



*Original Article*

## Effect of encapsulation of selected probiotic cell on survival in simulated gastrointestinal tract condition\*

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### Abstract

The health benefits of probiotic bacteria have been led to their increasing use in foods. Encapsulation has been investigated to improve their survival. In this study, the selection, encapsulation and viability of lactic acid bacteria (LAB) with probiotic properties in simulated gastrointestinal tract (GIT) condition were investigated. One hundred and fifty isolates of LAB were obtained from 30 samples of raw cow and goat milk and some fermented foods. Nine isolates could survive under GIT condition and only 3 isolates exhibited an antimicrobial activity against all food-borne pathogenic bacteria. Among them, 2 isolates (CM21 and CM53) exhibited bile salt hydrolase activity on glycocholate and glycodeoxycholate agar plates and were identified as *Lactobacillus plantarum*. CM53 was selected for encapsulation using 1-3% alginate and 2% Hi-maize resistant starch by emulsion system. Viability and releasing ability of encapsulated CM53 in simulated GIT condition was increased in accordance to the alginate concentration and incubation time, respectively.

**Keywords:** encapsulation, probiotic, lactic acid bacteria, simulated gastrointestinal tract, survival

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### 1. Introduction

The consumption of definite species of probiotic microorganisms is beneficial for reducing the duration and severity of diarrhea symptoms. Supplementing a diet with food containing beneficial bacteria can be used as a strategy for preventing diarrhea. Probiotics are defined as living microorganisms, which upon ingestion in certain number exert health benefits beyond inherent basic nutrition. For obtaining health benefits, a minimum of one million probiotic organisms per gram of food is recommended (Capela *et al.*, 2007). The most common commercial bacteria are of the genera *Lactobacillus* spp. These bacteria are typically saccharolytic (capable of metabolizing sugars), gram-positive,

rod shaped and reside in the large bowel (Cook *et al.*, 2012). The key criteria involved in the selection of probiotic microorganisms include the origin and biosafety of the strains, survival during passage through the gastrointestinal tract (GIT), ability to adhere and colonize on the epithelial cell surface of the host GIT, and inhibitory activity against enteric pathogens (Petsuriyawong and Khunajakr, 2011)

For processing and storage of foods, probiotic microorganisms may be exposed to low pH, high osmotic pressure and high levels of oxygen. These factors may have deleterious effects on probiotics. The survival of probiotic is also affected by acid encountered in the stomach and the bile salts in the intestine tract. Microencapsulation is the process of applying a shell to sensitive probiotic to protect them from their external environment (Capela *et al.*, 2007) and the probiotic cells are retained within an encapsulating matrix. Microencapsulation of probiotics has been examined for its ability to increase the viability of probiotic in food products and the intestinal tract. The most widely used encapsulating material is alginate, a linear heteropolysaccharide of D-

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mannuronic and L-guluronic acid extracted from various species of algae. Alginate beads can be formed by extrusion and emulsion methods. The use of alginate is privileged because of its simplicity, biocompatibility and inexpensive (Krasaekoopt *et al.*, 2004). Shah and Ravula (2000) found that calcium alginate beads formed by the emulsion technique can increase the viability of probiotic during processing and storage of frozen yoghurt. However, there are several disadvantages of using the emulsion technique including a wide distribution of bead size, difficulty in the automating technique and the large proportion of large beads with diameters ranging from 200 to 1000  $\mu\text{m}$  (Poncelet *et al.*, 1992).

The aim of this work was to screen lactic acid bacteria (LAB) with probiotic potential from raw cow and goat milk and some fermented foods. Encapsulation of probiotic with alginate by emulsion technique, the survival of encapsulated cell in simulated GIT condition, the efficiency of encapsulation and morphology of encapsulated cell were then evaluated.

## 2. Materials and Methods

### 2.1 Screening of LAB with probiotic potential

#### 2.1.1 Isolation of LAB

Ten raw cow milk, ten raw goat milk and ten fermented food samples were collected from five markets in Singhanakhon, Meuang, Jana, Hatyai and Sadao districts in Songkhla province, Thailand. To isolate the LAB, 25 g of each sample was taken aseptically, transferred to sterile 0.85% NaCl (J.T. Baker, USA) and then homogenized using a stomacher (Seward, England). One ml of homogenized samples was removed for dilution ten-fold with 0.85% NaCl. Appropriate concentrations were pour plated in MRS agar containing 0.5%  $\text{CaCO}_3$  (Finechem, Australia) and incubated at 37°C for 48 h. Different colonies with clear zone from agar plate were randomly selected from countable plates and then streaked onto MRS agar containing 0.5%  $\text{CaCO}_3$ . Strains with Gram positive and catalase-negative were selected and stored at -20°C in MRS broths containing 20% glycerol (VWR International, England). All strains were subcultured twice prior to use.

