated using agar well diffusion method. The overnight culture (200 μL) of each indicator microorganism was mixed with 20 mL of molten tryptic soya agar supplemented with 0.6% yeast extract (approximately 10^6 CFU/mL) and poured into a sterile petri dish. Wells (7-mm diameter) were punched out of the solid agar with a sterile cork borer. The overnight culture of freeze-dried *L. plantarum* (50 μL) was inoculated into the wells and the plates were incubated at 37°C for 24 h. The diameter of inhibition zones was measured by vernier caliper. Each experiment was done in triplicate.

Determination of cell surface hydrophobicity

Cell surface hydrophobicity of freeze-dried probiotic was evaluated by the method of Lapsiri et al. (2011) and with minor modifications. Freeze-dried probiotic *L. plantarum* TISTR 2075 were prepared in sterile 0.85% NaCl solution to give an optical density of 0.5 at 600 nm (OD_0). Equal volumes (3 mL) of each cell suspension were transferred to sterile test tubes and 1 mL of xylenes (Ajax finechem, Victoria, Australia) was added. The mixtures were resuspended in vortex mixer for 90 s and then incubated at room temperature for 15 min. The aqueous phase was taken and its optical density at 600 nm (OD_1) was then determined. The percentage of cell surface hydrophobicity was calculated as $[1 - (\text{OD}_1/\text{OD}_0)] \times 100$.

Differential scanning calorimetry analysis

Differential Scanning Calorimetry (DSC) measurements of freeze-dried probiotic in various protective matrices were performed using a DSC machine (DSC 204F1 Phoenix[®], NETZSCH GmbH, Selb, Germany). Samples of 5–10 mg

were placed into hermetically sealed aluminum crucible pan. The N_2 flow rate was 50 mL/min. Samples were cooled down to $-10\text{ }^\circ\text{C}$ and then heated up to $180\text{ }^\circ\text{C}$ with a heating rate of $10\text{ }^\circ\text{C}/\text{min}$. An empty aluminum crucible was used as a reference. Indium was used as the internal standard. The DSC data analysis was evaluated with NETZSCH Proteus software.

Scanning electron microscopy

Microstructure of freeze-dried probiotic powders was visualized using a scanning electron microscopy (SEM). The powders were mounted on a brass stub with double-sided adhesive tape and sputter-coated with gold. Digital images were recorded with a scanning electron microscope (JSM 6400, JEOL, Tokyo, Japan) and captured at the required magnification (Savedboworn et al., 2017).

Measurement of water activity and moisture content

The water activity (A_w) of freeze-dried probiotic powders was measured at $25 \pm 1\text{ }^\circ\text{C}$ using a water activity meter (Aqua Lab, CX3TE, Pullman, WA, USA). The residual water content of the freeze-dried powders was carried out in hot air oven at $105\text{ }^\circ\text{C}$ until a constant weight was achieved.

Statistical analysis

All experiments were carried out in duplicate with duplication determination. The data were statistically analyzed for analysis of variance (ANOVA) in a completely randomized design. Significant divergences among mean values were established using Duncan's multiple range tests at 95% confidence interval (CI). All statistical analyses were performed using SPSS Software version 22 (SPSS Inc., Armonk, NY, USA).

Results and discussion

The survival of probiotic *L. plantarum* TISTR 2075 after freeze-drying with various protectants

