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Original Article

Comparison of synbiotic beverages produced from riceberry malt extract using selected free and encapsulated probiotic lactic acid bacteria

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ABSTRACT

There has been much recent interest in probiotic products for lactose-intolerant consumers. This research developed a non-dairy synbiotic beverage from riceberry malt extract (RME). The objectives of the study were to select suitable lactic acid bacteria (LAB) and to compare the stabilities of products produced from free and encapsulated cells. Five LAB (*Enterococcus faecalis* N1-33, *Lactobacillus acidophilus* TISTR450, *Lactobacillus johnsonii* KUN119-2, *Lactobacillus plantarum* TC24 and *Lactobacillus reuteri* KUB-AC5) were tested for their ability to grow in RME, their survival under simulated gastrointestinal tract conditions and their antimicrobial activity. *L. plantarum* TC24 had the greatest probiotic potential. Comparisons of free versus encapsulated cell growth rates and their respective stabilities were carried out at two storage temperatures (8 °C and 30 °C) for 31 d. Calcium alginate encapsulation improved the survivability of *L. plantarum* TC24 under gastrointestinal tract conditions. However, the same treatment did not affect survivability under fermentation and storage conditions. Both free and encapsulated cell products could be stored at 8 °C for 15 d and the products retained viable cells at a level of 9 log colony forming units/mL. The encapsulated cell product was less preferable in consumer tests than the free cell product. The results suggested that RME has potential as a raw material for the production of nondairy synbiotic items for human consumption.

Introduction

Riceberry rice (*Oryza sativa L. indica* var. riceberry) was named for its dark purple color which cross-breed of Jao Hom Nin and Khoa Dawk Mali 105 (Rice Gene Discovery and Rice Science Center, 2017). Riceberry combines desirable features of two prominent rice varieties, namely the high antioxidative property of *Hom Nin* and the aromatic property of the well-known and fragrant *Khaw Dok Mali* (jasmine rice). Riceberry bran extract contains free fatty acids, their methyl ester derivatives and plant sterols; in addition, the cyanidin-3-glucoside and peonidin-3-glucoside found in riceberry have been shown to induce apoptosis in HL-60 and Caco-2 cancer cells (Leardkamolkarn et al., 2011). Reduction of oxidative stress and regenerative changes in the pancreas, kidney, and heart tissue of diabetic rats were reported after long-term treatment with riceberry bran oil (Posuwan et al., 2013). Pigmented rice has been studied extensively for its bioactive compounds; example properties of such compounds include anti-oxidative function (Jeng et al., 2012; Saikia et al., 2012) and immunomodulation and collagen synthesis in normal human dermal fibroblast cells (Phetpornpaisan et al., 2014). However, a functional beverage derived from riceberry has never been reported to date, despite the documented benefits of pigmented rice (Leardkamolkarn et al., 2011; Posuwan et al., 2013). Nonetheless, there has been recent interest in the potential of riceberry products as functional foods. The present study investigated the development of a non-dairy synbiotic beverage from riceberry malt extract. The beverage was developed for lactose-intolerant patients and for general health care purposes.

Probiotics are microorganisms that when administered in adequate amounts confer a health benefit to the host as defined by FAO/WHO (Hill et al., 2014). Health benefits of specific probiotic strains have been demonstrated for the following genera: *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Bacillus*, and *Escherichia coli* (Fijan, 2014). Generally, the existence of probiotics in the gastrointestinal tract of humans or animals depends on host behavior, the presence

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of antibiotics, and host dietary uptake (Rolfe, 2000). Fermentable ingredients that allow specific changes of composition or activity or both in the gut microbiota conferring benefits upon host health are defined as "prebiotics" (Gibson et al., 2004). Inulin and fructo-oligosaccharides are common prebiotics that help to improve the activity of probiotics. Probiotics have been used to improve the properties of fruit juice (Saavedra-Leosa et al., 2014), soy yoghurt (Bedani et al., 2013) and cream cheese (Alves et al., 2013).

Probiotic viability in the digestive tract and anti-pathogenic bacterial properties are considered vital for optimal probiotic function and probiotic products are considered beneficial when they contain at least 7 log colony forming units (cfu)/mL viable cell content (Shan, 2001). Unsuitable transportation methods, variation in shelf temperature and exposure to acid and bile salts in the gastrointestinal tract are among the various factors that can cause a decrease in the number of probiotic living cells (Soccol et al., 2010). Hence, the amount of probiotic content may be insufficient to overcome pathogenic microorganisms or even the local microflora. Therefore, cell encapsulation is used as a method of enhancing the cell survivability of probiotics in the digestive tract; microencapsulation techniques have been developed using various matrices for the protection of bacterial cells from damage due to the external environment and have been successfully applied (Rathore et al., 2013).

A probiotic riceberry malt extract (RME) beverage would be a novel product for vegetarians and for those who are allergic to lactose in dairy products. Therefore, the objective of the present research was to select suitable LAB and to compare product stabilities produced from free and encapsulated cells under various storage conditions.

Materials and methods

Microorganisms and inoculum preparation

The five lactic acid bacteria (LAB) used in this study were: *Enterococcus faecalis* N1-33, *Lactobacillus acidophilus* TISTR450, *Lactobacillus johnsonii* KUN119-2, *Lactobacillus plantarum* TC24 and *Lactobacillus reuteri* KUB-AC5.

The starter culture was prepared using inoculation of frozen stock culture in de Man, Rogosa and Sharpe (MRS) broth at a concentration of 10% (volume per volume; v/v) followed by incubation at 37 °C for 48 h.