#### 2.1.2 Simulated GIT condition tolerance

This analysis was based on the method described by Krasaekoopt *et al.* (2004). Freshly prepared cells were placed in a tube containing sterile simulated gastric juice (phosphate-buffer saline (PBS) pH 2.0 containing 3 mg/ml pepsin (Sigma, USA)) and incubated at 37°C for 3 h. After incubation, the cells were removed for counting the surviving cells and placed in sterile simulated intestinal juice (PBS pH 8.0 containing 3 mg/ml pancreatin and 1% bile salt (Sigma, USA)). The tubes were then incubated at 37°C for 4 h. After incubation, a 1 ml aliquot of each isolate was removed and survival

rate was counted by drop plate on MRS agar containing 0.5%  $\text{CaCO}_3$ . Isolates which showed survival rate higher than 50% were selected for subsequent test. Survival rate was calculated by the following expression:

$$\text{Survival (\%)} = \frac{\log \text{ cfu / ml at 3 h}}{\log \text{ cfu / ml at 0 h}} \times 100$$

#### 2.1.3 Adhesion to hydrophobic solvent

Selected isolates were measured for cell surface hydrophobicity by measuring microbial adhesion to hydrocarbons as described by Ocaña *et al.* (1999). Cells were harvested by refrigerated centrifugation at 8739  $\times g$  for 4 min, washed twice and suspended in 2 ml of PBS pH 7.2 with the optical density at 600 nm of 0.4-0.6. Then 1 ml of n-hexadecane (Merk, Germany) was added to the cell suspensions, mixed by vortex for 3 minutes and allowed to separate at room temperature for 20 min. After the separation, the optical density of the aqueous phase (lower phases) was determined at 600 nm. Isolates which showed high hydrophobicity were selected for subsequent testing. The index of hydrophobicity was the result of the decrease of turbidity of the aqueous phase calculated by the following expression:

$$\text{Hydrophobicity (\%)} = \frac{(\text{OD}_{\text{before}} - \text{OD}_{\text{after}})}{\text{OD}_{\text{before}}} \times 100$$

#### 2.1.4 Antimicrobial activity

Bacteria used as test organism in this study consisted of 4 pathogenic bacteria (*Escherichia coli* DMST 4212, *Listeria monocytogenes* DMST 1327, *Salmonella* Typhimurium DMST 562 and *Staphylococcus aureus* DMST 8840). They were obtained from Department of Medical Sciences, Ministry of Public Health, Thailand (DMST). The antimicrobial activity of selected isolates was determined by agar spot test as described by Buntin *et al.* (2008). Briefly, LAB were grown in MRS broth and then spotted on MRS agar. Plates were incubated at 37°C for 18 h. Pathogenic bacteria were grown in Brain Heart Infusion (BHI) (Difco, USA) at 37°C for 6 h (to obtain approximately 6 log cfu/ml). Then 1 ml of growth culture was suspended in 9 ml BHI soft agar (0.75%, w/v) and overlaid on MRS agar in which LAB were grown. The plates were incubated at 37°C for 24 h. The antimicrobial activity was recorded as growth-free inhibition zones (diameter) around the cell spot.

#### 2.1.5 Bile salt hydrolase (BSH) activity

The ability of strains to deconjugate primary and secondary bile salts was determined according to Taranto *et al.* (1996). Bile salt plates were prepared by adding 0.5% (w/v) sodium salts of taurocholate (TC), taurodeoxycholate (TDC), glycocholate (GC) and glycodeoxycholate (GDC) (Sigma, Germany) to MRS agar. All selected isolates were

grown in MRS broth for 18 h. The liquid culture of bacterial strains (10 µl) was spotted on agar plates and incubated at 37°C for 72 h in anaerobic condition. The presence of precipitated bile acid around colonies (opaque halo) was considered as a positive result.

### 2.1.6 Identification of LAB

Selected isolates with probiotic potential were identified by 16s rDNA sequencing method as described by Chen *et al.* (2008). Briefly, LAB were cultured for 12 h in 1.5 ml MRS broth at 37°C and cells were collected by centrifugation at 7602 ×g for 3 min at 4°C. MRS broth was removed from the tube and genomic DNA was extracted from the cell. The DNA extracted was amplified by forward Primer 28 F and reverse primer 519 R. The amplification was done in 50 µl reaction volumes. Each PCR reaction consisted of 11 µl water, 5 µl 28F (10 pmole), 5 µl 519R (10 pmole), 25 µl master mix (Econo Taq®PlusGreen, Lucigen) and 4 µl DNA sample. The thermal cycling included an initial denaturation step at 94°C for 5 min; 35 cycles of a denaturation step at 95°C for 30 s, an annealing step at 52°C for 1 min, an extension step at 72°C for 1 min; and a final extension at 72°C for 7 min. PCR products were purified and then submitted to sequence. The sequencing identities were determined by BLAST program with the GenBank database.