In the preliminary experiment, freeze-dried probiotic *L. plantarum* TISTR 2075 without addition of protectant was evaluated. A higher loss of viable cell number of approximately 2.54 log CFU/g with the survival rate of 0.29% was observed. The impact of 15% Rice protein and 10% Rice protein mixed with 5% prebiotics was determined for their effectiveness in protecting probiotic cell after freeze-drying process. According to the results, probiotic remained high viable cell number of $8.70\text{--}9.00\text{ log CFU/g}$ with the

reduction in viable cell number of $0.13\text{--}0.48\text{ log CFU/mL}$ after freeze-drying. Among all protective agents, RF, RX, and RP were significantly ($p < 0.05$) enhance the viable cell number of *L. plantarum* TISTR 2075 with the survival rate of 71.34, 66.96, and 61.01%, respectively. No significant difference in survival rate was observed when RG and RI were used comparing with R (Fig. 1). During freeze-drying process, the death of microorganisms is related to membrane injury and osmotic shock caused by intracellular ice formation and recrystallization (Broeckx et al., 2016; Rajam and Anandharamakrishnan, 2015). A variety of freeze-drying media have been developed to minimize the inactivation of membrane lipids or reduce the changes in the structure of sensitive protein (Lu et al., 2017). Rice protein exhibited a positive effect on the viability of probiotic *L. plantarum* TISTR 2075 after freeze-drying. Kalman (2014) revealed that rice protein contains essential amino acids and branched-chained amino acid which is similar to the amino acid profile of soy protein isolate. Amino acids play a crucial role in the protection of probiotic cells during freeze-drying process. The interaction between amino group of protective agents and carboxyl groups of the bacterial protein could maintain the protein structure of bacterial cells (Lapsiri et al., 2013).

Apparently, the combination of rice protein and prebiotic exert a great protective effect on the survival of the strain. Fructose from fructooligosaccharide interacting with the cell membrane prevented membrane damage during dehydration (Rajam and Anandharamakrishnan, 2015). Oligosaccharides could be able to penetrate the cell wall and induce plasmolysis of cells prior to freezing and provide mechanical protection (Jofré et al., 2015). According

Fig. 1 Viable cell number and survival rate of probiotic *L. plantarum* TISTR 2075 after freeze-drying with various freeze-drying media. Values with different lowercase letters are significant differences by Duncan's multiples range test ($p < 0.05$)

to Schwab et al. (2007), the presence of fructooligosaccharide correlated to the improvement of membrane integrity of *L. reuteri* TMW1.106 during freeze-drying. In addition, protective ability of polydextrose is similar to non-permeable cryoprotectants which adsorb on the cell surface forming a viscous layer, cause partial efflux of water from the cell, inhibit the growth of ice crystals and maintain the structure of ice amorphous in the close proximity of the cell (Hubálek, 2003). Moreover, xylitol, sugar alcohol, appeared more effective due to their hydroxyl group allowing them to interact with polar sites of phospholipid bilayers of bacterial cell envelopes (Dianawati et al., 2013).

Gum arabic, also known as gum acacia, has been extensively used as an encapsulating agent for the retention and protection of chemically reactive and volatile oils and flavor compounds (Burnside, 2014). It is a complex mixture of glycoproteins and polysaccharides. In drying process, Desmond et al. (2002) reported that gum arabic may offer protection against oxidative stress or reduce the rate of water removal from the immediate environment of live cells by the presence of a semipermeable wall. It could be indicated that gum arabic could form a semipermeable wall around the other components that make the liquid feed going into the drier. The use of gum arabic as drying medium resulted in the increasing of probiotic survival rate of *L. paracasei* NFBC 338 which exhibited tenfold greater survival than 20% reconstitute skim milk.

Water activity and moisture content also play a key role in the maintenance of cell activity and viability. The results of the water activity and moisture content values were shown in Table 1. Water activity of freeze-dried probiotic powders varied from 0.237 to 0.273. Furthermore, the scanning electron microscope photomicrographs revealed the morphological characteristics of the freeze-dried probiotic powders (Fig. 2). Without protective agent during freeze-drying process, probiotic cells were packed and

porous flakes were detected. Rice protein formed a protective coating on probiotic cells and probiotic cells may randomly allocate throughout the matrix. All freeze-dried probiotic powders showed the similar particle shape with porous and spongy flakes structure. Consistent with the reports of Rajam and Anandharamakrishnan (2015) that freeze-dried microencapsulated *L. plantarum* MTCC 5422 in fructooligosaccharide and whey protein isolate showed flake-like structure with smooth surface whereas fructooligosaccharide and denatured whey protein isolate exhibited a porous and spongy flakes surface. Moreover, Savedboworn et al. (2017) suggested that the SEM micrographs of freeze-dried *L. plantarum* TISTR 2075 in plant protein supplemented with different protectants (maltodextrin, trehalose, and inulin) illustrated a porous-sheet-like structure.

