FOOD
RESEARCH

Storage stability of freeze-dried *Lactobacillus plantarum* S20 starter culture as affected by various formulations of drying medium, and its fermentation characteristics in mung bean (*Vigna radiata* L.) slurry

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Abstract

Development of an active starter culture is essential in the production of safe and high-quality fermented foods. Freeze-drying is an effective microbial cell immobilization technology intended to produce active and stable starter cultures for long-term storage. To determine the influence of various drying medium formulations with mung bean powder and disaccharides on the viability of *L. plantarum* S20 during freeze-drying and subsequent storage at 6±2°C and 30±1°C, three treatments of drying medium consisting of mung bean powder without disaccharides (MBP), MBP with 5% (w/v) trehalose (MBP+T), and MBP with 5% (w/v) sucrose (MBP+T) were evaluated. Results showed that *L. plantarum* S20 viability after freeze-drying ranged from 9.56 to 10.03 log (CFU/g) with viability loss ranging from 0.20 to 0.51 log (CFU/g), with MBP being the least effective ($p<0.05$) in minimizing viability loss. Supplementing MBP with either 5% sucrose or 5% trehalose improved ($p<0.05$) survival of freeze-dried *L. plantarum* S20; and both disaccharides exhibit similar ($p>0.05$) cryoprotective effects on *L. plantarum* S20 during freeze-drying and subsequent storage. Storage at 6±2°C for 180 days resulted in significant decreases of 2.15, 2.17, and 3.27 log (CFU/g) in *L. plantarum* S20 population in MBP+T, MBP+S, and MBP, respectively. A more drastic decline [>6.0 Log (CFU/g)] in *L. plantarum* S20 population was observed in freeze-dried culture stored at 30±1°C for 60 days. Fermentation characteristics of freeze-dried *L. plantarum* S20 in mung bean slurry were also determined. Freeze-dried *L. plantarum* S20 in MBP with 5% (w/v) sucrose was able to acidify mung bean slurry from pH 6.8 to pH 3.8 after 8 hrs with maximum LAB population of 9.2 log (CFU/mL). Acidification of mung bean slurry by fresh culture (non-freeze-dried) on the other hand was relatively slower where pH dropped from 6.8 to pH 3.72 after 24 hrs. Current work suggested that lyophilized *L. plantarum* S20 could be used as a starter culture for mung bean-based fermentations but further research on this area is needed.

1. Introduction

Fermentation is an old but useful form of food preservation, and it is valuable in developing functional foods. It is commonly used in food processing to enhance the safety, nutritional content, flavor, and texture of food. The most popular fermented foods are those achieved by lactic acid bacteria (LAB) (Rezac *et al.*, 2018), which is a diverse group of Gram-positive bacteria capable of converting carbohydrates into lactic acid as the major end product (Paul *et al.*, 2017). A variety of LAB species, particularly those that belong in the genus *Lactobacillus*, can be found in a variety of

fresh produce, fermented foods, and mammalian digestive tracts. The species *Lactobacillus plantarum* is the most studied *Lactobacillus* due to its ability to thrive in a wide range of environmental niches. Different strains of *L. plantarum* have emerged as functional LAB exhibiting a long history of safe use. It is considered as "generally recognized as safe" (GRAS) and has qualified presumption of safety (Ray and Joshi, 2014; Ricci *et al.*, 2017). The versatility of *L. plantarum* makes it a good functional organism to be used in a wide range of applications in the food industry, especially as a starter culture.

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The use of starter cultures plays an essential role in controlled fermentations to ensure the safety and quality of commercially produced fermented foods. A starter culture is composed of either a single species or a mixture of microorganisms that are added directly into a food matrix to start the fermentation process, and to obtain a desired and consistent quality of the finished product (Durso and Hutkins, 2003). The production of starter cultures aims to ensure long-term delivery of viable and stable cultures, which can be achieved through the application of preservation technologies (Carvalho, 2003a). Freeze-drying is a cell immobilization technology that can be used to preserve active and stable starter cultures. It has been established in the food industry as the best method of preserving bacterial cultures in a powdered/crystalline form for long-term storage. However, freeze-drying can result in cell injury that could lead to cell death. Thus, selecting appropriate drying medium as a protective matrix can produce a stable final formulation of freeze-dried cell culture with minimal viability loss. Several studies have investigated the use of various protective agents during feeze-drying consisting of additives (Carvalho *et al.*, 2002), disaccharides, and skim milk (Carvalho *et al.*, 2003b; Jalali *et al.*, 2012; Mendoza *et al.*, 2013; Gisela *et al.*, 2014; Jofré *et al.*, 2015) in order to minimize viability loss of LAB during cryopreservation. In addition, drying media also serve as carriers of freeze-dried starter cultures for fermentation applications. The use of skim milk supplemented with disaccharides as drying medium for dairy starter cultures has been successful in minimizing bacterial cell death during freeze-drying and subsequent storage (Carvalho *et al.,* 2004). Food-grade plant-based materials were also utilized as components of freeze-drying media for probiotic bacteria and non-dairy starter cultures and have shown to reduce microbial viability loss. A number of these plant-based materials include plant protein powder (Savedboworn *et al.*, 2017), fruit powders (Trachoo *et al.*, 2008; Nualkaekul *et al.*, 2012), soybean flour (Trachoo *et al.*, 2008), plant extracts and fibers (Hongpattarakere *et al.*, 2012). Starchy materials like flours of rice, cassava, yam, millet, maize, and sorghum have been utilized as cryoprotective drying media for non-dairy starter cultures for gari and beer fermentations (Okafor *et al.,* 1999; N'Guessan *et al.,* 2016). Mahidsanan *et al.* (2017) also reported that a novel soybean flour as an inexpensive cryoprotectant option showed enhanced protection on *Bacillus subtilis* SB-MYP-1 starter culture for Thu-nao (fermented soybean) processing.

Mung bean (*Vigna radiata* L.) or munggo, locally known in the Philippines, is a pulse crop with high protein content (21-31%) and considered as a poor man's meat (Anwar *et al*., 2007). Mung bean is also known for its high carbohydrates content (64.2%) with small portions of dietary fiber (14.6%), ash (3.9%) and some traces of vitamins and minerals (BAFS, 2012). In food processing applications, mung bean has established its popularity due to its good quality starch, which comprises a majority of total carbohydrates. Starch in mung bean comprises more than 50% of the total seed weight (Li *et al.*, 2011). It has the excellent gelling capability, pasting property, and cohesiveness, becoming it a better choice for film-forming biopolymers and starch noodles (Kim *et al.*, 2007; Li *et al.,* 2015; Rompothi *et al.,* 2017). Fermentation of mung bean with *L. plantarum* strains was associated with the release of angiotensin I-converting enzyme (ACE) inhibitory peptides, which are drugs used to lower blood pressure. Fermentation of pea with *L. plantarum* 299 has produced bioactive metabolites that facilitated the release of angiotensin I-converting enzyme (ACE) inhibitory peptides during *in vitro* gastrointestinal process (Torino *et al.*, 2013). *L. plantarum* B1-6-fermented mung bean milk was shown to produce significantly higher ACE inhibitory activity (67.5%) at the end of fermentation (Wu *et al*., 2015). In addition, Wu *et al*. (2015) also demonstrated that mung bean milk could be a carrier matrix of probiotics. Thus, it is interesting to explore the potential of utilizing of mung bean as starter culture drying medium and fermentation substrate for *L. plantarum*.

