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Full Paper

Viability and probiotic properties of *Lactobacillus plantarum* TISTR 2075 in spray-dried fermented cereal extracts

Wanticha Savedboworn [1] and Penkhae Wanchaitanawong [2, *]

[1] Department of Agro-Industry Technology and Management, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi Campus, Prachinburi Province 25230, Thailand
[2] Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand
* Corresponding author, e-mail: fagipkw@ku.ac.th

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Abstract: Spray-dried cereal extracts containing probiotic *Lactobacillus plantarum* TISTR 2075 was produced. Sterile soya milk extract and Job's-tears extract fortified with sesame (1-2%) and glucose (1-20%) were fermented by *L. plantarum* TISTR 2075. After 24 hr, the strain was found to grow well both in soya milk and Job's-tears extracts with viable cells of 8.28 and 7.73 log CFU/mL respectively. Higher viable cells were observed with addition of 1% glucose and 1% sesame. Sesame was also found to significantly increase soluble calcium after fermentation due to lowered pH due to the production of organic acids. Each fermented cereal extract was then mixed with 20% maltodextrin prior to spray-drying at 130°C and 70°C (inlet and outlet air temperature respectively). After spray-drying, the survival of *L. plantarum* TISTR 2075 in spray-dried soya milk and Job's-tears extract powders was 79.0 and 85.4% respectively. The functional properties of the probiotic including pathogenic inhibition of foodborne pathogens (*Escherichia coli* O157:H7 DMST 12743 and *Salmonella typhimurium* ATCC 13311) and the tolerance to simulated gastric juice (pH 2.0) and small intestinal juice (pH 8.0) were not affected by the spray-drying process.

Keywords: probiotics, *Lactobacillus plantarum*, fermented spray-dried cereal extracts, spray-drying, probiotic properties, soluble calcium

INTRODUCTION

Recently, consumer demand for cereal-based probiotic products has increased due to a combination of high nutritive values from cereals and health benefits from probiotics. Many studies have used cereals for developing non-dairy probiotic foods since they can alleviate some of disadvantages associated with dairy products like lactose intolerance, allergy to milk protein and the

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impact in cholesterol levels [1-4]. Furthermore, cereals are suitable substrates for the growth of beneficial probiotic bacteria since it contains sucrose, raffinose and stachyose used as energy sources for probiotics [2, 5-9]. For example, soya milk is a suitable substrate for the growth of lactic acid bacteria *Lactobacillus* and *Bifidobacteria* [10-13]. According to Chou et al. [14], soya milk can support the growth of *B. infantis* CCRC 14633 and *B. longum* B6 with viable cell counts of 8.5 and 7.1 log CFU/mL respectively after fermentation for 48 hr. *L. plantarum* NCIMB 8826 and *L. acidophilus* NCIMB 8821 grow well in malt, barley and barley-malt media with viable cell numbers of 8.59, 7.91 and 8.53 log CFU/mL respectively after 24 hr of fermentation [2]. Oat-based substrate can be used as a growth medium of *L. reuteri*, *L. acidophilus* and *B. bifidum* with viable cell counts of greater than 8 log CFU/mL after 30 days of storage [15]. In addition, the fermentation of cereals or vegetables by lactic acid bacteria enhances the availability of minerals such as calcium and iron, which correlate with the effect of pH reduction [11, 16]. According to Tang et al. [11], *L. acidophilus* ATCC 4962 and *L. casei* ASCC 290 exhibit the highest increase in soluble calcium after calcium-fortified soya milk is fermented for 24 hr. Also, a 2.5-fold increase in soluble calcium is observed in fermented soya milk with sucrose addition [12]. Bergqvist et al. [16] further reported that the level of soluble iron is markedly enhanced during fermentation of carrot juice by *L. pentosus* FSC 1.