Effect of freeze-drying media on the survival of probiotic *L. plantarum* TISTR 2075 during subsequent storage

The viability of freeze-dried probiotic *L. plantarum* TISTR2075 in different protectants was determined during storage at 4 °C for 180 days and 30 °C for 90 days. The protective ability of freeze-drying media was considered in terms of a specific rate of cell death (k value). As shown in Table 2, the k values were depending on different storage conditions and type of protective agents. Regarding to the results, the k values were in the range of 1.20×10^{-2} – 5.39×10^{-2} 1/day and 5.79×10^{-2} – 2.24×10^{-1} 1/day during subsequent storage at 4 and 30 °C, respectively. The reduction in viable cell number of 1.02 and 2.30 log CFU/g was achieved at the end of storage at 4 and 30 °C, respectively. The addition of RF provided the best stability of the strain with the lowest k values at both storage temperatures. However, the viable cell number of freeze-dried cells without protectant decreased to undetectable number

Table 1 Water activity and moisture content of freeze-dried probiotic *L. plantarum* TISTR 2075 in various protective agents after freeze drying process and storage at 4 °C for 180 days and 30 °C for 90 days

Protective agents	Water activity $\pm$ SD	Storage temperatures	
		After freeze-drying	
		4 °C	30 °C
R	0.262 ± 0.001^{bc}	0.302 ± 0.008^b	0.299 ± 0.011^b
RF	0.237 ± 0.003^a	0.274 ± 0.012^a	0.271 ± 0.004^a
RG	0.258 ± 0.003^b	0.298 ± 0.015^b	0.309 ± 0.007^{bc}
RI	0.264 ± 0.007^{bc}	0.310 ± 0.010^{bc}	0.307 ± 0.007^{bc}
RP	0.255 ± 0.003^b	0.299 ± 0.012^b	0.302 ± 0.002^{bc}
RX	0.273 ± 0.009^c	0.323 ± 0.002^c	0.331 ± 0.006^d
Control	0.267 ± 0.004^{bc}	0.317 ± 0.006^{bc}	0.313 ± 0.004^c

Values with different lowercase letters in each column are significant differences by Duncan's multiples range test ($p < 0.05$)

Fig. 2 Scanning electron micrographs of freeze-dried *L. plantarum* TISTR 2075 powders with different protective agents (A) R (B) RF (C) RG (D) RI (E) RP (F) RX and (G) control

Table 2 Specific rate of cell death (k , 1/day) and glass transition temperatures (T_g) of freeze-dried probiotic *L. plantarum* TISTR 2075 in various protective agents during storage at 4 °C for 180 days and 30 °C for 90 days

Protective agents	Specific rate of cell death at storage temperature				T_g (°C)	
	4 °C		30 °C		Storage temperature	
	k_4 (1/day)	R^2	k_{30} (1/day)	R^2	4 °C	30 °C
R	5.39×10^{-2}	0.946	1.39×10^{-1}	0.792	20.8	43.9
RF	1.20×10^{-2}	0.849	5.79×10^{-2}	0.947	22.3	49.4
RG	3.04×10^{-2}	0.923	6.81×10^{-2}	0.864	14.3	42.9
RI	5.10×10^{-2}	0.842	2.24×10^{-1}	0.705	22.4	49.4
RP	3.34×10^{-2}	0.907	9.26×10^{-2}	0.898	25.4	50.1
RX	1.48×10^{-2}	0.959	1.52×10^{-1}	0.948	14.2	46.1
Control	1.01×10^{-1}	0.947	5.47×10^{-1}	0.832	− 0.8	37.8

