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Survival of probiotics *Lactobacillus acidophilus* TISTR1338 in a synbiotic-supplemented plant-based protein powder under various pH and temperatures

Panisa Ardsiri[1] , Araya Chaoruangrit[1] , Jaksuma Pongsetkul[2] , Sri Charan Bindu Bavisetty[3] , Kullapapruk Piewthongngam[4] , Amporn Sae-eaw[1] and Kantiya Petsong[1,*]

[1]Department of Food Technology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand
[2]School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand
[3]Department of Fermentation Technology, School of Food-Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand
[4]Faculty of Economics, Khon Kaen University, Khon Kaen, Thailand
[*]Corresponding author: kantpe@kku.ac.th

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Abstract

The demand of plant-based protein has been remarkably increased, leading to the development of various plant-based protein products. Supplementation of synbiotic is an effective approach to improve food function. This study aimed to develop the formulation of plant-based protein product supplemented with synbiotic which provides high survivability of probiotic through various harsh conditions. Pea protein isolate (PPI), inulin, and glycerol at ratio 2:1:1 was supplemented with probiotic strain (*Lactobacillus acidophilus* TISTR1388) to develop as encapsulated synbiotic plant-based protein powder (SPBP). The developed formulation provided probiotic survival rate after freeze-drying and gastro-intestinal (GI) tract conditions at 95.0±2.1% and 62.4±6.1%, respectively. Whereas, probiotic as free-cells showed survival rate after freeze-drying at 78.9±0.4% and no survival cell was observed after GI tract digestion. Scanning Electron Microscope (SEM) analysis demonstrated SPBP's remarkable capacity to perfectly entrap probiotic cells with no visible cell damage. Herein, the stability of the SPBP was tested through various pH levels (3, 5, 7, and 9) and temperature levels (6±2°C, 26±2°C, and 55±2°C) in order to investigate the possibility of applying this product to various food categories. Results showed that developed SPBP survived through all tested pH and temperature levels for 3 h at least 88.9±6.6%. Shelf-life evaluation showed that SPBP provided survival rate of probiotic cells up to 96.2±2.4% an a_w of 0.09±0.01 after 12 weeks of refrigerated storage. Overall, results revealed that SPBP had a strong possibility of being employed as a probiotic stabilizer that may be further developed for a functional food product.

Keyword: Plant-based protein powder, Synbiotic, Encapsulation, Harsh conditions, Stability

1. Introduction

Probiotics have been recognized as the provider of health benefits and recommended to use as metabolic syndrome disorder treatment [1]. Moreover, synbiotics (combination of probiotics and prebiotics) have been reported to provide more potential than using of probiotics alone, since prebiotics could improve the survivability of probiotics, and stimulate the growth of beneficial microorganisms leading to improve of gut health [2]. In addition, using of encapsulation is the effective approach to improve the stability of probiotics through the harsh conditions [3]. In the past two decades, plant-based protein products have been increasingly in demand [4,5]. Plant-based protein have gained much of interest as highly nutritious and sustainable source of essential amino acids [4,5]. The advantages of plant-based proteins over protein from animal origin have been reported as 38-91% less agricultural area, 53-95% less water utilization, and 69-92% less carbon emissions compared to meat-based proteins [6]. Nowadays, pea protein has gained increased interest by the food industry worldwide due to the richness of its nutritional values, and providing health benefits, availability, and low cost [7]. In terms of wall

material for encapsulation, proteins act as one of the most effective materials to protect living cells from harsh conditions [8]. Inulin is well-known as a prebiotic with various putative health benefits [9]. Supplementation of inulin in the developed plant-based protein powder could be useful for health beneficial microbes, leading to improve health and well-being of consumers. Glycerol is considered as a high potential cryoprotectant for bacteria [10]. By the kosmotropic properties, glycerol can form hydrogen bonding with water molecules, resulting in the difficulty of ice crystals formation which acts as the main cause to destroy microbial structure [11]. Combination of pea protein, inulin, and glycerol as wall material to protect probiotic through the harsh conditions is highly interesting to develop as a high value functional food. This study thus aimed to develop a plant-based protein powder containing synbiotic by encapsulation, to improve the stability of probiotic and health functions. Synbiotic supplemented plant-based protein in this study provided high stability of probiotic in various harsh conditions such as severe pH and digestive enzymes, as well as various temperature levels. Suggesting that this study achieved the goal of developing a synbiotic plant-based protein powder (SPBP) which could maintain effective probiotics to provide potential health benefits to consumers.