In recent years, spray-drying is considered as a useful technique for preserving probiotics in dried form due to its relatively inexpensive cost and availability of the process [17-20]. Viability and stability of probiotics are a technological challenge because the probiotics bacteria are susceptible to high temperature during the spray-drying process [21]. A prerequisite for probiotic products is that a sufficiently large number (at least 10^7 CFU/g or mL) of viable probiotic bacteria survive in the final product at the time of consumption [22, 23]. In addition, it is important that the probiotic should maintain its properties after spray-drying in order to give the health beneficial effect to the host [24]. Spray-drying does not affect bacteriocin production of *L. salivarius* UCC 118 [25]. Also, spray-dried *L. plantarum* 83114 and *L. kefir* 8321 do not lose their capacity to adhere to Caco-2/TC-7 cells [26]. *L. plantarum* CFR 2191 was found to retain its acid-tolerance property up to 95% in the cell suspensions spray-dried with maltodextrin. However, a significant loss of viable cells was observed in the case of *Pediococcusacidilactici* CFR 2193 spray-dried with non-fat skimmed milk [27].

Soya bean, sesame and Job's-tears were found to enhance the viability of *L. plantarum* TISTR 2075 during exposure to simulated gastrointestinal tract conditions [28]. In the present study the ability of *L. plantarum* TISTR 2075 to use the sesame-supplemented extracts of soya bean and Job's-tears as substrate is investigated. The effect of glucose is also studied in order to obtain a high cell density. Furthermore, the effects of spray-drying on cell survival with emphasis on retention of probiotic properties, viz. gastrointestinal tract tolerance and pathogenic inhibition, are evaluated.

MATERIALS AND METHODS

Bacterial Strain and Preparation of Culture

L. plantarum TISTR 2075 isolated from fermented vegetables was obtained from Microbiological Resource Centre, Thailand Institute of Scientific and Technological Research (TISTR). The strain was preserved in de Man, Rogosa, Sharpe (MRS) broth (Merck, Germany) with

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20% (v/v) glycerol at -20°C. The strain was subcultured twice in MRS broth by incubating at 37°C for 24 hr under microaerobic-static condition and then used as inoculum [29].

E. coli O157:H7 DMST 12743 and *S. typhimurium* ATCC 13311 were purchased from Department of Medical Science, Ministry of Public Health. These strains were grown in tryptic soya broth (Difco Laboratories, USA) supplemented with 0.6% yeast extract (Difco Laboratories, USA) at 37°C. Both strains were subcultured twice by incubating at 37°C for 24 hr under microaerobic-static condition and then used as indicator strains.

Preparation and Fermentation of Cereal Extracts

Cereal extracts were prepared as described by Wang et al. [30]. Dried soya bean and Job's-tears (Thai Cereals World Co., Bangkok) were washed and soaked in distilled water for 5 hr, then mixed with distilled water (cereal:water = 1:10 w/v) and sesame (Thai Cereals World Co., Bangkok) at 0%, 1% or 2% w/v. The mixture was comminuted in a blender for 3 min. and the resultant slurry was filtered twice through double-layered cheesecloth to yield the cereal extract (filtrate). Each of the extracts was dispensed into a container, added with glucose solution to make 1, 5, 10, 15 and 20% w/v concentrations and sterilised by heating at 121°C for 15 min. The sterile cereal extract was inoculated with an overnight culture of 1 *L. plantarum* TISTR 2075 to make 1% concentration (initial cell number = 10^7 CFU/mL). Fermentation was carried out at 37°C for 24 hr and viable cell counts were performed by the standard plate count method with MRS medium containing 0.5% $CaCO_3$ as indicator for the acid-producing strain at 37°C. The pH was measured with a pH meter.

Spray-Drying of Fermented Cereal Extracts

Prior to spray-drying, the overnight culture of fermented cereal extracts was mixed with 20% w/v maltodextrin (MD) (DE = 10; Du ZhiXue, China). The suspension was then spray-dried with a pilot-scale spray-drier (GEA Niro A/S, Denmark) at a constant air inlet and outlet temperature of 130°C and 70°C respectively. Viable cell count was done by standard plate count.