after 105 and 40 days storage at 4 and 30 °C, respectively. It was observed that storage freeze-dried probiotic powders at 4 °C maintained high viable cell number of probiotic strain. At low temperature, metabolism of cells decreases resulted in lower rate of detrimental chemical reaction (Heidebach et al., 2010). Additionally, storage temperatures could affect the physical state of freeze-dried probiotic powders in terms of glass transition temperature (T_g). As shown in Table 2, T_g values of freeze-dried probiotic in different protective agents were 14.2–25.4 and 42.9–50.1 °C at storage temperature of 4 and 30 °C, respectively. T_g values of freeze-dried probiotic without protectants stored at 4 and 30 °C were lower than that of T_g values of the freeze-dried probiotic with protectants. Moreover, it was observed that there was a little difference in T_g values of various freeze-dried probiotic powders at the same temperature. This was probably because rice protein might form an incompatible mixture with prebiotic resulted in a little interaction between them. Thus, a little change in T_g values of protein was detected. Focusing on the differences between storage temperatures (T_{storage}) and T_g values of freeze-dried probiotic powders, T_g values of various matrices were 10.2–21.4 °C higher than T_{storage} . A great difference in $T_{\text{storage}} - T_g$ of materials could maintain the viable cell number of probiotic bacteria than cell in matrices stored at temperature close to T_g . The temperature differences above glass transition temperature have an impact on the nonenzymatic browning (Passot et al., 2012). A large increase in the nonenzymatic browning rate was reported at a range of 2–40 °C above T_g (Karmas et al., 1992). Another possible reason resulted in the decrease of viable cell number of freeze-dried probiotic during subsequent storage was lipid oxidation. Santivarangkna (2015) supported that the stability of probiotic decreases proportionally with the increasing storage temperature. The decrease in viable cell number during storage of freeze-dried probiotic powders was related to further membrane damage caused by phospholipid degradation (Albadran et al., 2015). The change in the fatty acid profile (the

unsaturated/saturated fatty acids index: u/s index) of the membrane was proposed to define the phospholipid degradation. Unsaturated fatty acids declined during the storage time (Castro et al., 1996). The increase in the proportion of saturated fatty acids causes an increase in the membrane phase transition temperature resulting in a decrease in membrane fluidity and an increase in membrane leakage during rehydration. In addition, free radicals from lipid oxidation may directly induce DNA damages during prolonged storage (Santivarangkna et al., 2008).

Moisture level in dry probiotic product also plays an important role in the maintenance of cell viability. The result of the water activity was given in Table 1. The water activities of freeze-dried probiotic *L. plantarum* TISTR 2075 with different protectants varied from 0.274 to 0.323 and 0.271 to 0.331 after storage at 4 and 30 °C, respectively. Santivarangkna (2015) revealed that the stability of probiotic increase with decreasing moisture content. Furthermore, Chávez and Ledebor (2007) suggested that water activity values ranged from 0.11 to 0.23 and the moisture content below 5% could be able to avoid cell inactivation during storage. Moreover, it has been reported that an increase in water activity can cause detrimental effects on probiotics during storage (Xu et al., 2016).