Freeze-drying at commercial scale is an expensive dehydration technology. However, it is still the most used method at commercial scale for the production of starter cultures. Thus, utilizing freeze-drying media formulated with low-cost ingredients like mung bean should be able to reduce a portion of the cost. To date, no studies have been conducted in utilizing mung bean as a drying medium component that will also serve as a carrier matrix for *L. plantarum* starter culture. This study aimed to evaluate the influence of various protective agents formulated with mung bean powder and disaccharides on the freeze- and post-freeze-drying survival of *L. plantarum* S20 starter, which can be used in mung bean-based fermentation. Results of the study will provide baseline data necessary to produce starter culture by utilizing cheap food-grade materials like mung bean as a component of drying medium for the production of novel functional non-dairy fermented foods.

2. Materials and methods

2.1. Preparation of starter culture

2.1.1 Cultivation of Lactobacillus plantarum S20

Lactobacillus plantarum S20 was acquired from the

Philippine National Collection of Microorganisms of the University of the Philippines Los Baños College, Laguna. It was isolated from Nem chua, a fermented meat product of Vietnam with 98% homology with *L. plantarum* WCFS1 (Nguyen *et al.,* 2010). It was isolated using streak plating technique and identified by its 16s rDNA sequence. *L. plantarum* S20 was maintained in 7.0 mL deMann Rogosa Sharpe (MRS) broth (HiMedia, Mumbai, India) at 4±1°C and sub-cultured every 2 weeks.

2.1.2 Preparation of mung bean powder

Whole green mung bean seeds (NSIC MG-14) were purchased from the Bureau of Plant Industries-National Crop Research and Development Center of Los Baños, Laguna. The seeds are characterized to be dull-green and can be planted and harvested during the wet and dry season. Seeds were dried at 70°C for 2 hrs (moisture content= 9.33±0.40%) and were then pulverized with the fabricated stainless steel pin mill machine (50 Kg capacity; Alameda, Tondo, Manila, Philippines) and screened through 80-mesh sieve. Mung bean powder was then packed and vacuum-sealed in Polyethylene bags and stored at room temperature until use.

2.1.3 Preparation of drying medium

A total of three treatments of drying medium formulated with mung bean base mixture and two types of disaccharides (sucrose and trehalose) were prepared. The treatments include mung bean with 5% (w/v) trehalose (MBP+T), mung bean with 5% (w/v) sucrose (MBP+S), and mung bean powder only (MBP). A mixture of mung bean powder and sterile 0.85% (w/v) NaCl solution was used as base mixture ingredients of the freeze-drying medium. The proportion used in preparing the base mixture of drying medium was based on the optimized cryoprotectant formulation using skim milk described by Gisela *et al.* (2014) with some modifications. About 25 g of mung bean powder was diluted with 0.85% (w/v) NaCl solution to 100 mL. The mixture was then transferred to a sterile media bottle and pasteurized at 71±1°C for 66±1 mins in order to inactivate pathogenic bacteria and reduce population spoilage microorganisms. The hot mixture was then assigned to one of the three treatments: MBP, MBP+T, and MBP+S. Hot mixture assigned to MBP with disaccharides was prepared by supplementing it with either trehalose (Hayashibara, Japan) or sucrose (Ajax-FineChem Univar, Australia) at 5% (w/v) inclusion rate. All treatments were then allowed to cool down at room temperature.

2.1.4 Preparation and freeze-drying of starter culture

The preparation of starter culture was based on the procedures described by Yao *et al.* (2009) with major modifications. About 1 mL of overnight (16-18 hrs at 37°C) MRS-cultured *L. plantarum* S20 was propagated in fresh 100 mL MRS broth at 37°C for 18 hrs. The *L. plantarum* S20 cells were then harvested by centrifugation at 4300 x *g* at 4°C for 15 mins and washed with sterile 0.85% NaCl solution twice. Washed cells were then re-suspended in 5 mL of sterile 0.85% NaCl solution. The resulting cell suspension was then aseptically transferred onto a media bottle containing one of the previously prepared freeze-drying media. All mixtures were then aseptically transferred onto sterile petri dishes and were immediately frozen for 24 hrs at -20°C. All treatments were then lyophilized at a vacuum pressure of 0.20 Torr (ULVAC DF-01H, ULVAC Corp.), and secondary drying temperature set at 40°C for 72 hrs. Preparing a freeze-drying medium devoid of any of the cryoprotectants and *L. plantarum* S20 was used as a negative control.

2.1.5 Packaging and storage of freeze-dried starter culture

Under a sterile laminar flow cabinet, freeze-dried cultures were pulverized aseptically using a sterile spatula and transferred onto pre-sterilized amber glass vials. Sample vials were immediately sealed with sterile rubber stopper and stored either at 6±2°C for 180 days in the chiller or at 30±°C for 60 days in the incubator.

2.2. Evaluation of fermentation performance of the starter culture on mung bean slurry

2.2.1 The mung bean slurry preparation

Whole green mung bean seeds (NSIC MG-14) were soaked in water for 9-10 hrs. Soaked beans were manually de-hulled and a 200 g portion was transferred onto a clean stainless steel stockpot. About 1.5 L of sterile distilled water was added into the beans and the mixture was heated to boiling at approx. 100°C. Immediately after boiling, the heat was adjusted to the medium low setting and the mixture was heated to approx. 100°C for another 20 mins or until beans become soft. The mung bean mixture was then transferred into a blender and an additional 500 mL of sterile distilled water was used to wash the remaining mixture. The mixture was then homogenized for 2 mins and transferred into a clean stainless stockpot. The mixture was then added with 500 mL sterile distilled water and was brought to boil. About 250 mL portion of mung bean slurry was immediately transferred into a

sterile screw-capped media bottle glass container and was supplemented with sucrose at 5% (w/v) inclusion rate. The mixture was then transferred to the boiling water bath and was allowed to be pasteurized at 71±1°C for 66 mins and was allowed to cool at room temperature.

2.2.2 Fermentation of mung bean slurring using freeze-dried Lactobacillus plantarum S20 culture

Three fermentation treatments containing 250 mL mung bean slurry were prepared for fermentation experiment. The first treatment of mung bean slurry was supplemented with fresh *L. plantarum* S20 culture. This was done by harvesting *L. plantarum* S20 cells from 10 mL overnight (37°C for 18 hrs) culture of *L. plantarum* S20 in MRS broth, using the procedure described in the preparation of starter culture for freeze-drying (Section 2.1.4). Harvested cells from 10 mL of overnight culture (approx. 10^{10} CFU/mL) in MRS broth were re-suspended in 9 mL 0.85% NaCl. About 5 mL of the cell suspension was added to previously prepared mung bean slurry to contain initial LAB population of 7 log (CFU/mL). The second fermentation treatment of mung bean slurry was supplemented with freeze-dried *L. plantarum* S20 culture. This was done by rehydrating 1 g of 2-day chilled freeze-dried *L. plantarum* S20 culture (approx. 10^{10} CFU/g) in 9 mL 0.85% NaCl solution for 15 min. About 5 mL of the rehydrated cells were transferred onto 250 mL pasteurized mung bean slurry to contain initial LAB population of 7 log (CFU/mL). Lastly, the third fermentation treatment of mung bean slurry was not supplemented with *L. plantarum* S20 culture and served as the control of the experiment.

2.2.3. Sample collection from the fermentation mixture

During fermentation, sample aliquots for LAB viability test, pH, and total soluble solids (TSS) measurements were aseptically drawn from the fermenting mung bean slurry at 0, 4, 8, 16, 24, and 32 h of fermentation at 37°C.