Simulated Gastrointestinal Tract Tolerance

Simulated gastric juice at pH 2.0 was prepared by suspending pepsin from porcine gastric mucosa (P-7000, Sigma, UK) in sterile 0.5% NaCl to a final concentration of 3 g/L and adjusting to pH 2.0 with concentrated HCl [31]. Simulated small intestinal juice at pH 8.0 was prepared by suspending pancreatin USP (P-1500, Sigma, UK) in sterile 0.5% NaCl to a final concentration of 1 g/L, adding with 0.45% bile salt (Oxoid, UK) and adjusting to pH 8.0 with sterile 0.1M NaOH [32].

For the determination of tolerance to simulated gastric juice and small intestinal juices, spray-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. or after 240 min. for the simulated gastric juice or small intestinal juice tolerance determination respectively.

Determination of Pathogenic Inhibition of Spray-Dried *L. plantarum* TISTR 2075

Each spray-dried fermented cereal extract (1% w/v) was inoculated into MRS broth and cultured at 37°C for 24 hr. The inhibitory activity against *E. coli* O157:H7 DMST 12743 and *S. typhimurium* ATCC 13311 was determined using agar well diffusion method. The overnight culture

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(200 μL) of each of the two bacteria 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 spray-dried *L. plantarum* (50 μL) was inoculated into the wells and the plates were incubated at 37°C for 24 hr. The diameter of inhibition zones was measured. Each experiment was done in triplicate.

Enumeration of Viable Cells

The spray-dried powder (1 g) was suspended in sterile 0.85% NaCl solution (9 mL) for 1 hr at room temperature. Appropriate serial dilutions were prepared before pour plating onto MRS agar (with added 0.5% $CaCO_3$) and incubated at 37°C for 24 hr. The percentage of cell survival is defined as follows: survival rate (%) = (log N/log N_0) × 100, where N represents the number of viable cells (CFU/g) after exposure and N_0 denotes the initial viable cell count (CFU/g) prior to exposure [33].

Determination of Soluble Calcium

The fermented cereal extract was centrifuged at 10,000 *g* for 30 min. After centrifugation, the supernatant was collected and then filtered through a 0.2-μm membrane filter (Minisarts®, Satorius, Germany) before measurement of soluble calcium content by an atomic absorption spectrophotometer (Avanta M1, GBC Scientific Equipment, USA).

Scanning Electron Microscopy

The spray-dried powder was coated with sublimated 1% osmium tetroxide for 3 hr and kept in a desiccator for a week. The rehydrated spray-dried sample was filtered through 0.2-μm sterile membrane filter (Minisarts, Sartorius, Germany). The sample adhered to the filter was first fixed with a 2.5% glutaraldehyde in sodium phosphate buffer (pH 7.2) for 12 hr. After washing three times with the phosphate buffer, the sample was fixed with 1% osmium tetroxide for 1 hr followed by washing with distilled water three times. The sample was then dehydrated by soaking with a graded series of ethanol (30, 50, 70, 90 and 100 %, the last being used three times) and drying with liquid carbon dioxide. It was then attached to a brass stub with double-sided adhesive tape, coated with a layer of gold and analysed using a scanning electron microscope (JMS 5600 LV, Jeol, Japan).

Statistical Analysis

Each result was expressed as mean ± S.D. The data were assessed using analysis of variance (ANOVA) with a level of significance at $P < 0.05$. Significant divergences among mean values were determined with Duncan's multiple range tests. All statistical analyses were performed using SPSS Software (IBM, USA), version 12.

RESULTS AND DISCUSSION

Fermentation of Cereal Extracts

As shown in Tables 1 and 2, *L. plantarum* TISTR 2075 grows well in the extracts of soya milk and Job's-tears after 24 hr of fermentation, giving viable cell numbers of 8.28 and 7.73 log CFU/mL respectively. Addition of 1% and 2% sesame improves the viable cell count by 0.02-0.12 log CFU/mL in fermented soya milk extract and 0.66 log CFU/mL in fermented Job's-tears extract.