2. Materials and methods

2.1 Chemicals and reagents

Materials used for encapsulation comprised of pea protein isolate (PPI; Ingredion, Singapore), inulin (Fuji Nihon Thai Inulin Co., Ltd.), and glycerol (diethylene glycol ≥ 98%, Carlo Erba, Italy). All reagents for simulating GI tract; including pepsin, bile salt, and porcine pancreatin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Bacterial strain and culture conditions

The probiotic strain used in this study was *Lactobacillus acidophilus* TISTR1338 (purchased from TISTR Culture Collection, Thailand). Working stocks were prepared by 20% glycerol mixed with the culture at log phase and stored at -30°C. De Man, Rogosa and Sharpe (MRS) (HiMedia Laboratories Pvt. Ltd., India) was performed as the culture media. The culture was prepared by inoculating an isolated colony in MRS broth and incubated in anaerobic jar at 37°C, for 48-72 h. To prepare bacterial suspension as core material for encapsulation, the culture was centrifuged and cells washed with 0.85% NaCl. Probiotic suspension (approx. 10^9 CFU/mL) was prepared by using 0.85% NaCl.

2.3 Encapsulation of encapsulated synbiotic plant-based protein powder (SPBP)

In this study, the mixture of PPI (10%), inulin (5%), and glycerol (5%) were used as wall material for encapsulation. PPI as the major component was prepared by rehydrating with sterile distilled water and incubated at 10°C for 18 h. Unfolding structure of protein was performed by heating at 80°C for 30 min in water bath. The sterile inulin and glycerol were then added and mixed by homogenizing at 5,000 rpm for 15 min using homogenizer (Nihon Seiki Kassha, Ltd, Japan). Probiotic suspension (10^{10} CFU/mL) was added to the mixture as a final step and homogenized with the same conditions. For free-cell form, 100 mL of probiotic suspension was homogenized at the same condition to SPBP for 30 min. The prepared SPBP and probiotic as free-cell form were transferred to plastic zip-lock, then transformed to solid phase and stored at -60°C for 48 h before freeze-drying at -50°C under vacuum by a laboratory scale freeze-dryer (Gamma 2-16LSC, Christ, Germany) to transform the samples as dry powder. The experiment was conducted for three separate batches of production.

2.4 Characterization of encapsulated synbiotic plant-based protein powder (SPBP) by scanning electron microscope (SEM)

Physical characterization of SPBP and free-cell form as dry powder after freeze-drying were examined by using Field-Emission Scanning Electron Microscope (FE-SEM) (Tescan: Mira, Czech Republic). The samples were fixed on the aluminum stubs before vacuum coated with a layer of gold. Visualization was observed under various magnifications (2kx and 20kx for SPBP; 60 kx and 150 kx for free-cell form) at acceleration voltage acceleration of 2 kV and 5 kV.

2.5 Stability studies of encapsulated synbiotic plant-based protein powder (SPBP) under harsh conditions

2.5.1 Survival rate of encapsulated synbiotic plant-based protein powder (SPBP) after freeze-drying

After freeze-drying, the fresh amount of SPBP (1 g) was sampled and mixed with 30 mL of phosphate buffer saline (PBS, pH 7.4). Standard plate count (spread plate technique) on MRS agar was performed to enumerate the number of living probiotic after transformation. The plates were incubated at 37°C in anaerobic jar for 48-72 h. The experiment was conducted in three biological replications. Survival rate (%) of probiotic after freeze-drying was calculated by the following formula which was adapted from a previous study [12]. Number of probiotic cells recovered from the powder divided by the initial number of probiotic cells added for encapsulation multiply by 100.

2.5.2 Survival rate of encapsulated synbiotic plant-based protein powder (SPBP) under gastro-intestinal (GI) tract

This study demonstrated survival rate (%) of SPBP under GI tract by simulating the continuous digestive system performed three replications. The method followed previous study with some modification [13]. An amount (3 g) of SPBP was mixed with saline solution (140 mmol/L KCl and 5 mmol/L NaCl; 20 mL). The mixture was incubated at 37°C in shaking water bath (95 rpm). After 10 min, the mixture was adjusted to pH 2 with 1 M HCl solution before adding pepsin solution (pepsin 0.04 g/mL in 0.1 M HCl, pH 2.0) 1 mL. The agitation and incubation conditions were continuously conducted for 1 h to perform gastric digestion. Thereafter, intestinal digestion was conducted by adjusting pH of the mixture to 5.3 using 0.9 M sodium bicarbonate then added 200 µL of bile salt (3 mg/L) and 100 µL of porcine pancreatin (80 mg/mL), respectively. The incubation time for the step of intestinal digestion was performed for 3 h. Survival rate (%) of probiotic at each stage of digestion was calculated by using the formula mentioned above. In this study, control treatment was performed by probiotic as free-cell form.