Rice malt extract preparation

Riceberry malt was ground to 0.5 mm particle size using a pin mill (21,952×*g*, Alpine model 1602; Augsburg, Germany) and then mashed with water (malt:water ratio = 1:3). The temperature of the mash was raised to 45 °C for 30 min to allow the riceberry malt particles to absorb water and to produce partial activation of protein hydrolysis. The temperature was then increased and held at 60 °C for 60 min for saccharification; this was followed by increasing the temperature to 70 °C for 30 min to produce complete starch hydrolysis. The mash was then held at 85 °C for 10 min to terminate the malt enzyme activity. The rice malt extract (RME) was obtained using filtration of the mash through a thin white cloth, after which the filtrate was sterilized in an autoclave (Tomy SX-700 Autoclave; Tomy Digital Biology Co. Ltd; Tokyo, Japan) at 121 °C for 15 min. The clarified RME was obtained using filtration through filter paper (Whatman No. 2).

Selection of probiotic lactic acid bacteria

Growth ability in rice malt extract

The growth ability of the five selected LAB in RME and the corresponding pH and acidity were evaluated in this experiment. The Five LAB were prepared as described in the section on inoculum preparation. The cell pellets of the five LAB were collected using centrifugation at 2044×*g* for 30 min at 4 °C (Spectrafuge 16 M; Labnet; Woodbridge, NJ, USA) and washed twice with RME. The pellets were re-inoculated in 5 mL of RME to obtain a final concentration of 1×10^7 cfu/mL followed by incubation at 37 °C for 48 h. The fermented RME samples were collected at 4 h intervals for the determination of cell growth, pH and acidity.

For viable cell counting, 10-fold serial dilutions of the fermented RME samples were made using quarter-strength Ringer's solution (Patel et al., 2004); the pour plate technique was employed, and the plates were overlaid with MRS agar. The viable cell number was determined by estimating the number of colony-forming units on the MRS-agar plates (medium pH 5.7) after incubation at 37 °C for 48 h; counts were expressed as colony forming units per milliliter. The pH of the fermented RME was measured using a pH meter, and acidity was determined using titration according to the Association of Official Agricultural Chemists (2000).

Tolerance of simulated gastrointestinal tract conditions

E. faecalis N1-33, *L. acidophilus* TISTR450, *L. johnsonii* KUN119-2, *L. plantarum* TC24 and *L. reuteri* AC5 were grown in MRS broth at 37 °C for 48 h before collection by centrifugation at 2044×*g* for 30 min at 4 °C. Cell pellets were washed twice with RME and re-suspended in 10 mL of 0.1 M phosphate-buffered saline at pH 2.5 (adjusted with concentrated hydrochloric acid) containing pepsin (3 g/L; Zhao et al., 2012) prior to incubation at 37 °C for 3 h. The cell pellets were harvested using centrifugation (2044×*g* for 30 min at 4 °C), washed twice with RME, and resuspended in 5 mL of RME before mixing with 5 mL of 3% (weight per volume; w/v) oxgall solution containing 1 g/L pancreatin USP (P-1500; Sigma; St. Louis, MO, USA). The assay mixture had a final bile concentration of 1.5% (w/v) at pH 7.5; the mixture was adjusted with 1N NaOH before incubation at 37 °C for 3 h. Initial viable cell concentration (0 h) and final viable cell concentration (3 h) were determined using the spread plate method with properly diluted samples on MRS agar plates. The survival percentage was determined according to Equation (1):

$$\%Cellsurvival = \frac{Finalcellconcentration}{Initialcellconcentration} \times 100 \quad (1)$$

Antimicrobial activity

Antimicrobial activity was determined using the agar well diffusion method (Tagg and McGiven, 1971). The selected pathogens (*Bacillus subtilis* TISTR025, *Escherichia coli* 010, *Samonella enteritidis* DMST17368, and *Staphylococcus aureus* TISTR118) were grown in nutrient broth at 37 °C for 24 h. Each pathogen was subsequently spread on the respective nutrient agar plate and then 5 mm wells were prepared using a sterile cork borer. A volume of 50 μL of LAB supernatant (after incubation at 37 °C for 16 h in MRS media) was aliquoted into the wells. All cultured agar plates were then incubated at 37 °C for 24 h to observe inhibition zones. The diameters of inhibition zones were measured and expressed in units of millimeters of exclusion of the well diameter.

Comparisons of viability in fermentation and gastrointestinal tract conditions for free and encapsulated selected probiotic bacteria

Encapsulation of selected probiotic cells with alginate hydrogel

The inoculum of *L. planetarium* TC24 at 10% v/v was initially cultured in MRS broth and incubated at 37 °C for 16 h. Cell pellets were collected using centrifugation (2044×*g*, 30 min, 4 °C) and

inoculated in RME solution at 7 log cfu/mL for reculturing under the conditions indicated above. Cell pellets were collected in the same manner for use in the following experiment.

The encapsulation of the selected probiotic bacterial culture was prepared by mixing cell pellets with sterilized 2% (w/v) sodium alginate, 5% (v/v) glycerol, 1% (w/v) inulin, 0.1% (v/v) Tween 80 and