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However, there is no significant difference ($P > 0.05$) in viable cell number between 1% and 2% sesame. To obtain a higher cell number, different conc. of glucose (1-20%) were applied, whereby a slight increase in viable cell number was observed in all fermented cereal extracts. With 1% glucose addition, viable cell numbers of 8.76 log CFU/mL in fermented soya milk extract and 8.67 log CFU/mL in fermented Job's-tears extract were achieved, which were significantly higher in comparison with controls. When higher glucose concentrations were applied, there was no significant difference ($P > 0.05$) in the viable cell count. Similarly, Timbuntam et al. [34] reported that there was no significant difference in viable cell number of *Lactobacillus* spp. FCP2 grown in 3 and 13% sugarcane juice. According to Shirai et al. [35], glucose concentration of higher than 10% resulted in an extended lag phase during shrimp waste fermentation by *Lactobacillus* spp. B2. This is probably because a higher initial substrate concentration gives rise to an increase in the lag phase and a decrease in the specific growth rate due to the decrease in water activity in the system, promoted by a large amount of the water-binding substance [35, 36].

Table 1. Effects of glucose content on viability of *L. plantarum* TISTR 2075 after 24 hr fermentation in soya milk extracts

Glucose concentration (% w/v)	Viable cell count (log CFU/mL)		
	Fermented soya milk	Fermented soya milk + 1% sesame	Fermented soya milk + 2% sesame
0%	8.28 ± 0.09^{e}	8.30 ± 0.16^{e}	8.40 ± 0.11^{e}
1%	8.69 ± 0.10^{bcd}	8.76 ± 0.04^{abcd}	8.79 ± 0.04^{abcd}
5%	8.87 ± 0.03^{ab}	8.69 ± 0.14^{bcd}	8.64 ± 0.01^{cd}
10%	8.95 ± 0.04^{a}	8.79 ± 0.04^{abcd}	8.84 ± 0.04^{abc}
15%	8.79 ± 0.03^{abcd}	8.60 ± 0.01^{d}	8.62 ± 0.02^{d}
20%	8.84 ± 0.02^{abc}	8.61 ± 0.04^{d}	8.79 ± 0.21^{abcd}

Note: Values with different letters (a-e) are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 2. Effect of glucose content on viability of *L. plantarum* TISTR 2075 after 24 hr fermentation in Job's-tears extracts

Glucose concentration (% w/v)	Viable cell count (log CFU/mL)		
	Fermented Job's-tears extract	Fermented Job's-tears extract + 1% sesame	Fermented Job's tears extract + 2% sesame
0%	7.73 ± 0.14^{g}	8.39 ± 0.13^{def}	8.39 ± 0.06^{def}
1%	8.33 ± 0.06^{ef}	8.67 ± 0.12^{ab}	8.37 ± 0.01^{def}
5%	8.23 ± 0.04^{f}	8.64 ± 0.03^{abc}	8.48 ± 0.05^{cde}
10%	8.35 ± 0.05^{def}	8.76 ± 0.04^{a}	8.52 ± 0.08^{bcde}
15%	8.37 ± 0.01^{def}	8.63 ± 0.13^{abc}	8.54 ± 0.07^{bcd}
20%	8.36 ± 0.06^{def}	8.62 ± 0.13^{abc}	8.46 ± 0.04^{cde}

Note: Values with different letters (a-g) are significantly different ($P < 0.05$) by Duncan's multiple range test.