2.3. Analytical procedures and LAB viability test

2.3.1 Freeze-drying and storage viability of L.actobacillus plantarum S20

The ability of *L. plantarum* S20 to survive freeze-drying and storage conditions in mung bean powder with either trehalose or sucrose was determined using standard spread plating technique. To determine the influence of cryoprotectants on *L. plantarum* S20 viability in mung bean powder during freeze-drying, about 1 g of freeze-dried culture was aseptically drawn from each freeze-drying medium treatment before and after the freeze-drying process. To assess the stability of

freeze-dried *L. plantarum* S20 during storage, the same amount of freeze-dried culture was aseptically drawn at 0, 60, 120, and 180 days of 6±2°C storage and at 0, 20, 40, and 60 days of 30±1°C storage. Each sample obtained was serially diluted in 0.1% peptone (HiMedia Sa, Mumbai, India). About 0.1 mL sample aliquot from the diluted sample was spread plated onto MRS agar and overlaid with 5.0 mL molten MRS agar. Plates were then incubated at 37°C for 48 hrs. Viable cell count was reported as Log (CFU/g).

2.3.2 Enumeration of LAB population during fermentation

Population of lactic acid bacteria during fermentation of mung bean slurry with *L. plantarum* S20 was enumerated using a standard pour plating technique on MRS agar. About 1 mL sample aliquot drawn from fermenting mung bean slurry (section 2.2.3) was serially diluted in 0.1% peptone. About 1 mL of the diluted sample was pour plated with molten MRS agar. Plates were then incubated at 37°C for 48 hrs. Viable LAB counts were reported as log CFU/mL.

2.3.3 Water activity of freeze-dried starter during storage

About 2 g of sample was obtained from freeze-dried *L. plantarum* S20 before and after 180 and 60 d storage at 6±2°C and 30±1°C, respectively. Water activities of freeze-dried samples were then measured using a water activity meter (LabSwift-a_w meter; Novasina, Hamilton, NJ, USA) at room temperature.

2.3.4 Total soluble solids and pH during mung bean slurry fermentation

About 20 mL of sample was used for TSS and pH measurement. The total soluble solids content of samples was measured using a handheld refractometer (Atago, Japan) and the results were expressed as °Brix. The pH of samples was measured using a hand-held pH meter fitted with spear-shaped probe (PH60S; Apera Instruments) calibrated with pH 7.00 and pH 4.00 buffer solution. All readings for each parameter were done in duplicate for each sample.

2.4 Statistical analysis

Analysis of variance (ANOVA) with repeated measures was used to determine treatments significantly differ from each other, using 5% level of significance. A pairwise comparison using Tukey's HSD (honestly significant difference) was used to compare means using 5% level of significance. The analysis was carried out using SPSS Statistics (Version 25; IBM, USA). Both freeze-drying and storage stability experiments were

independently replicated three times with two independent samples used in each replication. Fermentation experiment, on the other hand, was replicated four times with one independent sample used in each replication.

3. Results and discussion

3.1 Effect of protective agents on Lactobacillus plantarum S20 during freeze-drying

The population *L. plantarum* S20 in mung bean powder with or without cryoprotectants after freeze-drying is shown in Figure 1. It is shown that viability after freeze-drying ranged from 9.56 to 10.03 log (CFU/g) with viability loss ranging from 0.20 to 0.51 log (CFU/g). Addition of either sucrose or trehalose at 5% (wt/v) exhibit improved protection on *L. plantarum* S20 during freeze-drying where higher ($p<0.05$) level of surviving *L. plantarum* population remained after freeze-drying was observed.
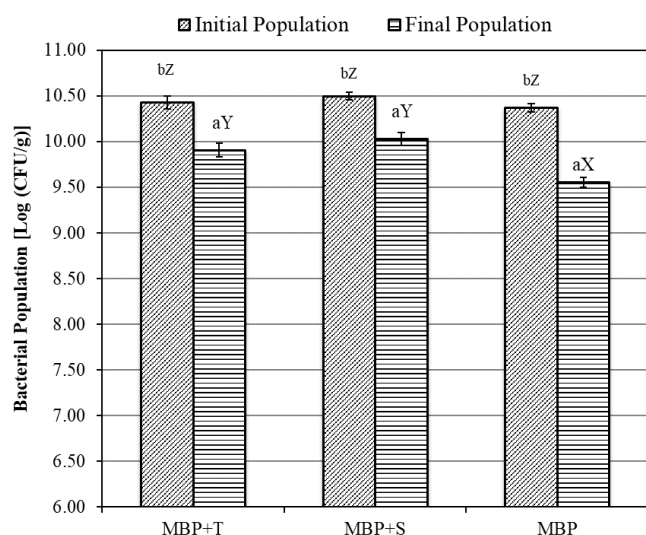

Figure 1. Population of *L. plantarum* S20 in various freeze-drying media before and after lyophilization. Data presented as mean Log (CFU/g) from 6 independent samples. Drying medium treatments: MBP+T= mung bean powder with 5% trehalose; MBP+S= mung bean powder with 5% sucrose; MBC= mung bean powder only. [ab]Values within each treatment not sharing the same letters are different from each other ($p<0.05$) analyzed using Tukey's HSD post hoc test; [XYZ]Values across treatments not sharing the same letters are different from each other ($p<0.05$) analyzed using Tukey's HSD post hoc test; Error bars represent standard error of the mean.

Viability loss observed in the current study was expected due to cell injury caused by the freezing and dehydration process. Several studies reported that cellular damage during freeze-drying was largely associated with cell membrane damage due to ice crystal formation that leads to high osmolarity of the cell (Telang *et al.*, 2003), macromolecule denaturation

(Thammavongs *et al.,* 1996), and the removal of water (Allison *et al.*, 1999; De Paz *et al*., 2002). Viability loss in *L. plantarum* S20 population observed in this study was almost similar to the values reported by Hongpattarakere *et al.* (2012). They reported that freeze-drying of *L. plantarum* suspended in MRS growth medium added with and without mung bean fiber has resulted in 0.25 and 0.51 log (CFU/g) viability loss. In the present work, all treatments were exposed at -20°C for 24 hrs freezing temperature prior to freeze-drying. This freezing temperature may have resulted in bigger ice crystals that resulted in cell injury. Hongpattarakere *et al.* (2012) and Perry (1998) noted that slow freezing occurring at temperatures above −30°C may result in poor survival due to loss of water from the cell while increasing the effects of solute concentration. Exposure of *L. plantarum* culture to extremely low freezing temperature of -196°C have shown to have the significantly lower freeze-drying viability loss of 0.18 log (CFU/g) as compared to -80 and -20°C with cell reduction of 0.57 and 0.38 log (CFU/g), respectively (Hongpattarakere *et al.*, 2012).

Current results showed that using mung bean powder only as drying medium exhibit significantly lower viability compared to those formulated with either sucrose or trehalose. The surviving *L. plantarum* S20 in mung bean drying medium after freeze-drying was 9.56 log (CFU/g). This survival level is equivalent to approx. 31% of the initial population level before freeze-drying. This survival rate was slightly better compared to the survival rate (23%) of *L. plantarum* TISTR 2075 when 15% plant protein was used as a protective and drying medium (Savedboworn *et al*., 2017). In the current study, the use of ground mung bean may have served as inert bulking agent forming viscous layer that provided some protection on *L. plantarum* S20 cells during the freeze-drying process. Carvalho *et al.* (2004) noted that some high molecular weight substances present in the drying medium like polysaccharides and proteins have no capability to penetrate the cell wall or interact with the cell wall or membrane. Their protective effect lies on their ability to be adsorbed on the surfaces of microorganisms forming viscous layer, which could cause partial removal of water from the cell to the surroundings. This would inhibit ice crystal growth while keeping the structure of amorphous ice in the close proximity of the cell (Hubálek, 1996). Consequently, this will decrease the amount of ice crystals inside the cell, hence could prevent cell death.