2.5.3 Survival rate of encapsulated synbiotic plant-based protein powder (SPBP) under various pH levels

To study the possibility of using SPBP as food supplement, various pH levels (3, 5, 7, and 9) were used to simulate the pH of food products. The protocol followed a previous study with minor modification [8]. Survival rate (%) of SPBP under various pH levels was compared to probiotic as free-cell form. PBS solutions represented various pH were prepared using 1 M HCl or 1 M NaOH. SPBP as dry powder 1 g or probiotic as free-cell form (1 mL), mixed individually with the solution and represented each pH level at ratio 1:10. The mixtures were incubated at room temperature (26±2°C) for 3 h. Each sample (1 mL) was taken every hour of incubation to enumerate the number of living probiotic cells after treated with various pH levels.

2.5.4 Survival rate of encapsulated synbiotic plant-based protein powder (SPBP) under various temperature levels

Temperature has been considered as the important factor to destroy probiotic survivability. This study demonstrated the survival rate (%) of SPBP at temperature 6±2°C, 26 ±2°C, and 55±2°C to perform refrigeration temperature, ambient temperature, and high temperature, respectively. SPBP (1 g) or probiotic as free-cell form (1 mL) was suspended in PBS solution (pH 7.4) at ratio 1:10. The mixtures were incubated at 6±2°C, 26±2°C, and 55±2°C individually. The samples were collected every hour for 3 h to enumerate the number of living probiotic cells.

2.6 Stability and chemical property change of encapsulated synbiotic plant-based protein powder (SPBP) during storage conditions

Stability and water activity (a_w) during storage of SPBP were studied at 26±2°C (represented ambient temperature) and 6±2°C (represented refrigeration temperature). The experiments were conducted for 12 weeks. Aluminum laminated foil bags were used as the packaging for storing the samples. Enumeration of living probiotic cells (plate count method) and a_w measurement (Meter Aqualab PRE; METER Group, Inc.) were conducted every week for 12 weeks.

2.7 Statistical analysis

All experiments were conducted in three biological replications. Data were analyzed using IBM SPSS Statistics 26. Mean comparison between (i) survival rate (%) of probiotic as SPBP form and free-cell form after freeze-drying, gastric digestion, and intestinal digestion, (ii) survival rate (%) of probiotic as SPBP form and free-cell form at the same temperature and time period, (iii) a_w of SPBP at refrigeration temperature and ambient temperature at the same time period, and (iv) number of probiotic from SPBP form and free-cell form at the same pH and time period were analyzed using Student's t-test. Analysis of Variance (ANOVA) was used to compare survival rate (%) of probiotic as SPBP form or free-cell form at different time periods (same temperature), a_w of SPBP at different time periods (same temperature), and number of probiotics from SPBP form or free-cell form at different time periods (same pH). Comparison of means was declared by Duncan's multiple range tests ($p < 0.05$).

3. Results

3.1 Stability of encapsulated synbiotic plant-based protein powder (SPBP) against various harsh conditions

In this study, the stability of SPBP against various harsh conditions (including freeze-drying, GI tract, various pH levels, and various temperature levels) was compared to *L. acidophilus* as free-cell form. For freeze-drying and GI tract conditions (Figure 1), SPBP showed survival rate at 95.0±2.1% and 62.4±6.1%, respectively, whereas free-cell form showed survival rate after freeze-drying only 78.9±0.4% and no living cell was detected after intestinal digestion. The stability of SPBP against various pH levels (pH 3, pH 5, pH 7, and pH 9) and temperature levels (refrigeration temperature; 6±2°C, ambient temperature; 26±2°C, and high temperature; 55±2°C) was also compared to free-cell form (Figure 2 and Figure 3). This study showed the remarkable results that SPBP provided high efficiency to protect probiotic through all tested pH and temperature levels. SPBP showed non-significant difference of probiotic survivability among tested period (3 h) at all pH levels. Especially, at pH 3 and pH 9, which represented severe acid and alkaline in this study. Whereas free-cell form showed no probiotic survival at pH 3 and pH 9 after 3 h of treatment. In addition, free-cell form showed the reduction of living probiotic at 2.2±0.0 log CFU/mL (at pH 5) and 1.2±0.3 log CFU/mL (at pH 7) after 3 h of treatment. Among tested temperature levels, the reduction of living probiotic from SPBP was detected only at the condition of high temperature (55±2°C). After 1 h, 2 h, and 3 h of treatment, the reduction of living probiotic was detected at 0.1± 0.0 log CFU/mL, 1.2±0.6 log CFU/mL, and 1.0±0.3 log CFU/mL, respectively. For free-cell form, the number of living probiotic at 1 h of treatment showed reduction at 3.0±0.4 log CFU/mL. In addition, the number of living probiotic from free-cell form was not detected since 2 h of treatment.