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As shown in Figure 1, with the addition of 1% glucose, a decrease in pH was observed in all cereal fermentations (pH < 3.34, < 3.28 and ~ 4.4 for soya milk extract, Job's-tears extract and control respectively). However, higher glucose concentrations do not seem to have any significant effect on pH. Increasing soluble calcium (73.02-112.0 mg/L in fermented soya milk extract and 2.27-5.26 mg/L in Job's-tears extract) can be clearly observed with increased addition of glucose (1-20%). Moreover, addition of sesame (1% and 2%) significantly improves soluble calcium content in the fermented cereals (67.1-145.3 mg/L in soya milk extract and 2.65-9.42 mg/L for Job's-tears extract). Tang et al. [11] reported a significant increase in soluble calcium in the fermentation of soya milk with *L. acidophilus* ATCC 4962 and *L. casei* ASCC 290 (89.3% and 87% respectively). Lopez et al. [37] established the degradation of phytic acid and the production of lactic acid leading to greater calcium solubility in the fermentation of *Leuconostoc mesenteroides* strain 38 in whole wheat flour medium. The increase in calcium solubility is related to a lowered pH due to, among other mechanisms, the production of organic acids such as lactic and acetic acids [11, 16, 37].

Soya milk and Job's-tears extracts supplemented with 1% sesame + 1% glucose were found to be suitable substrates for the growth of *L. plantarum* TISTR 2075 and were used as culture media for further study.

Effects of Spray-Drying

After spray-drying, the viable cell counts of *L. plantarum* TISTR 2075 in spray-dried soya milk and Job's-tears extract powders were 7.18 and 7.30 log CFU/g with survival rate of 79.0 and 85.4 % respectively (Figure 2). Although the stress caused by heat and dehydration results in permanent loss of viability [38], a high survival rate is observed in this experiment. This is probably because of the protein and carbohydrate in cereal, which play an important role in protecting the cells [39]. Protein can stabilise cell membrane constituents, resulting in improvement of viability [40]. MD as carrier has the ability to retain water, stabilise enzymes and prevent cellular injuries during drying [20, 41, 42]. Moreover, incorporation of MD could be beneficial due to its relatively high glass transition values (160°C) [41] and its amorphous form preventing protein unfolding during drying [43], and could thus result in less damage to the cells during drying at high temperature and desiccated condition [44]. As shown in Figure 3, the cells were found to localise within the microparticles after rehydration in 0.85% NaCl. According to Reddy et al. [27], *L. plantarum* CFR 2191, *L. salivarius* CFR 2158 and *Pediococcus acidilactici* CFR 2193 with 10% MD displayed more than 97% survival after spray-drying at 140°C (inlet air) and 40°C (outlet air). Fu and Chen [45] and Otero et al. [46] suggested that the survival rate is species-specific and depends on the drying method and protective agents. The survival rate of the strain in spray-dried Job's-tears extract is higher than that in spray-dried soya milk extract in the present study. The former extract containing *L. plantarum* TISTR 2075 was thus selected for further study.

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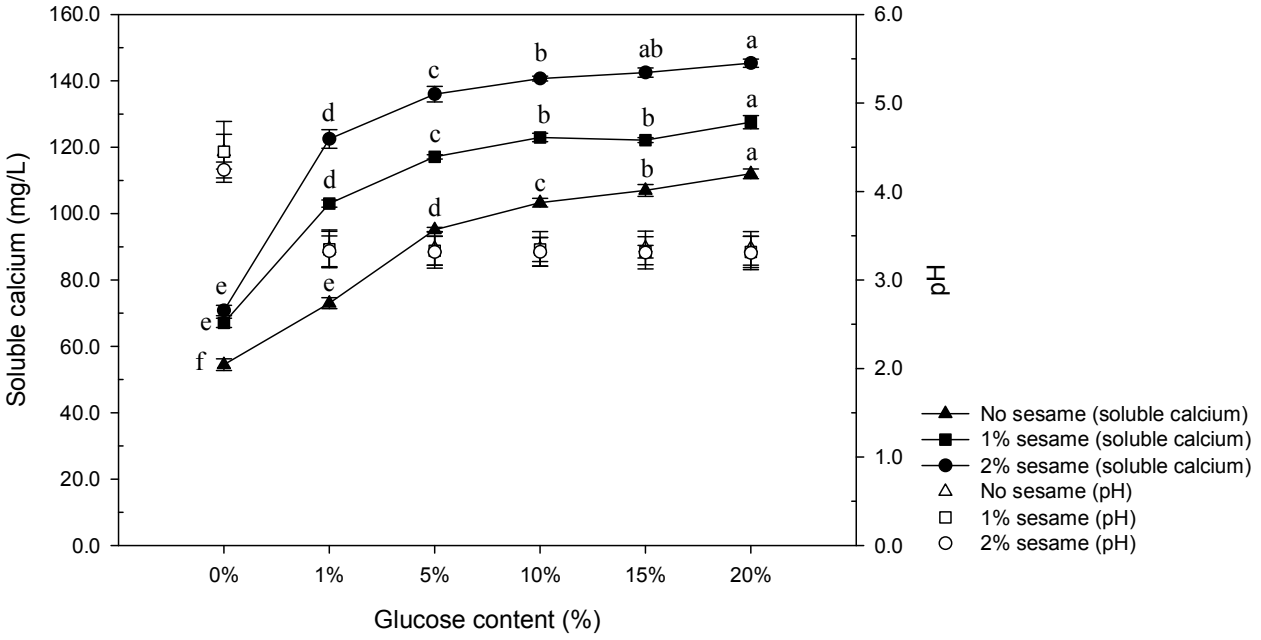
a)