The addition of either trehalose or sucrose at 5% (w/v) in the current study appeared to have increased the resistance of *L. plantarum* S20 to freeze-drying. Previous studies showed that these two disaccharides were

effective as protective agents for cryopreservation of lactic acid bacteria (Chavarri *et al.*, 1988; Carvalho *et al.*, 2002; Carvalho *et al.*, 2003b; Gisela *et al.*, 2014). Cryoprotectants in freeze-drying serve as antifreeze agents, which minimize cell death by lowering the melting point of freeze-drying media (Polge *et al.*, 1949). This would eventually freeze the medium surrounding the cell faster than the intracellular components, hence, preventing cell death. Carvalho *et al.* (2004) noted that trehalose and sucrose have the ability to decrease the transition temperature of dry membranes by replacing water between the lipid head groups. This phenomenon prevents phase transition, and its accompanying leakage upon rehydration (Leslie *et al.*, 1995; Castro *et al.*, 1997). An earlier study on cryopreservation of biomaterials suggested that trehalose was more effective cryoprotectant than other sugars (Crowe *et al.*, 1996). On the contrary, current work did not observe a significant difference between the cryoprotective ability of sucrose and trehalose. Current observation is supported by a previous study conducted by Strasser *et al.* (2009) where trehalose and sucrose demonstrated similar cryoprotective effects on *L. plantarum* strain IFA No. 278 during freeze-drying. In addition, Strasser *et al.* (2009) also reported that these two cryoprotectants were the most effective among the cryoprotectants tested in enhancing the survival of *L. plantarum* strain IFA No. 278 during lyophilization.

3.2 Stability of lyophilized Lactobacillus plantarum S20 during storage

Post-freeze-drying survival of *L. plantarum* S20 during chilled and ambient temperature storage is shown in Figure 2. It is shown that storage of freeze-dried cultures for either 180 days at 6±2°C or 60 days at 30±1°C resulted in a significant decrease on the viability of lyophilized *L. plantarum* S20 cells. At the end of 180 d

chilled storage period, viable *L. plantarum* population in MBP+T, MBP+S, and MBP decreased (*p*<0.05) by 2.15, 2.17, and 3.27 log (CFU/g), respectively (Figure 2A). Meanwhile, *L. plantarum* S20 did survive poorly during storage at a much higher temperature (Figure 2B). Ambient storage for 60 d resulted in >4 log CFU/g decrease in viability of *L. plantarum* S20. Results also show that for both storage temperatures, the addition of either 5% sucrose or 5% trehalose did improve survival of *L. plantarum* S20 during storage with lower population reduction as compared to drying medium containing ground mung bean only. No difference (*p*>0.05) in viability was observed in *L. plantarum* S20 population in MBP+T and MBP+S during ambient and chilled storage indicating that both cryoprotectants have similar protective effects on *L. plantarum* S20 survival at post-freeze-drying conditions. In addition, MBP exhibit lowest (*p*<0.05) survival after 180 d (cell reduction 3.78 log CFU/g) and 60 days (5.48 log CFU/g) storage at chilled and ambient storage, respectively. It appears that storage of freeze-dried cultures with mung bean powder and cryoprotectants at 6±2°C for 60 and 120 d manage to preserve *L. plantarum* S20 survival >8.0 Log (CFU/g) with only ≤0.70 and ≤1.70 log (CFU/g) reduction in cell population, respectively.

The drastic decline in *L. plantarum* S20 population during chilled and ambient storage could be attributed to relatively high water activities of the freeze-dried samples. Table 1 shows the water activity values of freeze-dried *L. plantarum* S20 in ground mung bean using different cryoprotectants before and after 180 and 60 d of storage at 6±2°C and 30±1°C, respectively. It is also shown that water activities slightly increase (*p*<0.05) after storage at 6±2°C and 30±1°C. Generally, dehydrated microorganisms survive better at much lower water activity. Stability of dried LAB upon storage is
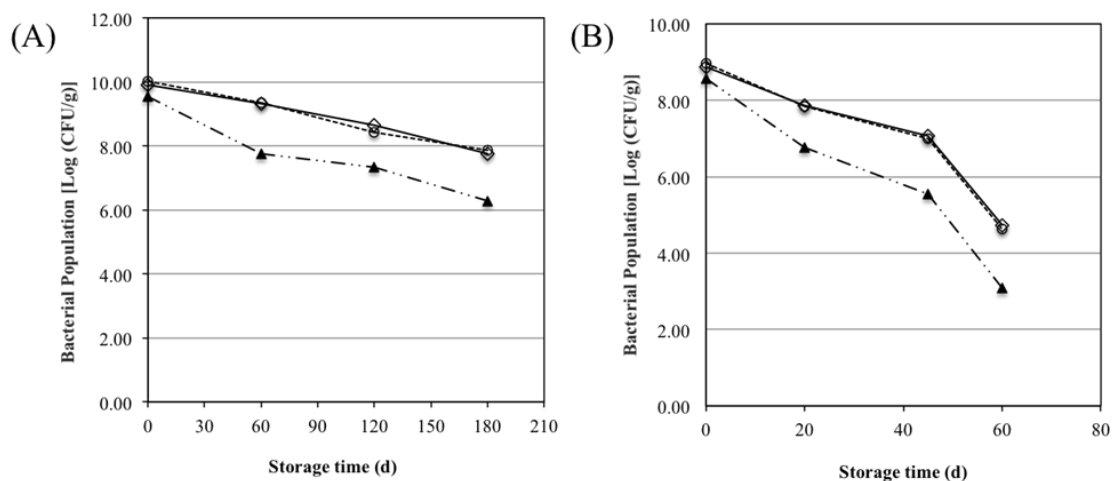

Figure 2. Population of lyophilized *L. plantarum* S20 culture in various freeze-drying media during storage at 6±2°C for 180 days (A) and at 30±1°C for 30 (B). Data presented as mean Log (CFU/g) from 6 independent samples. Drying medium treatments: Open diamond = MBP+T (mung bean powder with 5% trehalose); Open circle= MBP+S (mung bean powder with 5% sucrose); Closed triangle with dash line = MBP (mung bean powder only).

Table 1. Water activity of freeze-dried cultures throughout storage at 6±2°C for 180 days and at 30±1°C for 60 days.