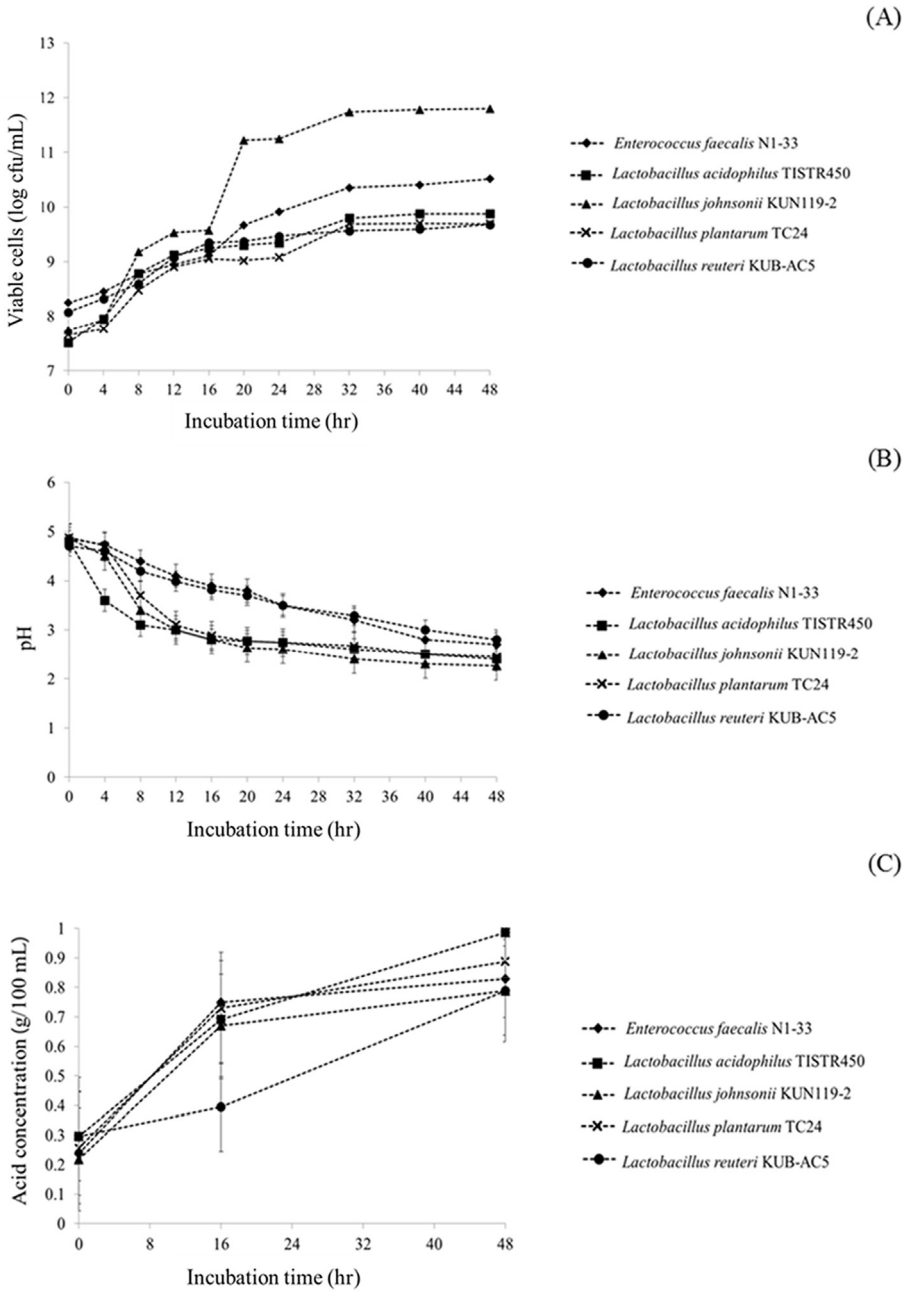

Fig. 1. Fermentation profiles of RME inoculated with five LAB at 37 °C for 48 h: (A) viable cell concentration; (B) pH; (C) titratable acidity, where cfu = colony forming units and error bars indicate standard deviation.

Table 1
Tolerance of simulated gastric and intestinal juices of five lactic acid bacteria.

	Viability in gastric juice (log cfu/mL)		% survival	Viability in bile juice (log cfu/mL)		% survival
	0 h	3 h		0 h	3 h	
E. faecalis N1-33	9.79 ± 0.03	9.38 ± 0.02	95.81^{c}	9.52 ± 0.03	8.31 ± 0.02	84.88^{b}
L.acidophilus TISTR450	9.92 ± 0.02	9.81 ± 0.01	98.89^{a}	9.90 ± 0.04	8.79 ± 0.05	88.60^{a}
L. johnsonii KUN119-2	10.83 ± 0.02	10.09 ± 0.01	93.16^{d}	10.11 ± 0.04	8.6 ± 0.10	79.40^{c}
L. plantarum TC24	10.98 ± 0.02	10.76 ± 0.00	97.99^{b}	10.76 ± 0.02	9.83 ± 0.02	89.52^{a}
L. reuteri KUB-AC5	10.26 ± 0.01	10.06 ± 0.02	98.05^{b}	10.0 ± 0.02	9.13 ± 0.09	88.98^{a}

Values show average cell viability and survival percentages of triplicate analyses.
Different lowercase superscript letters within the same column indicate significant ($p < 0.05$) differences.

Table 2
Mean (±SE)(±SD) antibacterial activity of cell-free supernatant of five lactic acid bacteria.

	Inhibition zone (mm)			
	B. subtilis TISTR 024	*E. coli* 010	*S. aureus* TISTR 118	*S. enteritidis* DMST 17368
E. faecalis N1-33	12 ± 0.1^{b}	14 ± 0.0^{b}	14 ± 0.0^{e}	14 ± 0.1^{c}
L. acidophillus TISTR450	11 ± 0.1^{c}	15 ± 0.0^{a}	17 ± 0.0^{b}	14 ± 0.0^{c}
L. johnsonii KUN119-2	11 ± 0.0^{c}	15 ± 0.1^{a}	15 ± 0.0^{c}	16 ± 0.1^{a}
L. plantarum TC24	14 ± 0.0^{a}	15 ± 0.3^{a}	20 ± 0.0^{a}	16 ± 0.1^{a}
L. reuteri AC5	12 ± 0.0^{b}	14 ± 0.0^{b}	15 ± 0.1^{d}	15 ± 0.0^{b}

Mean size of inhibition zones in millimeters excluding the well diameter of triplicate analyses. Different lowercase superscript letters within the same column indicate significant ($p < 0.05$) differences.

5.5% (v/v) RME. The encapsulation mixture at an initial viable cell concentration of 1×10^7 cfu/mL was extruded through a sterile needle (No. 25) at a height of 10 cm from the surface of a sterile 0.1 M calcium chloride solution. The gel beads were left in 0.1 M calcium chloride solution for 30 min before washing twice with RME.

The viable cell concentration in 1 g of fresh gel beads was determined to estimate the weight of beads required to achieve a certain number of viable cells. Alginate gel beads were depolymerized in sterile 1% (w/v) sodium citrate solution with gentle shaking for 20 min at room temperature (Tsen et al., 2003). The cell suspension was then serially diluted with Ringer's solution, and the viable cell count was determined the same as for free cells described in the experiment of growth ability in RME.