## 2.2 Encapsulation of probiotic bacteria

### 2.2.1 Preparation of probiotic LAB for encapsulation

Selected probiotic LAB were subcultured twice in MRS broth. Cells were harvested by centrifugation at 8739 ×g for 15 min at 4°C. Cell pellets were washed twice with 0.85% NaCl and were finally re-suspended in 5 ml UHT milk with initial load of approximately 9-10 log cfu/ml, and the initial population of probiotic was counted by pour plate in MRS agar containing 0.5% CaCO<sub>3</sub>.

### 2.2.2 Encapsulation procedure

All glassware and solutions used in the protocols were prepared in an aseptic condition. Alginate beads were produced using a modified encapsulation method originally reported by Homayouni *et al.* (2008). 1, 2 and 3% alginate (Fluka, USA) mixture in distilled water containing 2% Hi-maize resistant starch (Merck, Germany) and probiotic culture were prepared. Each mixture was added into 200 ml of soybean oil (Morakot, Thailand) containing 0.2% lecithin (MEGA Lifesciences Ltd., Australia). The mixture was stirred vigorously at 400 rpm for 20 min until it was fully emulsified. Then 200 ml of 0.1 M calcium chloride solution was added. The mixture was allowed to stand for 30 min to separate the prepared calcium-alginate beads at the bottom of the beaker as a calcium chloride layer. The oil layer was drained and beads were collected in calcium chloride solution which then

washed with 0.9% NaCl containing 5% glycerol and stored at 4°C.

### 2.2.3 Alginate bead morphology

The shape of alginate beads was examined according to the method of Allan-Wojtas *et al.* (2008). Samples were chemically stabilized by immerse fixation in 0.1 M PBS pH 5.0 containing 2.5% glutaraldehyde at 4°C for 4 h then dehydrated through an ethanol series as follows: 50% (1×15 min), 70% (1×15 min), 90% (1×15 min) and 100% (3×15 min). The alginate bead was then dried in a critical point drier (Quorum Technologies Ltd, UK.). Dried samples were mounted on aluminium supports using silver cement, sputter coated with gold/ palladium using a Hummer VII sputter coater (Anatech Ltd., USA) and observed in a FEI Quanta 400 SEM (FEI Company, USA) operated at 20 kV at ambient temperature. Images were captured on Polaroid Type 55 positive/negative film (Polaroid Corporation, USA).

### 2.2.4 Determination of encapsulation efficiency

Alginate beads (1 g) were added to 9 ml of PBS pH 7.2 and then incubated at 37°C for 2 h. 1 ml of homogenized samples was removed, diluted to appropriate concentrations and pour plated in MRS agar containing 0.5% CaCO<sub>3</sub>. The encapsulation efficiency (EE) was calculated by the following expression:

$$EE (\%) = (X_t / X_i) \times 100,$$

where  $X_t$  is the total amount of probiotic loaded in alginate beads and  $X_i$  represents the initial amount of probiotic added in the preparation process.

### 2.2.5 Simulated GIT condition tolerance

Alginate beads (1 g) were placed in a sterile tube and assayed using method described in section 2.1.2.

### 2.2.6 Release of encapsulated cells

Alginate beads (1g) were transferred into 9 ml PBS pH 7.2, mixed gently and then incubated at 37°C for 6 h. Samples were kept after exposed to PBS pH 7.2 at 0, 1, 2, 3, 4, 5 and 6 h (Sheu and Marshall, 1993). Released bacteria were counted using pour plate technique in MRS agar containing 0.5% CaCO<sub>3</sub>. The index of cell release was calculated by the following expression:

$$\text{Cell release } (\%) = \frac{\text{Released bacteria count at difference time}(\log \text{ cfu / ml})}{\text{Initial bacteria count}(\log \text{ cfu / ml})} \times 100$$

## 2.3 Statistical analysis

All experiments and analyses were run in triplicate. Data were recorded as mean ± standard deviation (SD) and

were subjected to one-way analysis of variance (ANOVA). Multiple comparisons were performed by Duncan's test. Statistical significance was set at  $P < 0.05$ . All analyses were performed using SPSS (SPSS Inc, USA).