b)

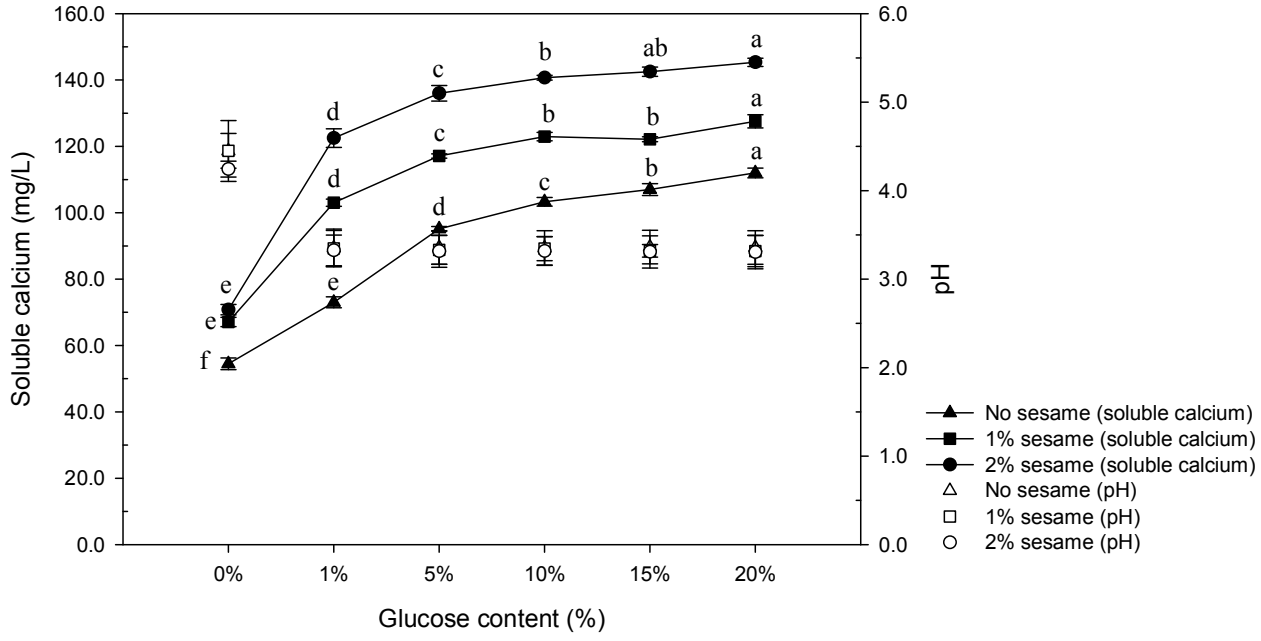


Figure 1. Effects of glucose content on pH and soluble calcium after 24-hr fermentation of: a) soya milk extract and b) Job's-tears extract. Values with different letters (a-f) in the same sesame content are significantly different ($P < 0.05$) by Duncan's multiple range test.