Growth of free and encapsulated probiotic cells in fermentation conditions

The inocula of *L. planetarium* TC24 were prepared in the forms of free and encapsulated cells as mentioned earlier. The initial cell concentrations were approximately 7 log cfu/mL for both of free and encapsulated cell fermentations. The RME fermentation was conducted at 37 °C for 48 h to monitor the growth of bacteria; cultures were observed every 4 h during the first 24 h and every 6 h thereafter.

Tolerance of free and encapsulated probiotic cells in simulated gastrointestinal tract conditions

The comparison of viable cell concentrations and survival percentages of free cell and encapsulated cell in simulated gastrointestinal tract conditions were conducted using the same method mentioned in the experiment of selection of probiotic LAB using an initial viable cell concentration of 9 log cfu/mL.

Morphology of encapsulated probiotic cells

The encapsulated probiotic alginate beads were observed under a scanning electron microscope (SEM; JSM-5600LV; JEOL; Peabody, MA, USA) (Kourkoutas et al., 2005). The beads were first washed with phosphate solution (15.25 g/L Na_2HPO_4, 5.85 g/L KH_2PO_4) prior to fixing in 2.5% (v/v) glutaraldehyde followed by dehydration with sequential ascending concentrations of ethanol solutions and drying in a critical point dryer operated at 35 °C, 1250 psi (K850; Quorum Technologies Ltd.; East Sussex, UK). The beads were then cut into thin layers and coated with gold before analysis under the SEM.

Product stability and sensory evaluation of the synbiotic beverage

Comparison of product stability under two storage conditions

Synbiotic beverage products fermented with free and encapsulated cells were prepared from RME and supplemented with 1.5% (w/v) inulin as a prebiotic. The products were incubated at 37 °C for 16 h order to achieve a cell viability at least 9 log cfu/mL. Honey from the Thailand Royal Chitralada project was added at a final concentration of 6% (v/v) prior to packaging in 50 mL sterile bottles, and the products were stored at 8 °C and 30 °C for 31 d. During this period, the viable cell count was enumerated every day for the first week of incubation and at 3 d intervals during subsequent weeks of incubation. The pH levels of both synbiotic beverage products were measured using a pH meter. Acidity was determined using the titration method according to the Association of Official Agricultural Chemists (2000).

Sensory evaluation test

Samples from both storage conditions were taken every 7 d. Sensory evaluation was conducted by inviting 50 consumers aged 15–60 years who had been trained to assess qualitative and quantitative differences between products to give personal ratings on overall satisfaction, color, odor and taste using a 9-point hedonic scale, with 1 representing disliked, 5 neither liked nor disliked (neutral), and 9 liked very much.

Statistical analysis

Analysis of variance was applied to test for significant effects, and the comparison of means was performed using Duncan's multiple range tests. Analyses were made using the SPSS software (version 14; SPSS Inc; Chicago, IL, USA) with significance set at $p < 0.05$.

Results and discussion

Selection of probiotic LAB

Growth in riceberry malt extract

The growth of each of the five selected LAB was significantly enhanced in the malt media (Fig. 1A). This could be attributed to the

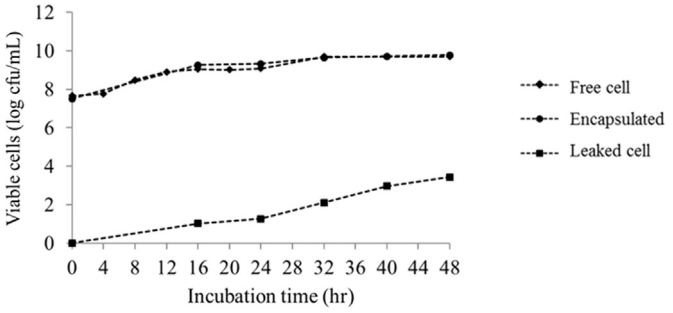

Fig. 2. Growth of *Lactobacillus plantarum* TC24 in riceberry malt extract (RME) under conditions of free cells and encapsulated cells (leaked cells were suspended individual cells in the RME fermented by encapsulated cells), where cfu = colony forming units.

Table 3
Tolerance of *L. plantarum* TC24 in simulated gastrointestinal tract conditions.

	Viability in gastric juice (Log cfu/mL)		% survival	Viability in bile juice (Log cfu/mL)		% survival
	Incubation time (h)			Incubation time (h)		
	0	3		0	3	
Free cells	9.47 ± 0.01	9.18 ± 0.01	96.93[b]	9.06 ± 0.05	8.73 ± 0.02	92.18[b]
Encapsulated cells	9.48 ± 0.01	9.37 ± 0.02	98.83[a]	9.40 ± 0.01	9.22 ± 0.02	97.25[a]
Leaked cells*	1.17 ± 0.03	1.93 ± 0.02	nd	1.36 ± 0.02	1.88 ± 0.01	nd

nd = not determined.
* suspended individual cells in the RME fermented by encapsulated cells.
Different lowercase superscript letters within the same column indicate significant ($p < 0.05$) differences.