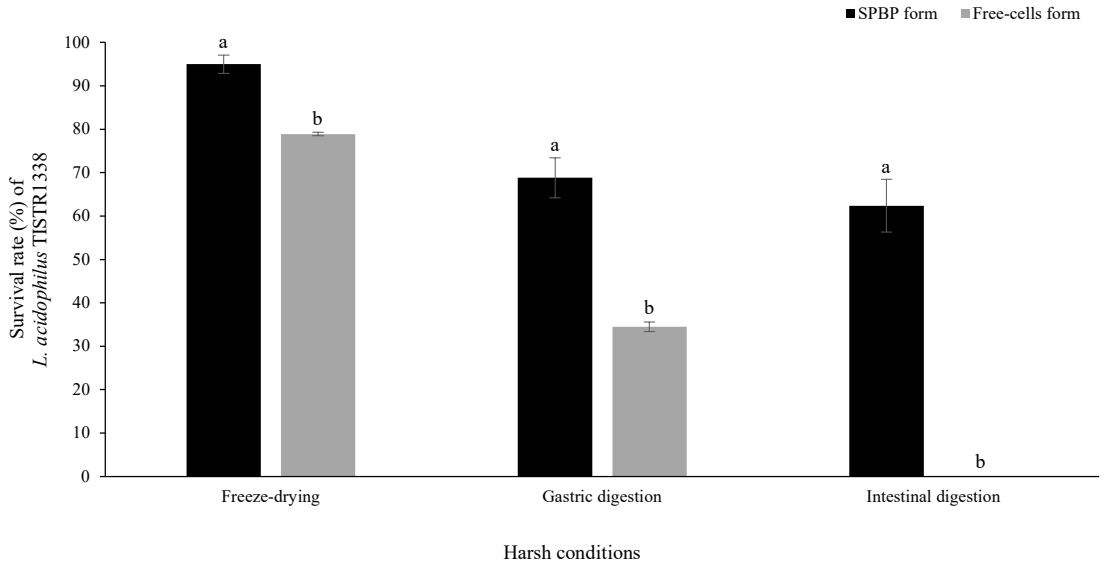

Figure 1 Survival rate (%) of probiotic as SPBP form (black bars) compared to free-cell form (grey bars) after freeze-drying, gastric digestion, and intestinal digestion. Lowercase letters on bars at the same condition indicate significant difference ($p < 0.05$) between SPBP form and free-cell form.