### 3. Result and Discussion

#### 3.1 Screening of LAB with probiotic potential

##### 3.1.1 Isolation of LAB

To isolate LAB, bacteria which showed clear zone on MRS agar containing  $\text{CaCO}_3$  were collected. Three hundred isolated bacteria obtained from the different sources were then identified on the basis of Gram-staining, morphology and catalase test. The isolates with Gram-positive and catalase-negative were collected. Table 1 shows the morphological classification of isolated LAB. These 150 isolates were classified into two groups based on cell morphology, including 76 cocci and 74 rod isolates.

##### 3.1.2 Simulated GIT condition tolerance

In order to act as probiotic in the GIT and to exert their beneficial effect on the host, the bacteria must be able to survive in the acidic conditions in the stomach and resist to bile acids at the beginning of the small intestine. Approximately 2.5 l of gastric juice and 1 l of bile are secreted into the human digestive tract every day. Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach, digestive enzymes and bile in the small intestine (Argyri *et al.*, 2012). Table 2 shows the survival of selected LAB isolates under simulated gastric juice condition and further simulated small intestinal condition. Among 150 LAB isolates, only 9 isolates were able to survive more than 50% in simulated GIT condition. These 9 isolates were composed of 7 rods and 2 cocci bacteria. Argyri *et al.* (2012)

reported the majority of *L. plantarum* and *L. pentosus* strains showed high resistance to low pH.

##### 3.1.3 Adhesion to hydrophobic solvent

Hydrophobicity has been considered as one of the main physical interactions during bacterial adhesion to epithelial cells. The hydrophobicity of a bacterial surface has been determined by direct methods, such as measuring the contact angle and indirect method such as the microbial adhesion to n-hexadecane (MATH) assay, which was used in this work.

MATH assay was employed to evaluate the hydrophobic character of the bacterial cell surface as shown in Figure 1. CM21, a rod-shape LAB, showed the highest hydrophobicity among other strains. However, it can be observed that adhesion to n-hexadecane of all isolates was lower than 50%.

The adhesion ability of LAB has been linked with their surface properties, which in turn is reflected by the composition, structure and organization of the cell wall. The Gram-positive cell wall of lactobacilli consists of a thick peptidoglycan layer, which is decorated with various surface components, including (lipo-)teichoic acids, polysaccharides,

Table 1. The morphological characteristic of isolated LAB

Samples	Number of LAB strains		
	Cocci	Rod	Total strains
Cow milk	1	53	54
Goat milk	43	11	54
Pla-Som	16	2	18
Fermented Jing-Jang Fish	6	2	8
Kimji	10	6	16
Total strains	76	74	150

Table 2. Survival rate of selected LAB strains under simulated gastric juice conditions at pH 2.0 for 3 h and further in simulated small intestinal tract condition at pH 8.0 for 4 h.

Isolates	0 h (log cfu/ml)	pH 2 for 3 h (log cfu/ml)	Survival (%)	pH 8 for 4 h (log cfu/ml)	Survival (%)
GF41	9.2 ± 0.7	6.1 ± 0.3	65.9	5.6 ± 0.2	60.1
CM7	8.9 ± 0.0	5.4 ± 0.1	61.0	4.5 ± 0.1	50.4
CM15	8.8 ± 0.1	5.3 ± 0.2	59.8	5.1 ± 0.2	58.3
CM21	9.6 ± 0.1	6.0 ± 0.1	62.7	5.9 ± 0.1	61.2
CM46	9.2 ± 0.9	6.1 ± 0.1	65.5	5.5 ± 0.3	59.3
CM47	8.6 ± 0.0	5.6 ± 0.4	64.8	5.2 ± 0.2	59.8
CM53	9.6 ± 0.1	5.8 ± 0.1	60.1	5.4 ± 0.3	55.9
JJ4	8.6 ± 0.1	5.8 ± 0.5	67.8	5.5 ± 0.1	64.7
KJ9	8.8 ± 0.0	5.4 ± 0.1	59.2	5.0 ± 0.0	56.9

Values are given as mean ± SD from triplicate determinations ( $n = 9$ ).