Drying medium	Chilled storage (6±2°C)		Ambient storage (30±1°C)	
	0 days	180 days	0 days	60 days
Mung bean with 5% (w/v) trehalose	0.409±0.013[aX]	0.458±0.008[aY]	0.413±0.238[aX]	0.438±0.253[aY]
Mung bean with 5% (w/v) sucrose	0.396±0.010[aX]	0.461±0.004[aY]	0.401±0.231[aX]	0.421±0.243[aY]
Mung bean	0.429±0.009[aX]	0.472±0.006[aY]	0.412±0.238[aX]	0.441±0.255[aY]

Data presented as mean ± standard error of the mean from 3 independent samples (n=3).
[ab]Values within the same column not sharing the same superscript are different (p<0.05) analyzed using Tukey's HSD post hoc test.
[XY]Values within the same row and within the same storage temperature not sharing the same superscript are different (p<0.05) analyzed using Tukey's HSD post hoc test.

shown to be optimal at 0.1 to 0.2 aw (Champagne *et al.*, 1996). In the current study, the slightly higher water activity of the dried cultures may have largely affected the viability of the *L. plantarum* cells during storage. Previous studies indicated that lipid oxidation of the membrane fatty acid was predominantly responsible for cell death during storage (Teixeira *et al.*, 1996; Kurtmann *et al.*, 2009). Thus, decreasing water activity and oxygen in the packaging have shown to stabilize freeze-dried cultures (Kurtmann *et al.*, 2009). In addition, the temperature can also influence the rate and magnitude of lipid oxidation. Current work observed higher mortality in *L. plantarum* population during storage at ambient temperature. A number of studies have shown that storage of freeze-dried cultures at higher temperatures resulted in a higher loss in viability a shorter shelf life. For instance, storage of vacuum-packaged freeze-dried *P. aglomerans* stored at 4°C for 90 d showed no more than 0.5 log viability loss, while those that were stored at 25°C resulted in 3 log decrease in viability after 28 d (Costa *et al.*, 2002). Moreover, previous work of Champagne *et al.* (1996) showed that viability loss of a freeze-dried culture containing *Bifidobacrium longum* at 20°C were 100x greater than those that were stored at 4°C. Similar observation was reported by Dhewa *et al.* (2014) where a considerable reduction in viability of *L. plantarum* was observed in freeze-dried symbiotic formulation stored at 25°C for 90 d while those that were stored at 4°C exhibit higher *L. plantarum* survival. Selmer-Olsen *et al.* (1999) recommended 0-5°C storage temperature for freeze-dried cultures because low temperatures maintain lower metabolic rate, thus increases storage stability.

3.3 Fermentation characteristics of freeze-dried Lactobacillus plantarum S20

Changes in lactic acid bacteria (LAB) population, pH, and total soluble sugar (TSS) of mung bean slurry during 32 hrs of aerobic fermentation at 37°C are presented in Figure 3. Results show that LAB population changes in mung bean slurry with fresh and freeze-dried starter culture showed a similar trend (Figure 3A). It

appears that LAB in both treatments entered exponential growth phase any time between 0 and 4th hr of fermentation due to the added *L. plantarum* S20 inoculum. Exponential growth phase for mung bean slurry with freeze-dried *L. plantarum* S20 extended up to 8 hrs of fermentation where about 2.0 log cycle increases in LAB population was observed, from an initial LAB population of approx. 7.0 log (CFU/mL) to 9.30 log (CFU/mL). It is evident that the maximum LAB population in mung bean with freeze-dried culture was reached faster compared to mung bean slurry with fresh culture. The LAB population in mung bean slurry with freeze-dried culture peaked after 8 hrs with cell population of 9.30 log (CFU/mL), while LAB in mung bean with fresh culture of *L. plantarum* S20 peaked after 16 and 18 hrs with cell population 9.07 and 9.12 log (CFU/g). The significant increases in LAB population in both treatments caused a significant drop in mung bean slurry pH and TSS. Highest pH drop (from pH 6.78 to pH 3.82) was observed after 8 hrs of fermentation in mung bean slurry inoculated with freeze-dried starter, while pH drop in treatment with fresh starter culture was slower from pH 6.81 to pH 3.72, which extended up to 16 and 24 hrs. It is observed that TSS in mung bean slurry with either fresh or freeze-dried *L. plantarum* S20 starter decreased (*p*<0.05) exponentially over time from 0 to 16 hrs and leveled off until the end of fermentation (Figure 3C).

Higher (*p*<0.05) drop in TSS was observed in mung bean slurry with a freeze-dried starter. The LAB growth curve trend corresponding to pH and TSS drop in mung bean slurry with *L. plantarum* S20 indicates sugar utilization of *L. plantarum* inoculum added in the mung bean slurry. This trend was not observed in the control where natural fermentation was used. The LAB population present in the natural microflora of mung bean was only observed after 4 hrs of fermentation and exponential growth phase was extended up to 16 hrs where the maximum population of 7.15 log (CFU/mL) was reached. This population level is significantly lower than the maximum population achieved by mung bean slurry with *L. plantarum* S20. This trend also
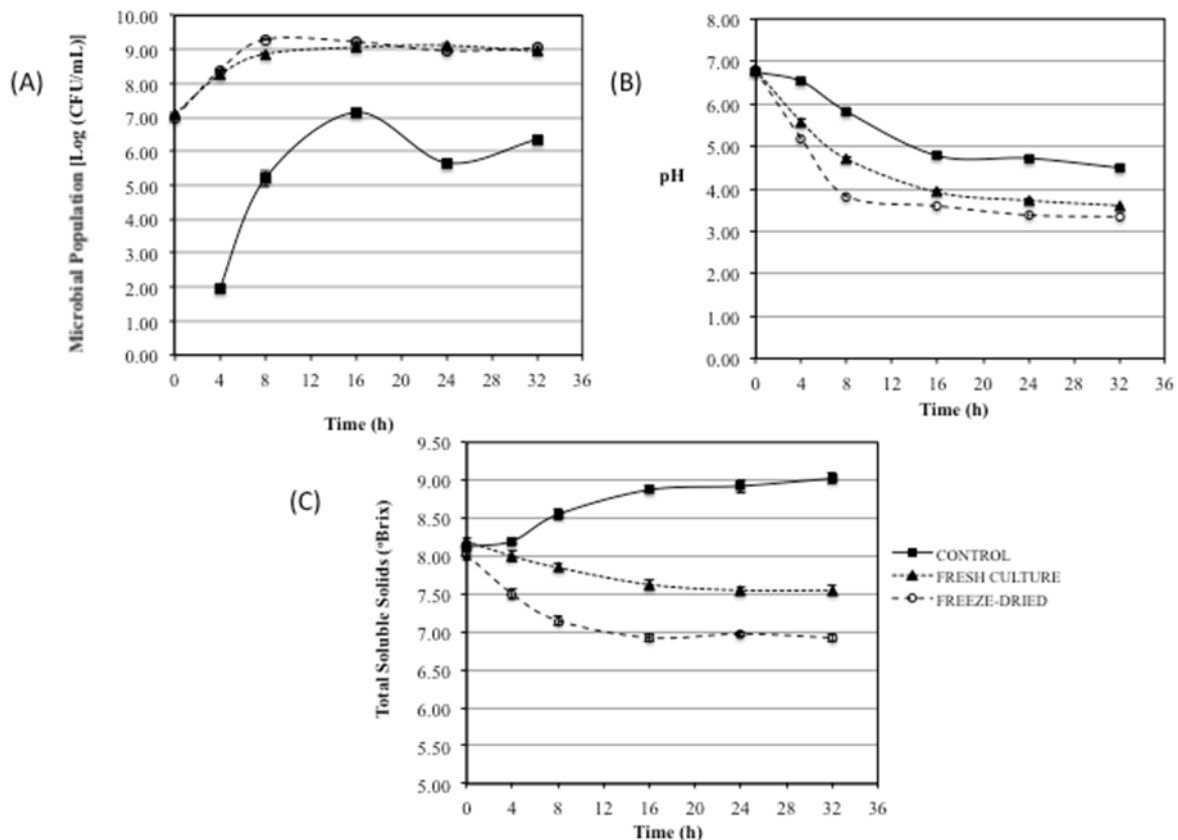
Figure 3. Changes in lactic acid bacteria population (A), pH (B), and total soluble sugars (TSS) (C) during 32 hrs mung bean slurry fermentation at 37°C with lyophilized culture, fresh, and without culture. Data presented as means from 4 independent samples. Error bars represent standard error of the mean (n=4).

corresponds to higher minimum pH achieved by mung bean slurry without starter culture as compared to mung bean inoculated with fresh or freeze-dried *L. plantarum* S20 starter. Interestingly, unlike the other treatments, TSS gradually increased in mung bean slurry without a starter culture. This can be attributed to biological activities by some LAB species naturally present in mung bean that survived pasteurization. For instance, heat-resistant, spore-forming, and lactic acid-producing *Bacillus* spp. (e.g., *Bacillus subtilis*) was reported to be the most predominant fermenting species in soybean and mung bean (Shrestha *et al.,* 2013; John and Olusegun, 2016). *Bacillus* species can produce a variety of enzymes capable of hydrolyzing macromolecules like starch into soluble sugars like oligosaccharides and simple sugars (Neway, 1989; Shrestha *et al.,* 2013), and this may have contributed to the increasing trend in TSS observed in the current study. This additional step could have slowed down the conversion of soluble sugars (sucrose, glucose, etc.) into lactic acid, hence resulted in a much slower pH drop.