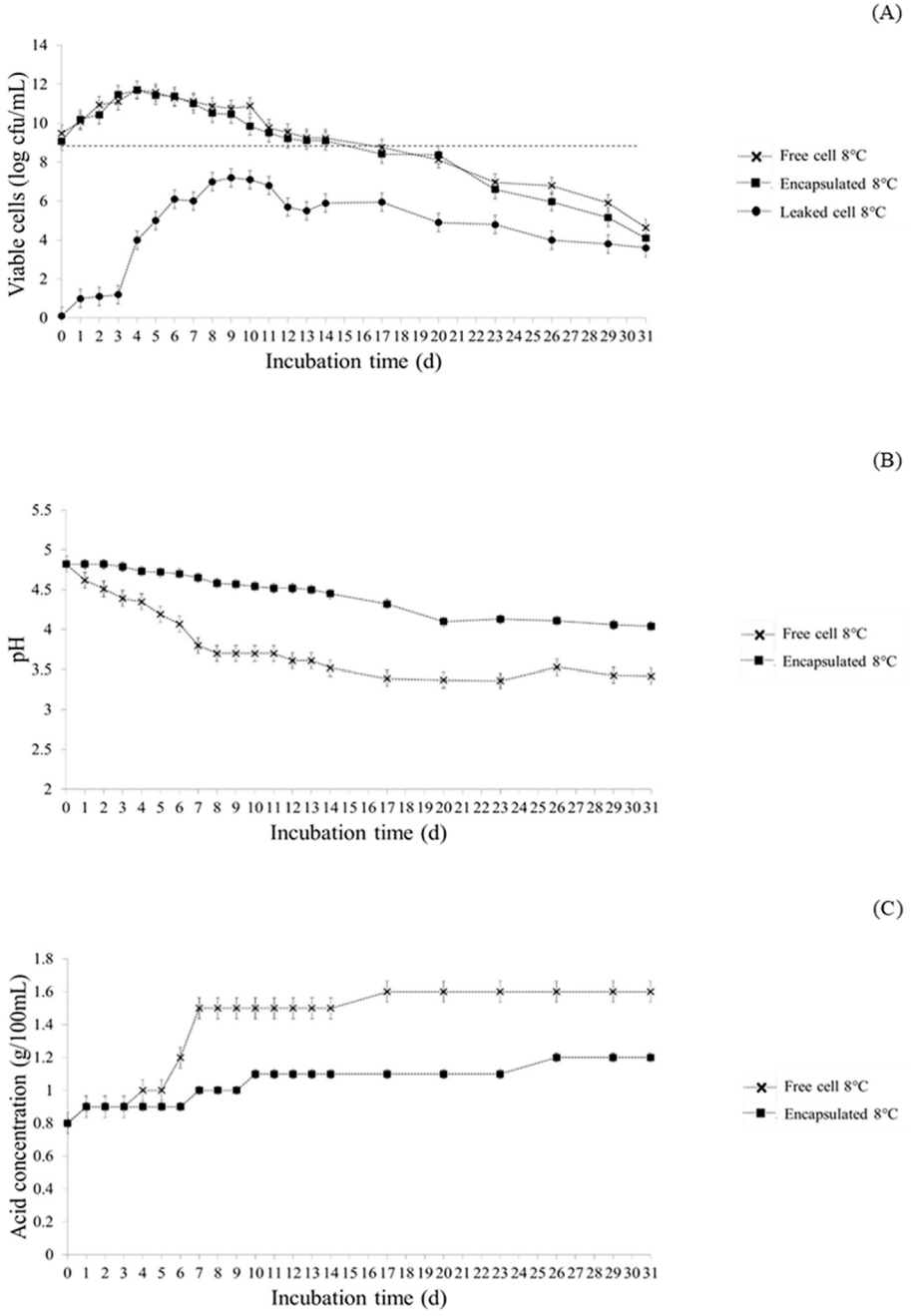

Fig. 3. Storage test of synbiotic beverages at 8 °C for 31 d after fermentation with free and encapsulated *L. plantarum* TC24: (A) viable cell concentration of free and encapsulated cells of *L. plantarum* TC24; (B) pH; (C) titratable acidity, where cfu = colony forming units.

simultaneous presence of monosaccharides (glucose and fructose), disaccharides (maltose and sucrose) and amino acids in the RME medium, as has been noted in other cereal-based probiotic beverages (Charalampopoulos et al., 2004; Rathore et al., 2012). The cell viability of the five LAB were approximately 9 log cfu/mL after 8–16 h of incubation, which was above the standard of viable cell numbers required for functional probiotics (6–7 log cfu/mL) (Galdeano and Perdigón, 2004; Kosin and Rakshit, 2006; Shan, 2001). However, the recommended number of viable cells that are delivered into the intestine has been stated as 9 log cfu per day (Heydari et al., 2011). Therefore, the viable cell number in the product at 9 log cfu/mL was preferred to ensure a health benefit for the host. Different rates of decrease in the pH with concomitant increases in titratable acidity were observed here. The different fermentation rates could be attributed to strain specificity. The pH measures of the RME after fermentation for 16 h using *E. faecalis* N1-33, *L. acidophilus* TISTR450, *L. johnsonii* KUN119-2, *L. plantarum* TC24 and *L. reuteri* AC5 were 3.9, 2.8, 2.8, 2.89, and 3.82, respectively (Fig. 1B). During fermentation, the lactic acid bacteria utilized sugar, and their metabolic activities resulted in the production of organic acids. Thus, the titratable acidities of the fermented beverages were increased in the range 0.4–0.7 g/100 mL within 16 h of incubation (Fig. 1C). The increase in acidity with a decrease in pH as fermentation proceeded might have eliminated or discouraged most of the spoilage by pathogenic microorganisms, especially spoilage due to those microorganisms that cannot withstand such conditions; this will result in probiotic non-dairy beverages being safer for consumption (Mohammadi et al., 2012: Rathore et al., 2012). Moreover, lactic acid has been reported to be an important flavor compound (Onyango et al., 2000); thus, it can influence the organoleptic responses of consumers (Kedia et al., 2007).