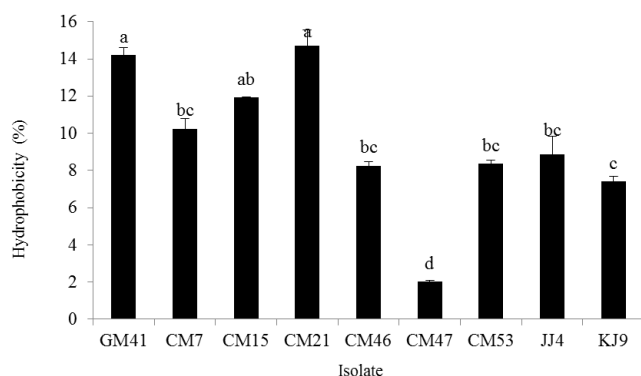


Figure 1. Cell surface hydrophobicity of selected lactic acid bacteria. The error bars represent standard deviations. Different superscripts indicates significant difference ( $p < 0.05$ ).

covalently bound proteins and S-layer proteins. Several studies have shown that these components are likely to contribute to the surface properties of a bacterium (Deepika *et al.*, 2009).

### 3.1.4 Antimicrobial activity

The bacteria used as indicators in this study included Gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and Gram-negative bacteria (*E. coli* and *S. Typhimurium*). Only 3 isolates, CM21, CM47 and CM53 showed inhibition effect against all pathogenic bacteria (Table 3). Among them, CM53 showed the highest inhibitory effect against all pathogenic bacteria. Maldonado *et al.* (2012) reported that natural inhibitor substances of LAB can be organic acids, hydrogen peroxide or bacteriocin. The lactic acid produced by LAB and the low pH is inhibitory to susceptible microorganisms such as Enterobacteriaceae. The results indicated that LAB

isolates may inhibit the pathogens mainly by production of lactic acid.

### 3.1.5 BSH activity

BSH is an enzyme that catalyzes the hydrolysis of conjugated bile salts resulting in free bile acid and amino acid. The white precipitates around colonies and the clearing of the medium are indicative of BSH activity. Moreover, BSH activity has been reported to correlate with cholesterol lowering.

Out of the 3 selected LAB isolates, based on antimicrobial activity against all pathogens, two isolates (CM21 and CM53) displayed precipitation zones on GC and GDC agar plate as shows in Table 4. Dashkevicz and Feighner (1989) reported that *L. plantarum* showed the BSH activity on GDC-MRS agar after being incubated anaerobically for 48 h.

### 3.1.6 Identification of LAB

In this work, 3 LAB isolates (CM21, CM47 and CM 53) presented probiotic properties and showed antimicrobial activity against all tested bacteria. Thus they were selected to identify by 16s rDNA sequencing and were determined by BLAST comparison of the obtained sequences with the nucleotide database in the GenBank database. 16S rDNA sequences of CM21, CM47 and CM53 identified as the species in the Genus *Lactobacillus* with the similarity between CM21, CM47 and CM53 and *L. plantarum* subsp. *plantarum* ATCC 14917<sup>T</sup> of 99.17, 99.79 and 98.91%, respectively. In addition, CM21, CM47 and CM53 showed most closely related to *L. pentosus* JCM 1558<sup>T</sup>, *L. plantarum* subsp. *argenteratensis* DKO 22<sup>T</sup> and *L. paraplantarum* DSM 10667<sup>T</sup>, *L. fabifermentans* DSM 21115<sup>T</sup> and *L. xiangfangensis* 3.1.1<sup>T</sup> with similarity higher than 97% as

Table 3. The antimicrobial activity of selected LAB strains against food-borne pathogenic bacteria

Isolates	Radius of inhibition zone around colony (mm)			
	<i>E. coli</i>	<i>S. Typhimurium</i>	<i>S. aureus</i>	<i>L. momocytogenes</i>
GF41	5.7 ± 0.4 <sup>b</sup>	—	2.46 ± 0.22 <sup>c</sup>	—
CM7	4.1 ± 0.5 <sup>b</sup>	—	—	—
CM15	6.3 ± 1.2 <sup>b</sup>	—	2.50 ± 0.49 <sup>c</sup>	—
CM21	6.4 ± 0.9 <sup>b</sup>	3.49 ± 0.44 <sup>b</sup>	5.23 ± 0.32 <sup>b</sup>	3.38 ± 0.88 <sup>a</sup>
CM46	5.2 ± 1.2 <sup>b</sup>	—	2.42 ± 0.20 <sup>c</sup>	1.19 ± 0.30 <sup>c</sup>
CM47	8.6 ± 0.3 <sup>b</sup>	5.44 ± 0.13 <sup>a</sup>	4.59 ± 0.72 <sup>b</sup>	3.56 ± 0.02 <sup>a</sup>
CM53	9.7 ± 0.8 <sup>a</sup>	5.05 ± 0.02 <sup>a</sup>	7.61 ± 0.18 <sup>a</sup>	3.06 ± 0.02 <sup>ab</sup>
JJ4	4.3 ± 0.2 <sup>b</sup>	—	2.32 ± 0.62 <sup>c</sup>	—
KJ9	5.7 ± 1.0 <sup>b</sup>	—	2.58 ± 0.11 <sup>c</sup>	2.11 ± 0.02 <sup>bc</sup>

Values are given as mean ± SD from triplicate determinations ( $n = 9$ ).