Previous work by Wu *et al*. (2015) on *L. plantarum* fermentation of mung bean milk showed slightly different results due to different mung bean preparation, sucrose concentration, and *L. plantarum* strain used. They reported that *L. plantarum* B1-6 fermentation mung bean beverage (without okara) supplemented with 5.55%

sucrose and 5.19% inoculum concentration reached optimum acidity and highest LAB population of 8.96 log (CFU/mL) after 4.86 h of fermentation at 35.4°C.

Previous studies have demonstrated that mung bean could be used as a fermentation substrate for *L. plantarum*. *L. plantarum* has been found to dominate early stages of a 4-stage of fermentation of mung bean as a base material for lu-doh-huang (Taiwanese herbal medicine) (Chao *et al*., 2013). In addition, a number of studies have documented the use of *L. plantarum* as a starter culture for mung bean-based fermentations (Wu *et al*., 2015; Landete *et al*., 2015; Gan *et al*., 2017; Świeca *et al*., 2018). Current work showed that the use of freeze-dried *L. plantarum* S20 starter was able to rapidly decrease pH of fermenting mung bean below pH 4.0 corresponding to maximum population of 9.3 log after 8 hrs. This indicates that freeze-dried *L. plantarum* S20 has successfully started the fermentation process. Previous studies documented the ability of rapid acidification of freeze-dried *L. plantarum* starter for cassava fermentation has been previously documented (Yao *et al*., 2009). An important characteristic of potential starter strains is their ability to acidify their environment rapidly, which could inhibit the growth of contaminating spoilage and pathogenic microorganisms (Smulders *et al*., 1986). Leroy and De Vuyst (2004) reported that the direct addition of selected starter culture to raw materials

resulted in dominance by the best-adapted strains. Furthermore, it is worth noting that freeze-dried *L. plantarum* S20 starter culture developed in the current study showed faster substrate acidification and LAB growth compared to MRS-grown living cells. This finding was inconsistent to previous reports, which showed that freeze-drying could impair metabolic activities of microorganisms upon rehydration (Meng *et al.,* 2008; Mahidsanan *et al.,* 2017) and could decrease growth and acidification activity (Fonseca *et al.,* 2000; Santivarangkna *et al.,* 2007). In the current study, sublethal stress due to freezing and freeze-drying process may have a role in rapid acidification and growth observed when freeze-dried *L. plantarum* S20 culture was used to ferment mung bean slurry. Previous work on the pre-inoculation treatment of *Lactobacillus sakei* starter culture with sublethal stress (cold shock, heat shock, salt shock) resulted in increased growth and acid production during sausage fermentation (Hüfner and Hertel, 2008). However, the extent of damage to microbial cells exerted by sublethal stresses applied to *L. sakei* is not as damaging compared to freeze-drying. Since current work has limited scope, current findings on this area warrant further study.

4. Conclusion

Results of the current study were able to demonstrate that mung bean powder formulated with either trehalose or sucrose could be used as a freeze-drying medium for *L. plantarum* S20. Results indicate that drying medium formulation and storage temperature greatly affected *L. plantarum* S20 viability during freeze-drying and subsequent storage. Supplementing of mung bean powder with any of the two disaccharides at 5% (w/v) has improved tolerance of *L. plantarum* S20 to freeze-drying. In addition, no difference was observed between sucrose and trehalose in terms of their cryoprotective ability on *L. plantarum* S20 during freeze-drying and subsequent storage. Low temperature storage at 6±2°C performed better than ambient storage (30±1°C) in minimizing viability loss of *L. plantarum* S20. However, drying medium formulations used in the study was not able to maintain the stability of *L. plantarum* S20 throughout storage. A significant decline of 2.0 to >4.0 log (CFU/g) in *L. plantarum* S20 population was observed in all drying medium treatments during cold and ambient storage. In future research, it will be useful to optimize the formulation of freeze-drying medium preparations, packaging conditions, and freeze-drying parameters to achieve higher recovery and stability for *L. plantarum* S20. In addition to mung powder and disaccharides, the incorporation of additives must be considered to further improve the shelf stability of freeze-dried culture. When freeze-dried *L. plantarum* S20 was

used as a starter culture in mung bean slurry, it was able to acidify the slurry from pH 6.8 to pH 3.8 after 8 hrs with maximum LAB population of 9.2 log (CFU/mL). Acidification of mung bean slurry by fresh culture (non-freeze-dried) on the other hand appeared to be slower than the freeze-dried culture. This observation contradicts previous findings. While freeze-dried *L. plantarum* S20 culture developed in the current study could be used as a starter culture for mung bean-based fermentations, its ability to perform better than non-freeze-dried culture by rapidly growing and acidifying mung bean slurry warrant further research.

References

Anwar, F., Latif, S., Przybylski, R., Sultana, B. and Ashraf, M. (2007). Chemical composition and antioxidant activity of seeds of dif-ferent cultivars of mung bean. *Journal of Food Science,* 72(7), S503–S510. https://doi.org/10.1111/j.1750-3841.2007.00462.x

Allison, S.D., Chtang, B., Randolph, T.W. and Carpenter, J.F. (1999). Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Archives of Biochemistry and Physics*, 365(2), 289–298. https://doi.org/10.1006/abbi.1999.1175

BAFS [Bureau of Agriculture and Fisheries Product and Standards]. (2012). Philippine National Standard: Mungbeans—classification and grading. PNS/BAFPS 105:2012. ICS 67.080.01. Phillipines: BAFS.