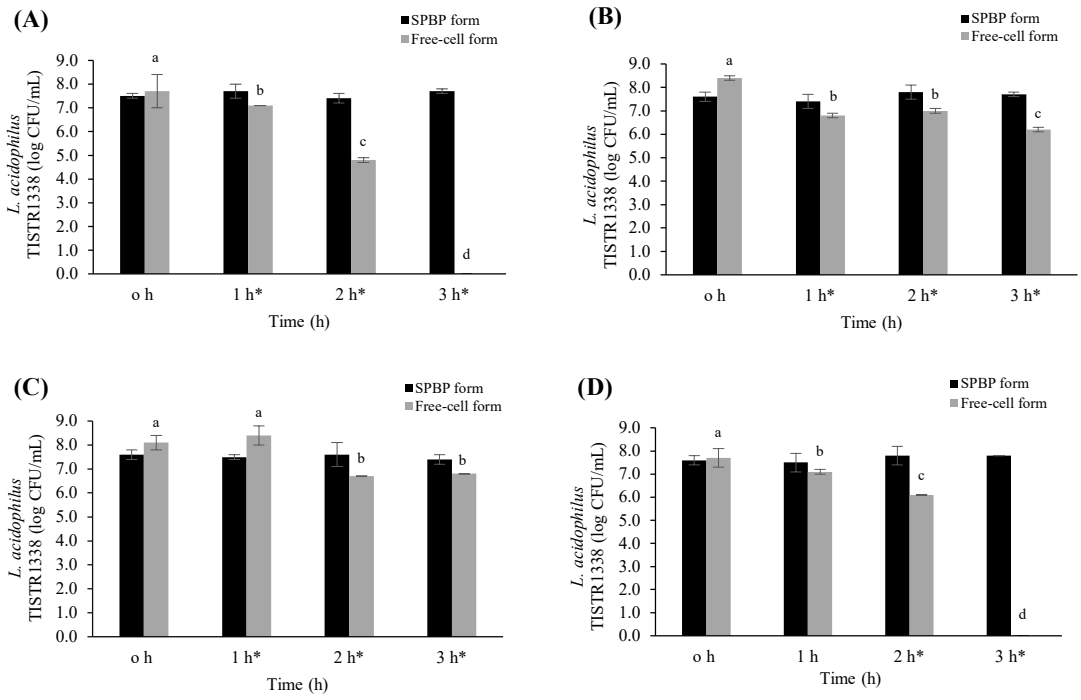

Figure 2 Stability of probiotic as SPBP form (black bars) and free-cell form (grey bars) in solutions at pH 3 (A), pH 5 (B), pH 7 (C), and pH 9 (D). The bars represent standard deviation (n = 3). Lowercase letters on bars indicate significant difference ($p < 0.05$) among free-cell form at the different time. The asterisk sign (*) indicate the difference between SPBP and free-cell form at a specific time. Bars without the letters indicate non-significant difference ($p < 0.05$) among SPBP form or free-cell form.

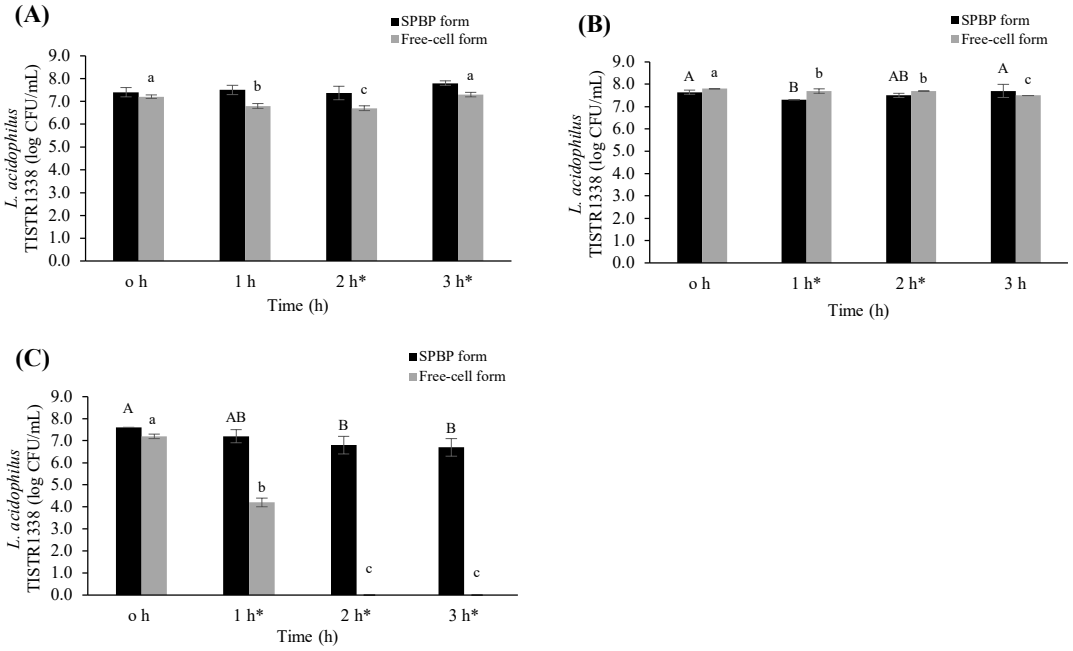

Figure 3 Stability of probiotic as SPBP form (black bars) and free-cell form (grey bars) in solutions at refrigeration temperature; 6±2°C (A), ambient temperature; 26±2°C (B), and high temperature; 55±2°C (C). Uppercase letters on bars indicate significant difference ($p < 0.05$) among SPBP form at the different time. Lowercase letters on bars indicate significant difference ($p < 0.05$) among free-cell form at the different time. The asterisk sign (*) indicate the difference between SPBP and free-cell form at a specific time. Bars without the letters indicate non-significant difference ($p < 0.05$) among SPBP form or free-cell form.

3.2 Physical characterization of encapsulated synbiotic plant-based protein powder (SPBP) after freeze-drying

The physical characteristics of SPBP and probiotic as free-cell after freeze-drying were revealed by SEM analysis (Figure 4). The images indicated the remarkable potential of developed wall material in this study to protect probiotic cells from processing condition (freeze-drying). SEM analysis showed the complete healthy cells of probiotic without shrinkage or broken cells. Moreover, the results showed that bacterial cells were entrapped in the surface of wall material structures, indicating physio-chemical interaction between wall materials and bacterial cells. The structure of probiotic as free-cell under SEM showed damaged cells with numerous of porous structure. In addition, the clumping of cell structure was also observed.