Survival of freeze-dried *L. plantarum* TISTR 2075 under exposure to simulated gastrointestinal tract conditions

Probiotic cells should be able to tolerate against acidic condition of the stomach as well as the bile salt and pancreatic enzyme in the small intestine to reach the colonization area in gut. Therefore, the protective capability of freeze-drying media on the survival of probiotic *L. plantarum* TISTR 2075 under exposure to simulated gastrointestinal tract conditions at pH 2.0 and 8.0 was carried out. As shown in Fig. 3, freeze-dried probiotic cells without protective agent were susceptible to gastric condition and the viable cell number reduced to undetectable levels after

Fig. 3 Viable cell number (solid lines) and survival rate (dotted lines) of freeze-dried probiotic *L. plantarum* TISTR 2075 in various protective agents during sequential exposure to simulated gastric and

small intestinal juice (A) R (B) RF (C) RG (D) RI (E) RP (F) RX (G) control and (H) active cells

exposure for 60 min, while active cells exhibited the viability loss of 4.25 log CFU/g (0.01% survival rate) after exposure to simulated gastric juice pH 2.0 for 180 min. The presence of freeze-drying media improved acid tolerance of the strain. All freeze-dried probiotic treatments exhibited certain resistance abilities to simulated gastric juice pH 2.0 for 180 min. RX and RF have a great protective effect with the loss of viable cell number of 0.09 and 0.13 log CFU/mL, respectively. The viability of freeze-dried cells in R, RG, RI, and RP declined proportionally with exposure time. The reduction in viable cell count of 0.50–2.41 log CFU/mL with the survival rate of 29.59–0.38% was achieved. This was in accordance with the report of Reddy et al. (2009) that the survival rate of freeze-dried *L. plantarum* with skim milk + fructooligosaccharide was more than 90% after exposure to simulated gastric juice pH 2.0. During freeze-drying process, osmotic fluxes may cause the rupture of cell membranes. This could probably affect the proton permeability of plasma membrane contributing to the regulation of intracellular pH which directly relates to the acid-stress response of microorganism (Chotiko and Sathivel, 2014). Furthermore, the buffering capacity of coating materials could temporarily increase the stomach pH resulting in lower loss of viability (Shamekhi et al., 2013).

The ability to survive under small intestinal juice in the human intestine so as to take up residence and proliferation in human large intestine is another influential characteristic of probiotic. Consequently, the survival of freeze-dried probiotic *L. plantarum* TISTR 2075 under exposure to simulated small intestinal juice pH 8.0 with 0.45% bile salt after sequential of simulated gastric juice was also determined. All treatments with freeze-drying media except for RG were capable to survive during exposure to simulated small intestinal juice condition for 240 min with the final survival rates ranged from 0.02 to 61.50%. Statistical

analysis revealed significant difference ($p < 0.05$) in the relative survival rate for the cell in simulated gastric and small intestinal conditions. RX and RF are effective materials to protect the probiotic cells from in gastrointestinal tract environment. Freeze-dried cells in RX and RF were equally stable. There was no significant difference in survival rate after sequential exposure to simulated gastric and small intestinal juice. Nevertheless, freeze-dried probiotic *L. plantarum* TISTR 2075 with RG and active cell were susceptible to simulated small intestinal juice pH 8.0 for 240 min. No viable cell number was detected after sequential exposure. Rajam and Anandharamakrishnan (2015) revealed that relative viability of *L. plantarum* MTCC 5422 encapsulated in fructooligosaccharide and denatured whey protein isolate remained stable in adverse simulated acidic and bile environments. The denaturation of proteins resulted in more exposure of hydrophobic sites on their surfaces which exhibited the poor permeation of gastrointestinal fluid.

Antimicrobial activity of freeze-dried cell

Freeze-dried *L. plantarum* TISTR 2075 with protectants showed pathogenic inhibition of *E. coli* O157:H7 DMST 12743 and *S. typhimurium* ATCC 13311, with inhibition zones of 15.57–15.83 mm and 15.90–16.10 mm, respectively (Table 3). While, freeze-dried probiotic cells without protectant exhibited the lowest antimicrobial activity. This agree with the work of Shobharani and Agrawal (2011) that freeze-dried culture of *Leuconostoc mesenteroides* MTCC 5209 retained the antimicrobial activity against foodborne pathogens *E. coli* and *Salmonella typhi*. It could be indicated that the inhibitory activity of probiotic could be maintained by protective agents during freeze-drying process.