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. (2003a). Effect of various growth media upon survival during storage of freeze-dried *Enterococcus faecalis* and *Enterococcus durans*. *Journal of Applied Microbiology,* 94(6), 947–952. https://doi.org/10.1046/j.1365-2672.2003.01853.x

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. (2003b). Effect of different sugars added to the growth and drying medium upon

thermotolerance and survival during storage of freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Biotechnology Progress*, 20(1), 248–254. https://doi.org/10.1021/bp034165y

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. (2002). Effect of additives on survival of freeze-dried *Lactobacillus plantarum* and *Lactobacillus rhamnosus* during storage. *Biotechnology Letters*, 24, 1587–1591. https://doi.org/10.1023/A:1020301614728

Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X. and Gibbs, P. (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. *International Dairy Journal,* 14(10), 835–847. https://doi.org/10.1016/j.idairyj.2004.02.001

Castro, H.P., Teixeira, P.M. and Kirby, R. (1997). Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze-drying. *Journal of Applied Microbiology,* 82(1), 87–94. https://doi.org/10.1111/j.1365-2672.1997.tb03301.x

Champagne, C.P., Mondou, F., Raymond, Y. and Roy, D. (1996). Effect of polymers and storage temperature on the stability of freeze-dried lactic acid bacteria. *Food Research International,* 29(5-6), 555–562. https://doi.org/10.1016/0963-9969(95)00050-X

Chao, S.-H., Huang, H.-Y., Chang, C.-H. Yang, C.-H., Cheng, W.-S., Kang, Y.-H., Watanabe, K. and Tsai, Y.-C. (2013). Microbial Diversity Analysis of Fermented Mung Beans (Lu-Doh-Huang) by Using Pyrosequencing and Culture Methods. *PLoS ONE,* 8, e63816. https://doi.org/10.1371/journal.pone.0063816

Chavarri, F.J., De Paz, M. and Nueez, M. (1988). Cryoprotective agents for frozen concentrated starters from non-bitter *Streptococcus lactis* strains, *Biotechnology Letters*, 10, 11–16. https://doi.org/10.1007/BF01030016

Costa, E., Usall, J., Teixido, N., Torres, R. and Vinas, I. (2002). Effect of package and storage conditions on viability and efficacy of the freeze-dried biocontrol agent *Pantoea agglomerans* strain CPA-2. *Journal of Applied Microbiology,* 92(5), 873–878. https://doi.org/10.1046/j.1365-2672.2002.01596.x

Crowe, L.M., Reid, D.S. and J. H. Crowe. (1996). Is trehalose special for preserving dry biomaterials? *Biophysical Journal,* 71, 2087–2093. https://doi.org/10.1016/S0006-3495(96)79407-9

De Paz, R.A., Dale, D.A., Barnett, C.C., Carpenter, J.F., Gaertner, A.L. and Radolph, T.W. (2002). Effects of drying methods and additives on the structure, function, and storage stability of subtilisin: role of protein conformation and molecular mobility. *Enzyme and Microbial Technology,* 31(6), 765-774. https://doi.org/10.1016/S0141-0229(02)00173-4

Dhewa, T., Pant, S. and Mishra, V. (2014). Development of freeze dried synbiotic formulation using a probiotic strain of Lactobacillus plantarum. *Journal of Food Science and Technology*, 51, 83–89. https://doi.org/10.1007/s13197-011-0457-2

Durso, L. and Hutkins, R. (2003). Starter cultures. In Caballero, B. (Ed.). Encyclopedia of Food Sciences and Nutrition. 2nd ed., p. 5583–5593. Cambridge, MA: Academic Press. https://doi.org/10.1016/B0-12-227055-X/01146-9

Fonseca, F., Beal, C. and Corrieu, G. (2000). Method of quantifying the loss of acidification activity of lactic acid starters during freezing and storage. *Journal of Dairy Research*, 67(1), 83-90. https://doi.org/10.1017/S002202999900401X

Gan, R.-Y., Shah, N.P., Wang, M.-F., Lui, W.-Y. and Corke, H. (2017). *Lactobacillus plantarum* WCFS1 fermentation differentially affects antioxidant capacity and polyphenol content in mung bean (*Vigna radiata*) and soya Bean (*Glycine max*) milks. *Journal of Food Processing and Preservation,* 41(1), 12944. https://doi.org/10.1111/jfpp.12944

Gisela, G., Leonardo, A.E., Lucia, P., Rodrigo, V., Eduard, G. and Angeles, C.M. (2014). Enhancement of the viability of *Lactobacillus plantarum* during the preservation and storage process based on the response surface methodology. *Food and Nutrition Sciences,* 5, 1746–1755. https://doi.org/10.4236/fns.2014.518188

Hongpattarakere, T., Rattanaubon, P. and Buntin, N. (2012). Improvement of freeze-dried *Lactobacillus Plantarum* survival using water extracts and crude fibers from food crops. *Food and Bioprocess Technology,* 6, 1885–1896. https://doi.org/10.1007/s11947-012-1018-z

Hubálek, Z. (1996). Cryopreservation of microorganisms. Prague, Check Republic: Academia.

Hüfner, E. and C. Hertel. (2008). Improvement of raw sausage fermentation by stress-conditioning of the starter organism *Lactobacillus sakei*. *Current Microbiology,* 57, 490–496. https://doi.org/10.1007/s00284-008-9274-x

Jalali, M., Abedi, D., Varshosaz, J., Najjarzadeh, M., Mirlohi, M. and Tavakoli, N. (2012). Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. tolerance and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules. *Research in Pharmaceutical Sciences,* 7(1), 31-36.

Jofré, A., Aymerich, T. and Garriga, M. (2015). Impact of different cryoprotectants on the survival of freeze-dried *Lactobacillus rhamnosus* and *Lactobacillus casei/paracasei* during long-term storage. *Beneficial Microbes*, 6, 381–386. https://doi.org/10.3920/BM2014.0038

John, K. and Olusegun, O. (2016). Effect of fermentation on the microbial, proximate and mineral composition of mung bean (*Vigna radiata*). *Journal of Applied Life Sciences International,* 5(4), 1–12. https://doi.org/10.9734/JALSI/2016/25298

Kim, S., Lee, B., Baik, M., Joo, M.-H. and Yoo, S.-H. (2007). Chemical structure and physical properties of mung bean starches isolated from 5 domestic cultivars. *Journal of Food Science,* 72(9), 471–477. https://doi.org/10.1111/j.1750-3841.2007.00525.x

Kurtmann, L., Carlsen, C.U., Risbo, J. and Skibsted, L. H. (2009). Storage stability of freeze-dried *Lactobacillus acidophilus* (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology,* 58(2), 175–180. https://doi.org/10.1016/j.cryobiol.2008.12.001

Landete, J. M., Hernández, T., Robredo, S., Dueñas, M., de las Rivas, B., Estrella, I. and Muñoz, R. (2015). Effect of soaking and fermentation on content of phenolic compounds of soybean (*Glycine max* cv. Merit) and mung beans (*Vigna radiata* [L] Wilczek). *International Journal of Food Sciences and Nutrition,* 66(2), 203–209. https://doi.org/10.3109/09637486.2014.986068

Leroy, F. and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science and Technology,* 15(2), 67-78. https://doi.org/10.1016/j.tifs.2003.09.004

Leslie, S.B., Israeli, E., Lighthart, B., Crowe, J.H. and Crowe, L.M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology,* 61, 3592–3597. https://doi.org/10.1128/AEM.61.10.3592-3597.1995

Li, W.H., Guo, H.M., Wang, P., Tian, X., Zhang, W., Saleh, A.S.M., Zheng, J., Ouyang, S., Luo, Q. and Zhang, G. (2015). Physicochemical characteristics of high pressure gelatinized mung bean starch during recrystallization. *Carbohydrates Polymers*, 131, 432 –438. https://doi.org/10.1016/j.carbpol.2015.05.076

Mahidsanan, T., Gasaluck, P. and Eumkeb, G. (2017). A novel soybean flour as a cryoprotectant in freeze-dried *Bacillus subtilis* SB-MYP-1. *LWT-Food Science and Technology,* 77, 152–159. https://doi.org/10.1016/j.lwt.2016.11.015

Mendoza, G.M., Pasteris, S.E, Otero, M.C. and Nader-Macías, M.E.F. (2013). Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. *Journal of Applied Microbiology*, 116(1), 157–166. https://doi.org/10.1111/jam.12359

Meng, X.C., Stanton, C., Fitzgerald, G.F., Daly, C. and Ross, R.P. (2008) Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry*, 106(4), 1406-1416. https://doi.org/10.1016/j.foodchem.2007.04.076

Neway, O. (1989). Fermentation process development of industrial organisms., p. 73-75. New York, USA: Marcel Dekker.