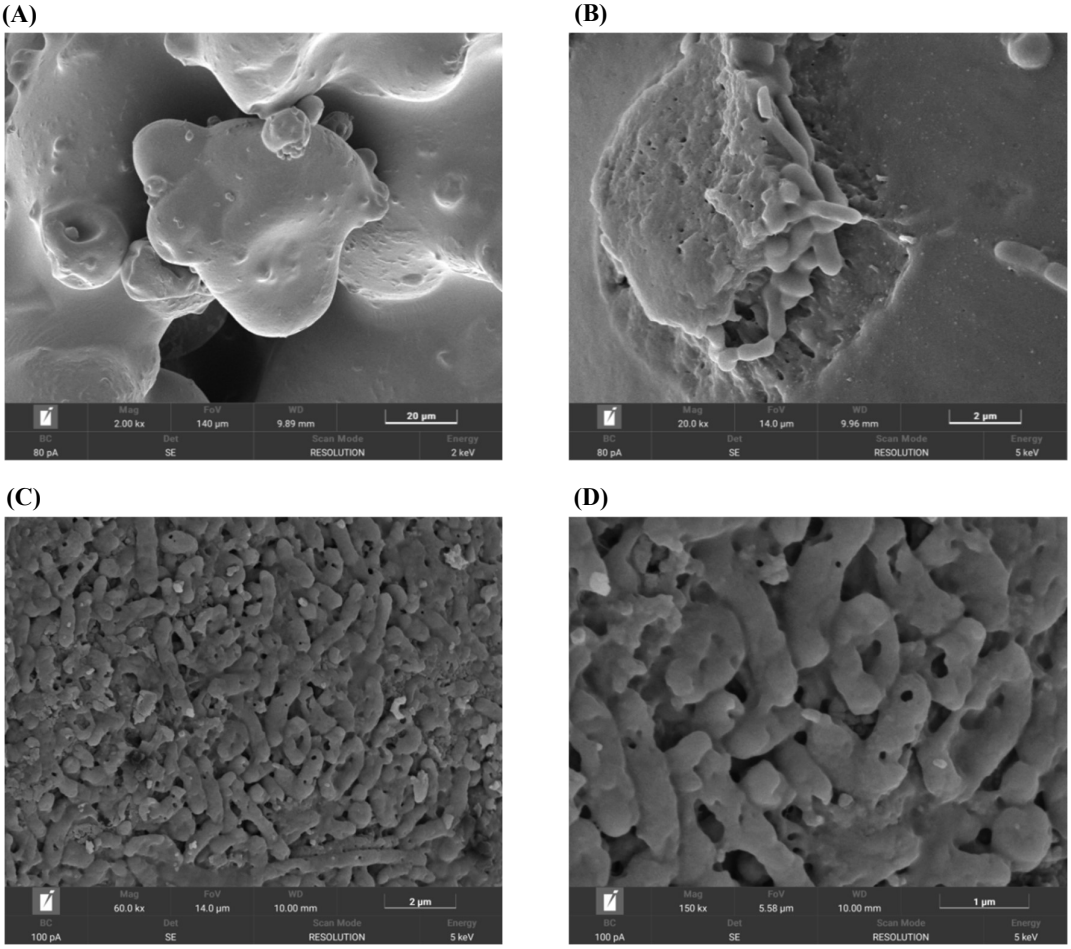

Figure 4 Images of SPBP (A; 2 kx and B; 20 kx) and freeze-dried probiotic free-cell (C; 60 kx and D; 150 kx) under scanning electron microscope.

3.3 Stability and chemical property change of encapsulated synbiotic plant-based protein powder (SPBP) during storage

In this study, stability (Figure 5) and a_w evaluation (Figure 6) of SPBP was performed by keeping SPBP in aluminum laminated foil bags at refrigeration temperature (6±2°C) and ambient temperature (26±2°C). Stability study showed that probiotic as SPBP form kept at refrigeration temperature was survived at 96.2±2.4% (9.3±0.2 log CFU/g) during 12 weeks of storage. At ambient temperature, the number of living probiotic was decreased continuously since the first week and no living probiotic was observed at week 12. In addition, the number of living probiotic at 6.0±0.6 log CFU/g was detected at week 6. Evaluation of a_w of SPBP at refrigeration temperature and ambient temperature presented 0.09±0.10 and 0.15±0.20, respectively, after 12 weeks of storage. This study suggests to keep SPBP in the refrigerator for long-term storage and only six weeks at ambient temperature.