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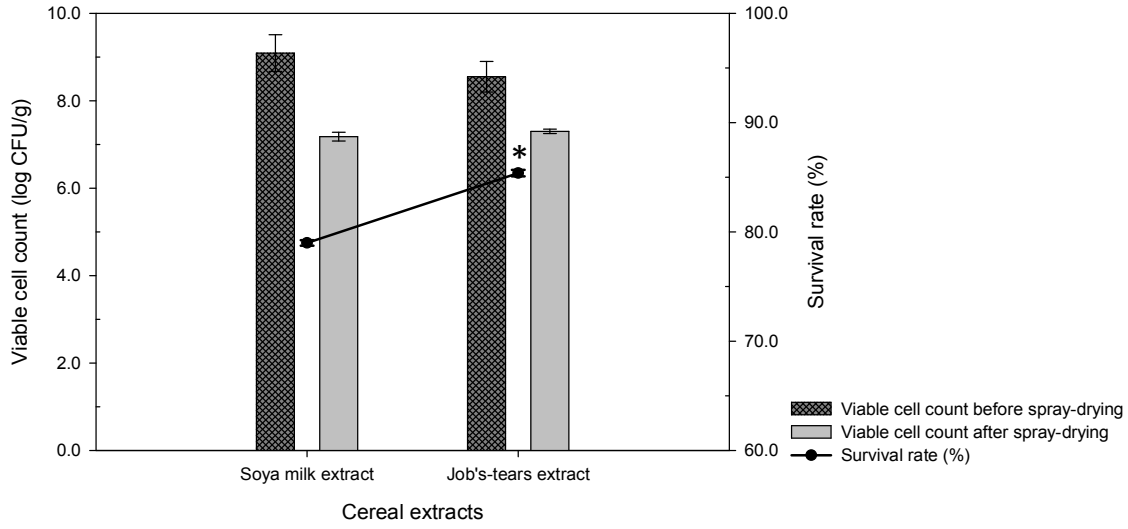
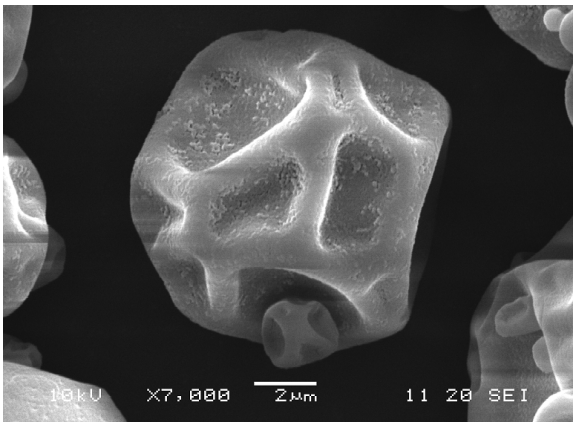


Figure 2. Viable cell counts of *L. plantarum* TISTR 2075 grown in soya milk and Job's-tears extracts before and after spray-drying. Survival rates of strain after spray-drying of cereal extracts were compared.
* $P < 0.05$ (Student's *t*-test, two tailed)

a)



b)

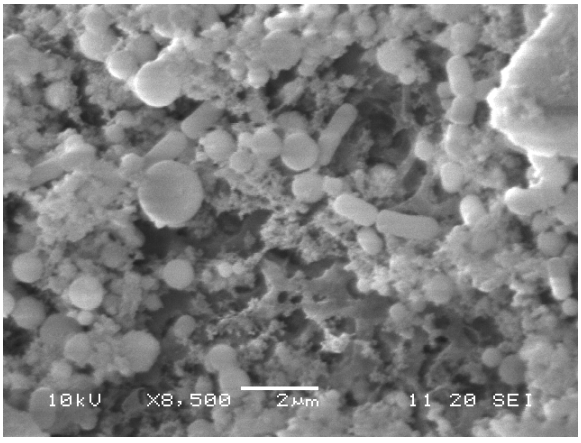


Figure 3. Scanning electron micrographs of spray-dried *L. plantarum* TISTR 2075: a) with MD and b) after rehydration in 0.85% NaCl