Tolerance of simulated gastrointestinal tract conditions

The viability of the five selected LAB under simulated gastrointestinal conditions was approximately 9 log cfu/mL (Table 1). This

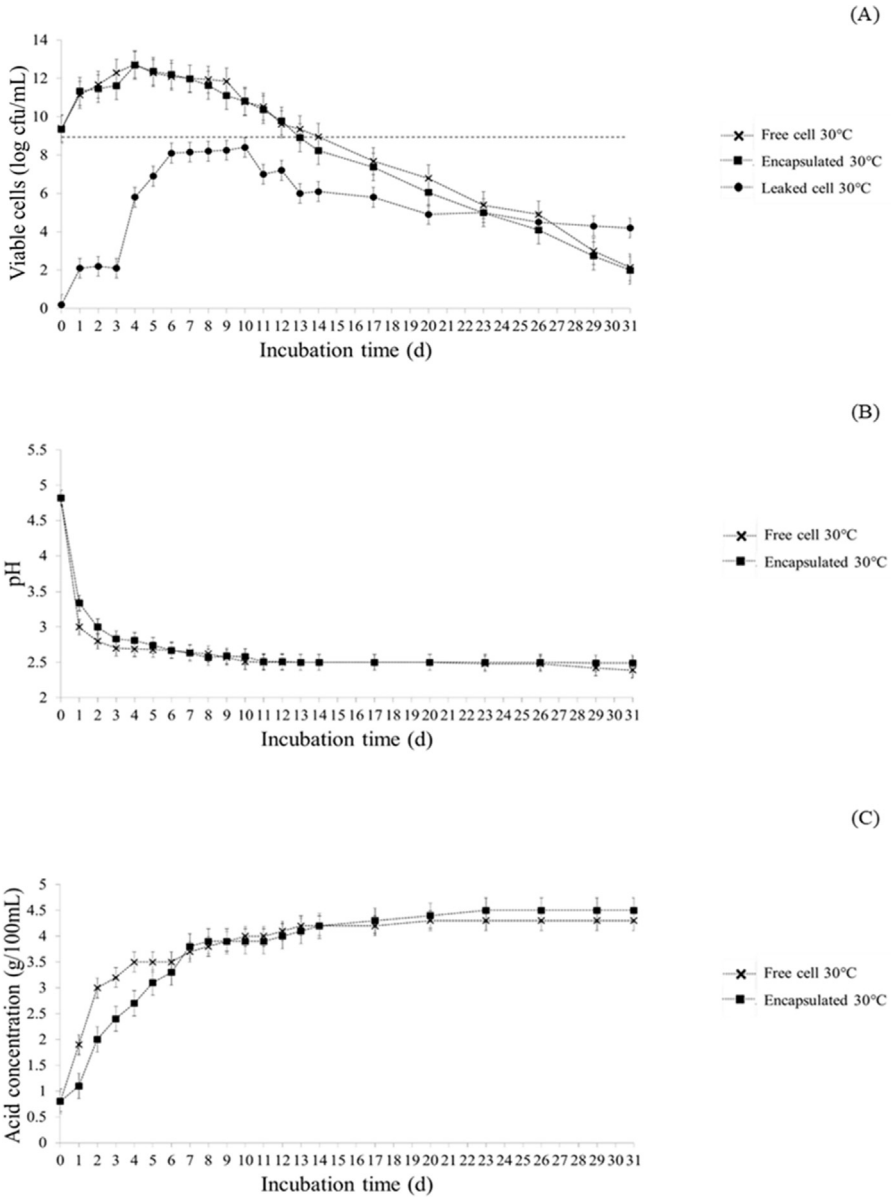

Fig. 4. Storage test of synbiotic beverages at 30 °C for 31 d after fermentation with free and encapsulated *L. plantarum* TC24: (A) viability of free and encapsulated cells of *L. plantarum* TC24; (B) pH; (C) titratable acidity, where cfu = colony forming units and error bars indicate standard deviation.

result indicated that the selected five LAB were able to tolerate the acidic environment of the gastrointestinal tract. Other studies have confirmed that *Lactobacillus* strains can tolerate acidic environments (Hassanzadazar et al., 2012; Jin et al., 1998; Mirlohi et al., 2009; Succi et al., 2005). The resistance to bile was an important characteristic enabling *Lactobacillus* to survive in the intestinal tract (Anatoly, 2001; Elli et al., 2006; Gilliland et al., 1984; Oozeer et al., 2006). All tested LAB showed approximately 1 log cfu/mL lower viability cell count when exposed to 1.5% (w/v) oxgall, resulting in the final viability in the range 8–9 log cfu/mL. The results of the acid and bile tolerance tests suggested that *L. acidpohilus* TISTR450, *L. reuteri* AC5 and *L. plantarum* TC24 were the most tolerant LAB. The survival percentages of these LAB under acidic conditions were 98.89, 98.05 and 97.99, respectively, and their survival percentages under bile conditions were 88.60, 88.98 and 89.52, respectively (Table 1).

Antimicrobial activity

The anti-pathogenic bacteria efficiency of the five selected LAB were reported in terms of the diameter of cleared zones (in millimeters) that appeared after incubation with bacterial culture supernatant for 24 h (Table 2). All tested LAB showed inhibitory activity, with *L. plantarum* TC24 demonstrating the strongest inhibitory effect. These results agreed with those of Selvamohan and Sujitha (2010) where *L. acidophilus, L. plantarum* and *L. casei* were reported to have inhibitory effects on pathogens (*Escherichia, Staphylococcus, Streptococcus, Klebsiella,* and *Pseudomonas*). The inhibitory ability of LAB has been reported to be attributed to the production of organic acids and bacteriocin, a proteinaceous compound exhibiting antimicrobial activity (Tran Van et al., 2011). Bacteriocins have exhibited a broad spectrum of inhibitory effects against pathogens, food spoilage organisms and various LAB (Ogunbanwo et al., 2003). Plantaricin from *L. plantarum* ZJ5 has been reported to be active at pH 2–6. However, the plantaricin was in an inactive form and was degraded at pH levels over 7 (Song et al., 2014). In comparison, *L. plantarum* TC24 had the highest antimicrobial activity in the current study; it also had high survival percentages under the simulated gastrointestinal tract conditions. Therefore, *L. plantarum* TC24 was selected as a high-potency probiotic bacteria for preparation of the riceberry malt synbiotic beverage.