Table 3 Cell surface hydrophobicity and antimicrobial activity of freeze-dried probiotic *L. plantarum* TISTR 2075 against *E. coli* O157:H7 DMST 12743 and *S. typhimurium* ATCC 13311

Protective agents	Cell surface hydrophobicity (%)	Antimicrobial activity (mm $\pm$ SD)	
		<i>E. coli</i> O157:H7 DMST 12743	<i>S. typhimurium</i> ATCC 13311
R	77.75 $\pm$ 3.54 ^{ab}	15.57 $\pm$ 0.12 ^{bc}	15.90 $\pm$ 0.14 ^{ab}
RF	79.57 $\pm$ 1.99 ^{ab}	15.73 $\pm$ 0.06 ^{ab}	16.07 $\pm$ 0.07 ^a
RG	76.12 $\pm$ 4.90 ^{ab}	15.83 $\pm$ 0.15 ^a	16.03 $\pm$ 0.21 ^a
RI	79.26 $\pm$ 1.45 ^{ab}	15.77 $\pm$ 0.15 ^a	16.07 $\pm$ 0.14 ^a
RP	80.11 $\pm$ 3.92 ^a	15.83 $\pm$ 0.06 ^a	16.03 $\pm$ 0.21 ^a
RX	79.99 $\pm$ 3.72 ^{ab}	15.80 $\pm$ 0.10 ^a	16.10 $\pm$ 0.14 ^a
Control	71.27 $\pm$ 2.43 ^b	15.43 $\pm$ 0.06 ^c	15.73 $\pm$ 0.07 ^b
Active cells	80.30 $\pm$ 2.71 ^a	15.83 $\pm$ 0.01 ^a	16.10 $\pm$ 0.01 ^a

Values with different lowercase letters in each column are significant differences by Duncan's multiple range test ($p < 0.05$)

Cell surface hydrophobicity

Cell surface hydrophobicity related to the adhesion ability to intestinal mucus layer. The retention of cell surface hydrophobicity of freeze-dried probiotic *L. plantarum* TISTR 2075 in different protective agents was shown in Table 3. The degree of cell surface hydrophobicity of freeze-dried probiotic was relatively high varied in the range of 76.12–80.11% which was not significant difference from active cells. However, freeze-dried cells without protectant showed the lowest degree of cell hydrophobicity of 71.27%. It could be indicated that protective agents could retain probiotic functional property after dehydration process. Dianawati and Shah (2011) suggested that phospholipids and glycolipids in cell membrane layer of bacteria were sustained resulted in high degree of cell surface hydrophobicity.

In conclusion, probiotic *L. plantarum* TISTR 2075 has the ability to survive after freeze-drying process. The protective agents could maintain not only the viability during freeze-drying and storage but also the probiotic functionality of the strain. Rice protein supplemented with prebiotics has the protective capability to protect probiotic cells during freeze-drying. Especially, RF could effectively be used as an appropriate protective agent for the preparation of freeze-dried *L. plantarum* TISTR 2075 and subsequent storage with high viability coupled with a high degree of retention of key functional properties. The T_g of the protective matrices varied with storage temperatures. Storage temperature above T_g maintained the viability of freeze-dried probiotic cells. The results indicated that this protective agent could be used as vehicle for delivery of high numbers of probiotic through the human gastrointestinal tract and also ready for incorporation into food products as potential probiotic culture. This research could further lead to the development of new synbiotic functional foods. The study of probiotic *L. plantarum* emphasized on the encapsulation should be carried out.