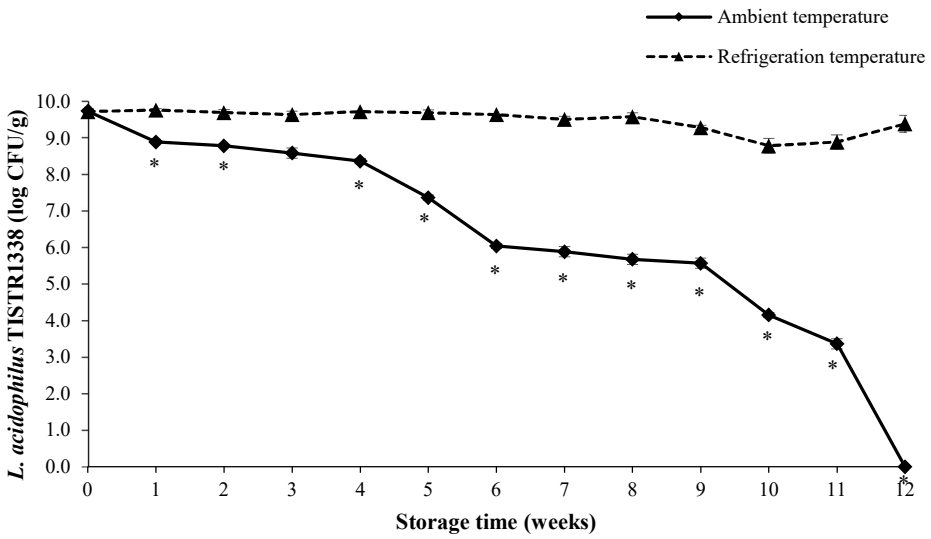

Figure 5 Stability of *L. acidophilus* TISTR1338 as SPBP form stored at refrigeration temperature; 6±2°C (--▲--) and ambient temperature; 26±2°C (—◆—) for 12 weeks. The bars represent standard deviation (n = 3). The asterisk sign (*) indicate the difference ($p < 0.05$) between stability of *L. acidophilus* TISTR1338 as SPBP form at refrigeration temperature and ambient temperature at a specific storage time.

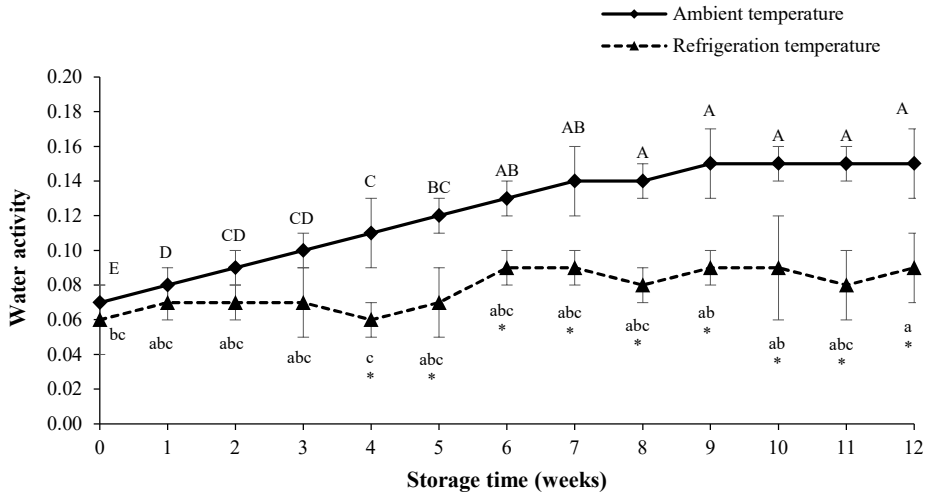

Figure 6 Water activity (a_w) of SPBP form at refrigeration temperature; 6±2°C (--▲--) and ambient temperature; 26±2°C (—◆—) during 12 weeks of storage. The bars represent standard deviation (n = 3). Uppercase letters on bars indicate significant difference ($p < 0.05$) among ambient temperature at the different time. Lowercase letters on bars indicate significant difference ($p < 0.05$) among refrigeration temperature at the different time. The asterisk sign (*) indicate the difference ($p < 0.05$) between water activity of SPBP form at refrigeration temperature and ambient temperature at a specific storage time.

4. Discussion

This study aimed to develop the formulation of synbiotic plant-based protein as dry powder via freeze-drying. A previous study reported that bacterial cell injury can be occurred by freezing via intracellular ice formation. Resulting in cell damages as clumping, shrinkage, and cavities [14]. Water activity (a_w) has been reported as one of the key parameters which affect the survivability of probiotic. Water activity approximately 0.1 to 0.3 has been reported to affect functional groups and block reaction sites, leading to the chemical reactions which cause the destruction of of lipids and proteins on probiotic cell wall, resulting in cell death [15]. Thus, the conditions of