Different superscripts in the same column indicate significant difference ( $p < 0.05$ ).

Table 4. The bile salt hydrolase activity of selected LAB strains

Isolates	GC	GDC	TC	TDC
CM21	+	+	-	-
CM47	-	+	-	-
CM53	+	+	-	-

+: The present of opaque halo around colonies, -: the absent of opaque halo around colonies.

GC: glycocholate, GDC: glycodeoxycholate, TC: taurocholate and TDC: taurodeoxycholate.

shown in Figure 2.

In this study, *L. plantarum* CM53 which showed the greater antimicrobial effect than CM21 and CM47 was selected for study the effect of encapsulation on survival in simulated GIT condition.

### 3.2 Encapsulation of probiotic bacteria

#### 3.2.1 Size, morphology and encapsulation efficiency of alginate bead

In this study, *L. plantarum* CM53 was selected for encapsulated by emulsion technique. A small volume of the cell CM53-alginate suspension (discontinuous phase) was added to a large volume of a soy bean oil (continuous phase). The mixture was homogenized to form a water-in-oil emulsion. Once the water-in-oil emulsion was formed, the water soluble polymer was insolubilized (cross-linked) to form tiny gel particles within the oil phase. The mean diameters of beads were measured, calculated and presented in Table 5. The diameter of 1% alginate beads could not determined by microscopy while the mean diameters of 2 and 3% alginate beads were determined and showed the different average diameters with the ranges of 65-127  $\mu\text{m}$  and 146-228  $\mu\text{m}$ , respectively. The size and spherical shape of the bead

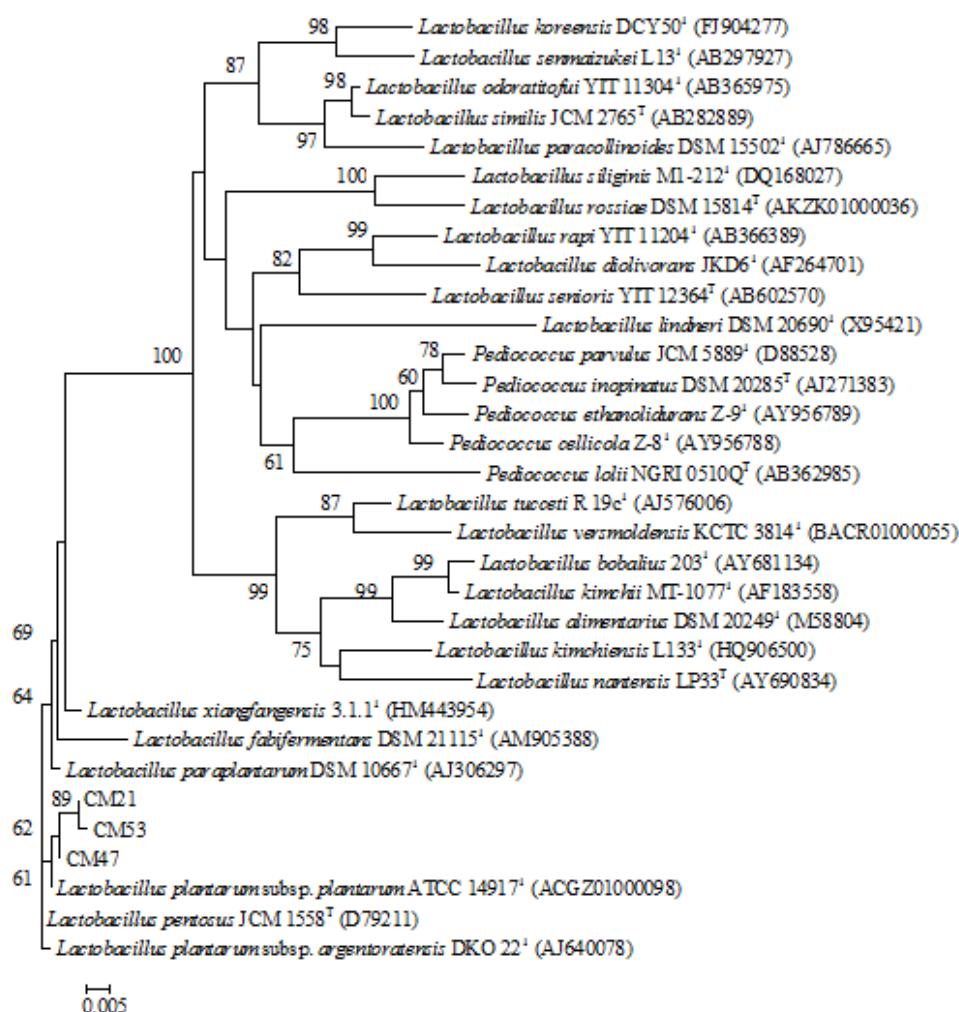


Figure 2. Neighbor-joining tree comprising 16S rDNA sequences of CM21, CM53, CM47 and related *Lactobacillus* species. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at the branch points. Bar, 0.01 substitution per nucleotide position.