Nguyen, H., Elegado, F., Librojo-Basilio, N., Mabesa, R. and Dizon, E. (2010). Isolation and characterization of selected lactic acid bacteria for improved processing of *Nem chua*, a traditional fermented meat from Vietnam. *Beneficial Microbes,* 1, 67–74. https://doi.org/10.3920/BM2009.0001

N'Guessan, F.K., Coulibaly, H.W., Alloue-Boraud, M.W.A., Cot, M. and Dje, K.F. (2016). Production of freeze-dried yeast culture for the brewing of traditional sorghum beer, tchapalo. *Food Science and Nutrition*, 4(1), 34-41. https://doi.org/10.1002/fsn3.256

Nualkaekul, S., Deepika, G. and Charalampopoulos, D. (2012). Survival of freeze dried *Lactobacillus plantarum* in instant fruit powders and reconstituted fruit juices. *Food Research International,* 48(2), 627 –633. https://doi.org/10.1016/j.foodres.2012.06.003

Okafor, N., Azubike, C. and Ibenegbu, C. (1999). Carriers for starter cultures for the production of garri, a fermented food derived from cassava. *World Journal of Microbiology and Biotechnology*, 15, 231 -234. https://doi.org/10.1023/A:1008867824975

Paul, E., Sasikumar, P., Gomathi, S., Abhishek, A. and Selvam, G.S. (2017). Recombinant lactic acid bacteria secreting OxdC as a novel therapeutic tool for the prevention of kidney stone disease. In Grumezescu, A.M. (Ed.). Multifunctional Systems for Combined Delivery, Biosensing and Diagnostics., p. 377-345. USA: Elsevier. https://doi.org/10.1016/B978-0-323-52725-5.00017-4

Perry, S.F. (1998). Freeze-drying and cryopreservation of bacteria. *Molecular Biotechnology*, 9, 59–64. https://doi.org/10.1007/BF02752697

Polge, C., Smith, A. U. and Parkes, A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature,* 164, 666. https://doi.org/10.1038/164666a0

Ray, R.C. and Joshi, V.K. (2014). Fermented foods:

FULL PAPER

Past, present and future scenario. In Ray, R.C. and Didier, M. (Eds.) Microorganisms and fermentation of traditional foods., p.1–36, Boca Raton, Florida, USA: CRC Press. https://doi.org/10.1201/b17307

Rezac, S., Kok, C.R., Heermann, M. and Hutkins, R. (2018). Fermented foods as a dietary source of live organisms. *Frontiers in Microbiology,* 9, 1785. https://doi.org/10.3389/fmicb.2018.01785

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., Koutsoumanis, K., Lindqvist, R., Nørrung, B., Robertson, L., Ru, G., Escamez, P.S.F., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Kuile, B.T., Threlfall, J., Wahlstrom, H., Cocconcelli, P.S., Peixe, L., Maradona, M.P., Querol, A., Suarez, J.E., Sundh, I., Vlak, J., Correia, S. and Herman, L. (2017). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 5: suitability of taxonomic units notified to EFSA until September 2016. *European Food Safety Authority Journal*, 15(7), e04884. https://doi.org/10.2903/j.efsa.2017.4884

Rompothi, O., Pradipasena, P., Tananuwong, K., Somwangthanaroj, A. and Janjarasskul, T. (2017). Development of non-water soluble, ductile mung bean starch based edible film with oxygen barrier and heat sealability. *Carbohydrates Polymers*, 157, 748–756. https://doi.org/10.1016/j.carbpol.2016.09.007

Santivarangkna, C., Kulozik, U. and Foerst, P. (2007). Alternative drying processes for the industrial preservation of lactic acid starter cultures. *Biotechnology Progress,* 23(2), 302–315. https://doi.org/10.1021/bp060268f

Savedbworn, W., Kerdwan, N., Sakorn, A., Charoen, R., Tipkanon, S. and Pattayakorn, K. (2017). Role of protective agents on the viability of probiotic *Lactobacillus plantarum* during freeze drying and subsequent storage. *International Food Research Journal,* 24(2), 787-794.

Selmer-Olsen, E., Birkeland, S.-E. and Sørhaug, T. (1999). Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. *Journal of Applied Microbiology,* 87(3), 429–437. https://doi.org/10.1046/j.1365-2672.1999.00839.x

Shrestha, A.K., Dahal, N.R. and Ndungutse, V. (2013). *Bacillu*s fermentation of soybean: a review. *Journal of Food Science and Technology Nepal,* 6, 1–9. https://doi.org/10.3126/jfstn.v6i0.8252

Smulders, F.J.M., Barendsen, P., van Logtestijn J.G., Mossel, D.A.A. and van Der Marel, G.M. (1986). Lactic acid: consideration in favor of its acceptance as a meat decontaminant. *Journal of Food Technology,* 21(4), 419-436. https://doi.org/10.1111/j.1365-2621.1986.tb00420.x

Strasser, S., Neureiter, M., Geppl, M., Braun, R. and Danner, H. (2009). Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. *Journal of Applied Microbiology,* 107(1), 167–177. https://doi.org/10.1111/j.1365-2672.2009.04192.x

Świeca, M., Kordowska-Wiater, M., Pytka, M., Gawlik-Dziki, U., Bochnak, J., Złotek, U. and Baraniak, B. (2018). *Lactobacillus plantarum* 299V improves the microbiological quality of legume sprouts and effectively survives in these carriers during cold storage and *in vitro* digestion. *PLoS OnE* 13, e0207793. https://doi.org/10.1371/journal.pone.0207793

Teixeira, P., Castro, H. and Kirby, R. (1996). Evidence of membrane lipid oxidation of spray-dried *Lactobacillus bulgaricus* during storage. *Letters in Applied Microbiology*, 22(1), 34-38. https://doi.org/10.1111/j.1472-765X.1996.tb01103.x

Telang, C., Yu, L. and Suryanarayanan, R. (2003). Effective inhibition of mannitol crystallization in frozen solutions by sodium chloride. *Pharmaceutical Research,* 20, 660-667. https://doi.org/10.1023/A:1023263203188

Thammavongs, B., Corroler, D., Panoff, J.M., Auffray, Y. and Boutibonnes, P. (1996). Physiological response of *Enterococcus faecalis* JH2-2 to cold shock: growth at low temperatures and freezing/thawing challenge. *Letters in Applied Microbiology*, 23(6), 398–402. https://doi.org/10.1111/j.1472-765X.1996.tb01345.x

Trachoo, N., Panomkorn, W., Moongngarm, A. and Suttajit, M. (2008). Stability of freeze-dried *Lactobacillus acidophilus* in banana, soybean and pearl barley powders. *Journal of Biological Science,* 8(1), 119-124. https://doi.org/10.3923/jbs.2008.119.124

Wu, H., Rui, X., Li, W., Chen, X., Jiang, M. and Dong, M. (2015). Mung bean (*Vigna radiata*) as probiotic food through fermentation with *Lactobacillus plantarum* B1-6. *LWT - Food Science and Technology,* 63(1), 445–451. https://doi.org/10.1016/j.lwt.2015.03.011

Yao, A.A., Dortu, C., Egounlety, M., Pinto, C., Edward, V.A., Huch, M., Mengu, M. and Thonart, P. (2009). Production of freeze-dried lactic acid bacteria starter culture for cassava fermentation into *gari*. *African Journal of Biotechnology,* 8, 4996-5004.