Comparisons of viability in fermentation and gastrointestinal tract conditions for free and encapsulated selected probiotic bacteria

Alginate hydrogel encapsulation is a crucial method of providing nutrient and oxygen transportation across the hydrogel membrane. Most importantly, it can protect bacterial cells from a harsh environment. In this study, previous experiments demonstrated that *L. plantarum* TC24 could resist acid-bile conditions, with an 89.52% survival percentage. However, the gastro-intestinal tract matrix and its pH could change according to the food matrix and host behavior (McClements et al., 2016). Thus, the effect of alginate hydrogel encapsulation for improvement of *L. plantarum* TC24 survival was determined in the current study. Cell growth in alginate beads was compared to free cells cultured in RME for 48 h. The *L. plantarum* TC24 viability in alginate beads was not significantly different from that of the free cells (Fig. 2). This implied that cells could consume nutrients that were both mixed inside the beads and that had diffused into the beads. The microporous structure of the alginate hydrogel allowed nutrient transportation into the encapsulated cells; this sustained cell growth. Despite some leakage of cells (approximately 4 log cfu/mL) during fermentation, the number of cells retained in the beads was sufficient for delivery through the digestive tract. This was due to the fact that the percentages of free cells and encapsulated cells surviving after 3 h of exposure and under exposure to simulated gastric juices with pH 2.5 were 96.93 and 98.18, respectively. Under the simulated bile juice conditions, the survival percentages of free and encapsulated cells were 92.18 and 97.25, respectively. These results indicated that alginate hydrogel encapsulation significantly improved the survival rate of bacteria under the gastrointestinal tract conditions (Table 3). Similarly, studies on the survivability of free and encapsulated probiotic bacteria grown in yoghurt reported that encapsulated probiotic bacteria survived better in simulated acid and bile juice in comparison to free probiotic cells (Kailasapathy, 2005; Sandoval-Castilla et al., 2010).

Product stability and sensory evaluation of the synbiotic beverage

The synbiotic beverage was formulated by the addition of 1% (w/v) inulin, a dietary fiber prebiotic that can improve the growth of probiotic bacteria in the colon (Shan, 2001). Viable cells of *L. plantarum* TC24 in the form of free and encapsulated cells were inoculated in the synbiotic beverage at a concentration of 1×10^7 cfu/mL, and the beverage was fermented at 37 °C for 16 h. Finally, 6% (v/v) honey was added before storage.

The viable cell counts during synbiotic beverage storage at 8 °C and 30 °C were conducted for 31 d (Figs. 3 and 4). The numbers of viable cells for both synbiotic products increased rapidly to maxima (approximately 12 log cfu/mL) during the first 4 d of incubation and decreased to the original concentration after 2 wk. The effective number of beneficial probiotic cells delivered to the intestine should be more than 9 log cfu. Thus, the storage time at a refrigerated temperature was longer than that at warmer temperature (15 and 12 days, respectively). Cell survivability was not significantly different between the free and encapsulated cells. The faster depletion rate of bacterial cells stored at 30 °C in comparison to 8 °C could be attributed to a higher metabolic rate. This was reflected in the rapid decrease in the pH and the sharp increase in acid production (Fig. 4). This in turn led to rapid depletion of the nutrients required for the growth of bacterial cells after 12 d of storage. Moreover, excessive post-acidification not only increased the titratable acidity or decreased the pH during storage but also decreased the viability of the LAB (Mortazavian et al., 2007). In addition, the formation of some metabolites such as short-chain

Table 4
Sensory evaluation of synbiotic beverages stored at 8 °C.

	Storage time (d)	Mean score (±SE) (±SD)	
		Free cells	Encapsulated cells
Aroma	0	6.5 ± 0.223^{a}	6.3 ± 0.335^{a}
	7	6.3 ± 0.260^{a}	6.2 ± 0.326^{a}
	14	4.3 ± 0.472^{b}	3.9 ± 0.406^{bc}
	21	4.2 ± 0.512^{bc}	3.6 ± 0.618^{bc}
	28	4 ± 0.632^{bc}	2.8 ± 0.592^{c}
Taste	0	6.1 ± 0.276^{a}	5.9 ± 0.433^{a}
	7	6 ± 0.298^{a}	5.8 ± 0.416^{a}
	14	4.1 ± 0.481^{b}	3.2 ± 0.553^{bc}
	21	3.7 ± 0.538^{bc}	2.6 ± 0.561^{c}
	28	3.6 ± 0.400^{bc}	2.4 ± 0.266^{c}
Overall	0	6.5 ± 0.166^{a}	5.3 ± 0.335^{a}
	7	6.3 ± 0.260^{a}	5.8 ± 0.442^{a}
	14	4 ± 0.494^{b}	3.6 ± 0.561^{bc}
	21	3.7 ± 0.495^{bc}	2.4 ± 0.635^{cd}
	28	3.2 ± 0.416^{bcd}	2.1 ± 0.233^{d}

Sensory evaluation conducted by 50 persons of trained panelist using 9-point hedonic scale tests.
Different lowercase superscript letters indicate different levels of statistically ($p < 0.05$) significant differences between mean scores of each testing attribute by inoculum preparation method and storage time.

fatty acids and hydrogen peroxide and bacteriocins, which had been reported to be highly toxic to bacteria cells, could also account for the observed difference (Mortazavian et al., 2007; Shafiee et al., 2010; Korbekandi et al., 2011). The changes in the pH and acidity of the synbiotic beverage stored at 8 °C were dissimilar for both free and encapsulated cells in comparison to those of the synbiotic beverage stored at 30 °C. The higher temperature stimulated bacterial metabolism, causing more acid to be released; as a result, there was a considerable decrease in the number of viable cells (Mortazavian et al., 2007). Encapsulated cells had lower acidity values, while the pH was slightly higher than that of the free cells; these results were related to the slower reduction of viable cells.

The synbiotic beverages prepared with free and encapsulated cells were stored at the refrigerated temperature to produce a cool and fresh feeling in the consumer. In the sensory evaluation of the samples during the first week of storage time, no significant differences in terms of taste, aroma and overall quality were found between the free and encapsulated samples. There were slight differences between samples for all attributes after 14 d (Table 4). The consumer preference test indicated that the synbiotic product stored up to 7 d was rated as slightly liked (Table 4), but slightly disliked after 14 d of storage. There was a slightly lower preference score for the beverage made from encapsulated cells. The application of microcapsules of probiotics in food has received poor sensory ratings due to the texture and size of the microcapsules (De Prisco and Mauriello, 2016). In the current study, the size of the alginate beads (200 μm) was controlled by the needle size and the distance between the needle set point and the surface of the calcium solution.