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References

- Albadran HA, Chatzifragkou A, Khutoryanskiy VV, Charalampopoulos D. Stability of probiotic *Lactobacillus plantarum* in dry microcapsules under accelerated storage conditions. *Food Res. Int.* 74: 208–216 (2015)
- Broeckx G, Vandenheuveld D, Claes IJJ, Lebeer S, Kiekens F. Drying techniques of probiotic bacteria as an important step towards the development of novel pharmabiotics. *Int. J. Pharm.* 505: 303–318 (2016)
- Burnside E. Microencapsulation in the food industry. Academic Press: San Diego. 241–252 (2014)
- Castro HP, Teixeira PM, Kirby R. Changes in the cell membrane of *Lactobacillus bulgaricus* during storage following freeze-drying. *Biotechnol. Lett.* 18: 99–104 (1996)
- Chávez BE, Ledebor AM. Drying of probiotics: Optimization of formulation and process to enhance storage survival. *Dry. Technol.* 25: 1193–1201 (2007)
- Chotiko A, Sathivel S. Effects of enzymatically-extracted purple rice bran fiber as a protectant of *L. plantarum* NRRL B-4496 during freezing, freeze drying, and storage. *LWT Food Sci. Technol.* 59: 59–64 (2014)
- Desmond C, Stanton C, Fitzgerald GF, Collins K, Paul Ross R. Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. *Int. Dairy J.* 12: 183–190 (2002)
- Dhewa T, Pant S, Mishra V. Development of freeze dried synbiotic formulation using a probiotic strain of *Lactobacillus plantarum*. *J. Food Sci. Technol.* 51: 83–89 (2014)
- Dianawati D, Mishra V, Shah NP. Survival of *Bifidobacterium longum* 1941 microencapsulated with proteins and sugars after freezing and freeze drying. *Food Res. Int.* 51: 503–509 (2013)
- Dianawati D, Shah NP. Survival, acid and bile tolerance, and surface hydrophobicity of microencapsulated *B. animalis* ssp. *lactis* Bb12 during storage at room temperature. *J. Food Sci.* 76: M592–M599 (2011)
- Gwak HJ, Lee, JH, Kim TW, Choi HJ, Jang JY, Lee S, Park HW. Protective effect of soy powder and microencapsulation on freeze-dried *Lactobacillus brevis* WK12 and *Lactococcus lactis* WK11 during storage. *Food Sci. Biotechnol.* 24(6): 2155–2160 (2015)
- Halim M, Mohd Mustafa N, Othman M, Wasoh H, Kapri M, Ariff AB. Effect of encapsulant and cryoprotectant on the viability of probiotic *Pediococcus acidilactici* ATCC 8042 during freeze-drying and exposure to high acidity, bile salts and heat. *LWT Food Sci. Technol.* 81: 210–216 (2017)
- Heidebach T, Först P, Kulozik U. Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. *J. Food Eng.* 98: 309–316 (2010)
- Hubálek Z. Protectants used in the cryopreservation of microorganisms. *Cryobiology* 46: 205–229 (2003)
- Jofré A, Aymerich T, Garriga M. Impact of different cryoprotectants on the survival of freeze-dried *Lactobacillus rhamnosus* and *Lactobacillus casei/paracasei* during long-term storage. *Benef. Microbes* 6(3): 381–386 (2015)
- Kalman DS. Amino acid composition of an organic brown rice protein concentrate and isolate compared to soy and whey concentrates and isolates. *Foods* 3: 394–402 (2014)
- Karmas R, Pilar Buera M, Karel M. Effect of glass transition on rates of nonenzymic browning in food systems. *J. Agric. Food Chem.* 40: 873–879 (1992)
- Lapsiri W, Bhandari B, Wanchaitanawong P. Stability and probiotic properties of *Lactobacillus plantarum* spray-dried with protein and other protectants. *Dry. Technol.* 31: 1723–1733 (2013)
- Lapsiri W, Nitisinprasert S, Wanchaitanawong P. *Lactobacillus plantarum* strains from fermented vegetables as potential probiotics. *Kasetsart J. (Nat. Sci.)* 45: 1071–1082 (2011)
- Lu Y, Huang L, Yang T, Lv F, Lu Z. Optimization of a cryoprotective medium to increase the viability of freeze-dried *Streptococcus thermophilus* by response surface methodology. *LWT Food Sci. Technol.* 80: 92–97 (2017)
- Michida H, Tamalampudi S, Pandiella SS, Webb C, Fukuda H, Kondo A. Effect of cereal extracts and cereal fiber on viability of *Lactobacillus plantarum* under gastrointestinal tract conditions. *Biochem. Eng. J.* 28: 73–78 (2006)