freeze-drying might disrupt the survivability of probiotic by the ice crystals and the less water activity. Gastro-intestinal (GI) tract presenting low pH and proteolytic enzymes in gastric stage, as well as pancreatic fluids and bile salt in intestinal stage. The harsh conditions may directly exposure to probiotic cells, resulting in the disruption of probiotic cells' membranes, internal imbalance, and cell death [16]. Encapsulated synbiotic plant-based protein powder (SPBP) developed in this study showed desirable efficiency to protect probiotic from various harsh conditions (freeze-drying, GI tract conditions, various pH levels, and various temperature levels), resulting in a high number of living probiotic cells entering to the colon and providing benefits to the host. PPI has been reported as one of the effective anti-freezing proteins (APFs) via adsorption-inhibition mechanism [17]. The efficiency of APFs to inhibit the growth of ice crystals has been described by the adsorption of AFPs at the ice-solution interface results in local surface curvature effects, providing inhibiting of ice crystal formation [17]. A previous study reported the efficiency of protein combined with prebiotic as an excellent encapsulation material, providing the high survivability of probiotic through GI tract. The structure of protein provided the ability to interact with prebiotic material as hydrophilic interaction, forming physical barrier as the mask to probiotic cells [16]. In addition, PPI has been reported as high resistance to gastric digestion [18] as well as prebiotic which presented high resistance in GI tract digestion [19]. In addition, present of prebiotic could improve the survivability of probiotics though the severe conditions [2]. The similar phenomenal might be occurred in this study since PPI and inulin provided the functions of protein and prebiotic, respectively. Moreover, SEM images clearly showed the physical interaction of developed wall materials and probiotic cells as core material. The images showed complete entrapment of healthy probiotic cells on the surface of the larger complex matrix. Probiotic used in this study was *L. acidophilus*, which is a Gram-positive bacterium with rod shape. The size of *L. acidophilus* generally presented as 0.6-0.9 μm in width and 1.5-6.0 μm in length [20]. PPI has been reported to interact with lipid structure as protein-fat in order to form three dimensional networks. PPI could provide excellent emulsifying property because of the abundance of reactive amino groups (such as lysine residue) and chemical reaction (such as acetylation or succinylation) [7,21]. Glycerol has been widely used as cryoprotectant by kosmotropic properties. Glycerol can form hydrogen bonding with water molecules, resulting in the difficulty of ice crystals formation [11]. Moreover, glycerol content in the developed formulation could play the role to form gelation and provide the emulsion system. Heat-induced gel formation at 75-85°C allowed protein content of PPI to form disulfide bond [7,22], which might provide a strong gel network, leading to high efficiency of wall material formulation developed in this study to protect probiotic cells from harsh conditions. Inulin structure contained abundance of hydroxyl groups [23] which could interact with protein gel networks, resulting in the formation of complex matrix to entrap bacterial cells. Gram-positive bacteria contained a number of peptide structures on the cell wall [24]. Peptides as protein molecules on the probiotic cell wall might interact with the larger protein molecules of PPI as protein-protein interaction, allowing the strong physical interaction of probiotic cells and protein structure of PPI, which was the major component of the developed wall material formulation. SPBP kept in aluminum laminated foil bags showed the longer stability at refrigeration temperature. This study showed similar result to previous study, which suggested to keep encapsulated microorganisms as dry powder at this condition, since aluminum laminated foil provides high potential to prevent light and moisture, which are a major factor to change material quality and reduce microbial load in encapsulated material [12]. SPBP kept at both refrigeration- and ambient-temperature showed a_w less than 0.6, indicating the desired characteristic serving food safety and food quality in terms of preventing microbial growth (bacteria, yeasts, and molds) caused food spoilage and chemical change [12,25]. However, color measurement (data not shown) of SPBP kept at ambient temperature (evaluated by L*a*b* system) showed the increased value of a* and b*, indicated the increase of redness and yellowness, respectively in the product. The results indicated that Maillard reaction might be slightly occurred in the product kept at ambient temperature.

5. Conclusion

This study developed synbiotic plant-based protein as dry powder which provided a high number of living probiotic cells. The remarkable highlights of this study include (i) developed product contained high healthy living probiotic cells, (ii) probiotic contained in the developed product could survive through GI tract, severe pH and temperature levels, and (iii) product provided long shelf-life. This developed product meets the need of world population in terms of future food supply as plant-based protein food product. Moreover, the characteristics of the developed product meet regulations of probiotic supplement, which presented the high probability to improve as supplement for health and wellness. In terms of cost effectiveness issue, this study attempted to use the ingredients with low cost, in order to expand the further product development in the larger scale. Thus, the formulation of developed product provided the less limitations to produce in the commercial scale. In addition, the formulation of developed synbiotic plant-based protein also showed the desired results when evaluated with *Bifidobacterium bifidum* TISTR2129 and *Bifidobacterium breve* TISTR2130 as preliminary testing. Indicating the possibility to use this formulation for other common probiotic strains.

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