The morphology of entrapped *L. plantarum* TC24 in sodium alginate beads was observed under an SEM (Fig. 5). The bead samples before storage at 8 °C (Fig. 5C and E) showed some clusters of bacterial cells coated with a thin layer of alginate hydrogel. However, the alginate hydrogel retained most of the bacterial population in the core structure and lost some bacterial cells from the beads into the RME solution after 1 d of storage. In addition, after storage at 8 °C for 14 d, disintegration of alginate hydrogel was observed under the SEM (Fig. 5D and F). Alginate hydrogel has been

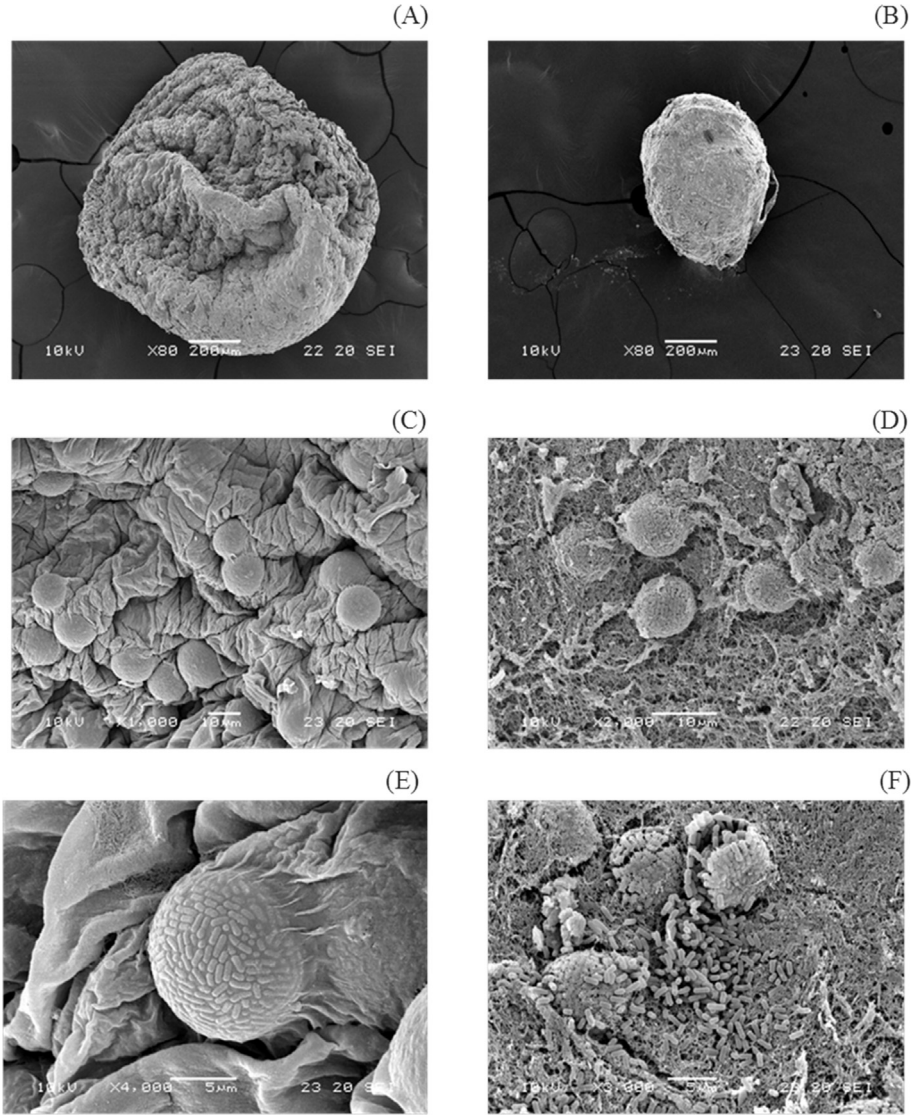

Fig. 5. Scanning electron micrographs of encapsulated *L. plantarum* TC24 in alginate beads under storage at 8 °C: (A) alginate beads before storage; (B) smooth surface of alginate beads before storage; (C) cluster of *L. plantarum* TC24 on the surface of an alginate bead before storage; (D) alginate beads after storage for 14 d; (E) disintegration on surface of alginate beads after storage for 14 d; (F) broken bacterial cluster found on the surface of alginate beads after storage for 14 d.

estimated to have a pore size of less than 17 nm (Klein et al., 1983), meaning that it can retain the microstructure of bacterial cells (0.5–4 μm). The type of core material can significantly limit the release of cells, and alginate hydrogel can release free cells even if it is coated with chitosan (Voo et al., 2011). However, it has been reported that the alginate gel is stable in low-acid environments. The pK_a values of mannuronic and guluronic acid are 3.6 and 3.7, respectively (Charalampopoulos et al., 2004). However, a high concentration of lactic acid produced by LAB may cause dissolution of the beads due to lactic acid competing to bind Ca^{2+} (Boyaval et al., 1985; Mokarram et al., 2009), or a chelating agent in the media could destabilize the gel network (Voo et al., 2011).

In conclusion, the RME medium supported the growth of the selected LAB quite well. Hence, it could serve as a nutritious medium for the production of novel non-dairy synbiotic beverages that have potential benefits in balancing the digestive system. Alginate hydrogel encapsulation significantly improved the survivability of the selected *L. plantarum* TC24 under GI conditions. For this reason, encapsulated *L. plantarum* TC24 could be employed to promote host gastrointestinal tract health, although the microcapsules may not be stable for long storage periods. Therefore, the recommended conditions appropriate for synbiotic products containing high concentrations of *L. plantarum* should combine a cool temperature with less than 2 wk of storage.

Conflict of interest

The authors declare there are no conflicts of interest.

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