Table 5. Size and encapsulation efficiency of alginate bead at different concentration of alginate

Treatments	Alginate beads size ( $\mu\text{m}$ )	Encapsulation efficiency (%)
1% Alginate	-	81.10 <sup>ns</sup>
2% Alginate	95.4	82.58 <sup>ns</sup>
3% Alginate	187.4	80.55 <sup>ns</sup>

<sup>ns</sup> No significant difference ( $p < 0.05$ ).

depend mainly on the viscosity of the sodium alginate solution, calcium chloride collecting solution and speed of agitation (Krasaekoopt *et al.*, 2004). Moreover, EEs for viable cell of *L. plantarum* CM53 at 1-3% alginate were not significantly different ( $P > 0.05$ ) (Table 5).

SEM micrograph of the alginate bead containing *L. plantarum* CM53 showed the spherical shape and the exterior surface of the bead covered with a network of small cracks and fissures (Figure 3A). The Figure 3B shows the internal structure of alginate bead. The bacteria were distributed randomly in the alginates matrix (Figure 3C) while the Figure 3D indicates that the alginate matrix entrapped *L. plantarum* CM53.

### 3.2.2 Survival of free and encapsulated *L. plantarum* CM53 in simulated GIT condition

Encapsulation in alginate containing 2% Hi-maize resistant starch bead improved the survival of *L. plantarum* CM53 in simulated GIT significantly ( $P < 0.05$ ) (Figure 4). The addition of Hi-maize resistant starch at 2% concentration in the encapsulation procedure was to provide prebiotic to the encapsulated probiotic bacteria (Sultana *et al.*, 2000). Cell survival after exposure to stomach gastric juice (SGJ) for 3 h was 92.97, 89.55, 77.55, and 69.82% of the initial population found in alginate beads produced by 3%, 2% and 1% alginate and free cells, respectively. Overall, the sequential exposure to SGJ (3 h) followed by simulated small intestine (4 h) resulted in significantly ( $P < 0.05$ ) higher number of *L. plantarum* CM53 surviving in the 2 and 3% alginate bead than were obtained for cells entrapped in 1% alginate bead and free cell. The cell survival after exposure to simulated small intestine for 4 h was 91.18, 89.09, 74.30 and 69.09% of the initial population found in alginate beads produced by 3, 2 and 1% alginate and free cells, respectively. After the initial losses, the populations of *L. plantarum* CM53 declined at the same rate for all treatments at the 7 h incubation period with final decreases of 2.96 log cfu/g for free cells and 0.70, 0.87 and 2 log cfu/g for 3, 2 and 1% alginate beads, respectively. The survival rate of *L. plantarum* CM53 in GIT was influenced by the concentration of alginate. Guérin *et al.* (2003) also found that an initial immobilized *Bifidobacteria bifidum* population of 10 log cfu/g in mixed alginate, pectin and whey protein matrix could reach the small intestine in

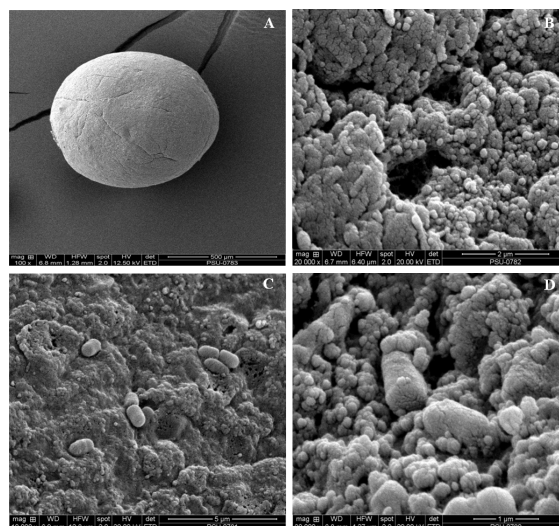


Figure 3. Scanning electron microscopy of calcium alginate beads. (A) Alginate bead (B) Fractured microcapsules without bacteria (an air pocket in dark area is visible) (C) Fractured microcapsule loaded with bacteria (D) Higher magnification view of microencapsulated bacteria. Magnification is indicated individually on data bar at bottom at the bottom of each micrograph