- Montel Mendoza G, Pasteris SE, Otero MC, Fatima Nader-Macías ME. Survival and beneficial properties of lactic acid bacteria from ranculture subjected to freeze-drying and storage. *J. Appl. Microbiol.* 116: 156–166 (2014)
- Passot S, Cenard S, Douania I, Tréléa IC, Fonseca F. Critical water activity and amorphous state for optimal preservation of lyophilized lactic acid bacteria. *Food Chem.* 132: 1699–1705 (2012)
- Rajam R, Anandharamakrishnan C. Microencapsulation of *Lactobacillus plantarum* (MTCC 5422) with fructooligosaccharide as wall material by spray drying. *LWT Food Sci. Technol.* 60: 773–780 (2015)
- Reddy KBPK, Awasth SP, Madhu AN, Prapulla SG. Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. *Food Biotechnol.* 23: 243–265 (2009)
- Romano N, Tymczyszyn E, Mobili P, Gomez-Zavaglia A. Prebiotics as protectants of lactic acid bacteria. In: Probiotics, Prebiotics, and Synbiotics. Watson R and Preedy VR (ed). Academic Press, San Diego, CA, USA (2016)
- Santivarangkna C, Kulozik U, Foerst P. Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. *J. Appl. Microbiol.* 105: 1–13 (2008)
- Santivarangkna C. *Advances in Probiotic Technology*. CRC Press, Boca Raton, FL. 286–310 (2015)
- Santos MI, Gerbino E, Araujo-Andrade C, Tymczyszyn EE, Gómez-Zavaglia A. Stability of freeze-dried *Lactobacillus delbrueckii* subsp. *bulgaricus* in the presence of galacto-oligosaccharides and lactulose as determined by near infrared spectroscopy. *Food Res. Int.* 59: 53–60 (2014)
- Savedboworn W, Kerdwan N, Sakorn A, Charoen R, Tipkanon S, Pattayakorn K. Role of protective agents on the viability of probiotic *Lactobacillus plantarum* during freeze drying and subsequent storage. *Int. Food Res. J.* 24: 787–794 (2017)
- Savedboworn W, Wanchaitanawong P. Viability and probiotic properties of *Lactobacillus plantarum* TISTR 2075 in spray-dried fermented cereal extracts. *Maejo Int. J. Sci. Technol.* 9: 382–393 (2015)
- Schwab C, Vogel R, Gänzle MG. Influence of oligosaccharides on the viability and membrane properties of *Lactobacillus reuteri* TMW1.106 during freeze-drying. *Cryobiology* 55: 108–114 (2007)
- Shamekhi F, Shuhaimi M, Ariff A, Manap YA. Cell viability of microencapsulated *Bifidobacterium animalis* subsp. *lactis* under freeze-drying, storage and gastrointestinal tract simulation conditions. *Folia Microbiol.* 58: 91–101 (2013)
- Shobharani P, Agrawal R. Enhancement of cell stability and viability of probiotic *Leuconostoc mesenteroides* MTCC 5209 on freeze drying. *Int. J. Dairy Technol.* 64: 276–286 (2011)
- Xu M, Gagné-Bourque F, Dumont MJ, Jabaji S. Encapsulation of *Lactobacillus casei* ATCC 393 cells and evaluation of their survival after freeze-drying, storage and under gastrointestinal conditions. *J. Food Eng.* 168: 52–59 (2016)