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Probiotic Properties

Results of simulated gastric juice tolerance of *L. plantarum* TISTR 2075 in spray-dried fermented cereal extracts are shown in Table 3. The survival rate of spray-dried strain (44.3%) is not significantly different ($P > 0.05$) from that of the free cells (46.8%) at 180 min. This is in agreement with the work of Reddy et al. [27], in which the acid tolerance of *L. plantarum* CFR 2191 was found to remain at a significant level even after spray-drying. However, the survival rates of the spray-dried *L. plantarum* TISTR 2075 are significantly lower than those of the free cells after exposure for 30, 60 and 90 min. These results are in accordance with those of Miciel et al. [47], in which heat-injured cells are more prone to inactivation in stress environments such as gastrointestinal conditions and high salt.

After exposure to the simulated small intestinal juice with 0.45% bile salt for 240 min., the TISTR 2075 strain exhibited a survival rate of 82.4%, which was not different from the control (77.5%), thus indicating that its acid and bile tolerance is not affected by the spray-drying process. According to Reddy et al. [27], there is no significant difference in bile tolerance between spray-dried *L. plantarum* CFR 2191 and active cell. Serrazanetti et al. [48] suggested that the small intestinal juice tolerance of probiotic bacteria is strain dependent.

Table 3. Survival rates of spray-dried *L. plantarum* TISTR 2075 exposed to simulated gastric juice at pH 2.0

Condition	Survival rate (% ± S.D.)			
	Exposure time			
	30 min.	60 min.	90 min.	180 min.
After spray-drying	78.72 ± 0.93*	68.67 ± 0.45*	60.90 ± 1.92*	44.33 ± 2.01
Control (free cell)	91.25 ± 3.36	82.21 ± 3.21	73.56 ± 3.52	46.83 ± 1.70

* $P < 0.05$ (Student's *t*-test, two-tailed)

Spray-dried *L. plantarum* TISTR 2075 was also found to exhibit pathogenic inhibition of *Escherichia coli* O157:H7 DMST 12743 and *Salmonella typhimurium* ATCC 13311, with inhibition zones of 12.6 ± 0.3 and 14.4 ± 0.4 mm respectively. These results were not significant different ($P > 0.05$) from the inhibition zones of the free cell, which were 12.2 ± 0.2 mm. and 14.3 ± 0.2 mm. respectively, again indicating that the pathogenic inhibition of *L. plantarum* TISTR 2075 is not affected by the spray-drying process. This agrees with the work of Gardiner et al. [25], in which spray-dried *L. salivarius* UCC 118 was found to retain its pathogenic inhibition of *Bacillus coagulans*. According to Silva et al. [24], spray-dried *L. sakei* CTC 494 and *L. salivarius* CTC 2197 were observed to retain the bacteriocinogenic activity against *Listeria innocua*, *L. monocytogenes* and *Staphylococcus aureus.*

CONCLUSIONS

Soya milk and Job's-tears extracts supplemented with sesame can be used as culture media for the growth of *Lactobacillus plantarum* TISTR 2075, giving a viable cell number above 8.6 log CFU/mL. A significant increase in calcium solubility was also observed after fermentation of the cereal extracts supplemented with 1% glucose and 1% sesame. After spray-drying, high numbers of viable cells (7.30 log CFU/g in Job's-tears extract powder and 7.18 log CFU/g in soya milk powder) could be achieved and their functional probiotic properties, viz. pathogenic inhibition of foodborne

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pathogens and tolerance to simulated gastrointestinal tract conditions were not affected. These findings make possible the development of new functional cereal beverages containing probiotics.

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