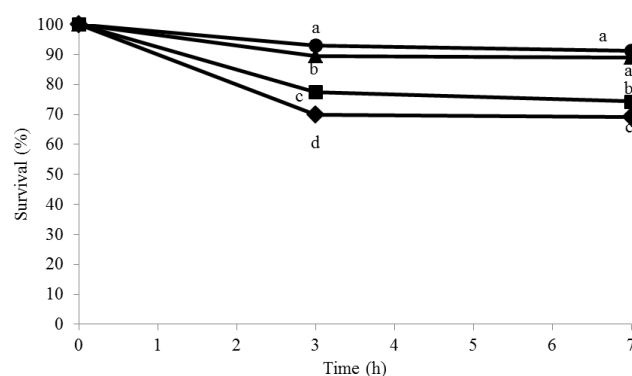


Figure 4. Survival of free and encapsulated *L. plantarum* CM53 during exposure to simulated gastrointestinal tract condition at 37°C for 7 h. Symbols: (◆) free cells, (■) 1% alginate, (▲) 2% alginate and (●) 3% alginate. Survival (%) represents the percentage of cells surviving relative to the initial population. Different superscript in the same time indicates significant difference ( $p < 0.05$ ).

numbers of 7.5 log cfu/g and hence provide the host with a beneficial health effect.

### 3.2.3 Release of encapsulated cells

While the ionotropic alginate gel formed by  $\text{Ca}^{2+}$  cross linking of carboxylate groups is insoluble in low pH, exposure to neutral pH or higher solubilises the alginate (Annan *et al.*, 2008). In our study, this pH-dependent behavior of the biopolymer was used to control the degradation of alginate bead and release of the microencapsulated cell load under the natural conditions found in the small intestine. The ability to release of *L. plantarum* CM53 from alginate beads in PBS pH 7.2 at the beginning of incubation (0 h) was  $48.15 \pm 0.06$ ,  $45.70 \pm 0.85$  and  $45.25 \pm 1.90\%$  for 1, 2 and 3% alginate beads, respectively as shown in Figure 5. When incubation time increased the release of cells was increased. Ability to release *L. plantarum* CM53 after exposure to PBS pH 7.2 for 2 h were 97.00, 90.25 and 87.81% of the initial population found in alginate beads produced by 1, 2 and 3% alginate, respectively. At 5-6 h of incubation, there were no significant differences ( $P > 0.05$ ) indicating no effect of alginate concentrations on the release of cells from microcapsules after 4 h. An efficient release of viable and metabolically active cells in the intestine is one of the aims of microencapsulation (Mandal *et al.*, 2006).

### 4. Conclusion

In conclusion, the results of this study showed that 3 strains of *L. plantarum* (CM21, CM47 and CM53), were found to possess desirable *in vitro* probiotic properties. These strains are good candidates for further investigation with *in vivo* studies to elucidate their potential health benefits as well as in fermentation studies to assess their technologi-

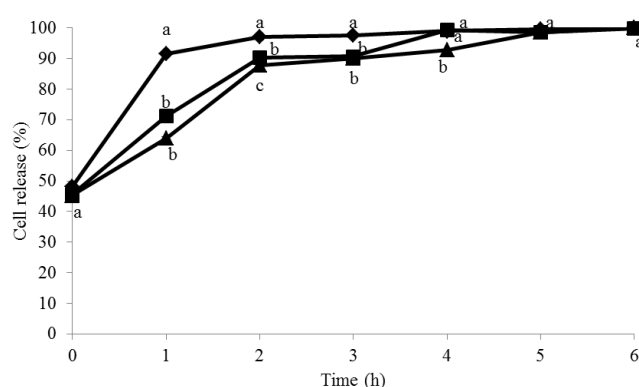


Figure 5. Release of *L. plantarum* CM53 from alginate microcapsules during exposure to simulated small intestine juice at different time intervals (0–6 h). Symbols: (◆) 1% alginate, (■) 2% alginate and (▲) 3% alginate. Cell release (%) represents the percentage of cells released relative to the initial population. Different superscript in the same time indicate significant difference ( $p < 0.05$ ).

cal characteristics for application as novel probiotic starters. Encapsulation of *L. plantarum* CM53 with alginate provided significant protection for viable cells from harsh acidic condition of simulated GIT conditions. As a result, significantly higher number of bacteria survived sequential incubation from the simulated gastric juice into the simulated small intestine due to the soluble of alginate beads in an appropriate condition.

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