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Evaluation of Microencapsulated Local Isolates *Lactobacillus casei* 97/L3 and *Lactobacillus delbrueckii* 94/L4 for Improved Probiotic and Yogurt Starter Culture Application

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The effect of microencapsulation on previously isolated *Lactobacillus delbrueckii* 94/L4 as starter culture for yogurt, and *Lactobacillus casei* 97/L3 as a probiotic candidate was investigated. Preliminary results showed that *L. delbrueckii* 94/L4 exhibited tolerance to bile, unlike *L. casei* 97/L3. Freeze drying significantly ($p < 0.05$) reduced the viability of both isolates by log 0.71–2.70. Although microencapsulation preserved the viability of *L. casei* 97/L3 cells exposed to simulated gastrointestinal tract conditions for 120 min, it did not impart significant ($p < 0.05$) protection against loss of viability during the first 30 min of exposure. Conversely, microencapsulated *L. delbrueckii* 94/L4 with the addition of *Streptococcus thermophilus* 24/S1 as starter culture was successfully incorporated into milk to form yogurt, yielding a significantly ($p < 0.05$) improved product quality.

Keywords: ***Lactobacillus casei*, *Lactobacillus delbrueckii*, microencapsulation, probiotic, starter culture**

Introduction

The demand for food containing probiotics or dairy products fermented with starter culture have been growing due to the continuous reports on their valuable effects to health and sensory preference [1, 2]. Common species that were identified as probiotics or starter culture belonged to the *Lactobacillus* and *Bifidobacterium* genera [3]. Nevertheless, other specific strains of the *Bacillus*, *Enterococcus*, *Escherichia coli*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, and *Saccharomyces* genera were claimed to impose benefit to consumers' wellbeing and fitness as well [4]. One of the prerequisite for probiotics to be effective in host, is that the microorganisms need to withstand the harsh condition along the GI tract reaching and colonizing the small intestine, where nutrients are being absorbed. These microorganisms need to sustain their viability throughout storage as well as withstanding the harsh condition of the GI tract upon consumption [5]. The convenience of storing whilst keeping the microorganism viable was reported to be achievable through microencapsulation which resulted in dried form of probiotic bacteria [6, 7]. Besides, microencapsulation confers protections of the embedded microorganisms along the GI tract, promoting their colonization [8]. This study was conducted to support the local market of starter cultures and probiotics in Asia for fermented products as the existing cultures were highly dependent on imports [9]. The aim of this study was to assess the propensity of two different *Lactobacillus* species: *Lactobacillus casei* 97/L3 and *Lactobacillus delbrueckii* 94/L4,

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previously isolated from local fresh milk on viability assessment to storage, tolerance towards bile and acids aiding the isolates application as functional starter cultures and probiotic, respectively [10]. This paper supported the application of the conventionally isolated local strains on storage through microencapsulation of both isolates by sodium alginate-chitosan using an extrusion-ionic gelation method [11, 12]. *L. casei* was reported to support the digestive health [13]. It is important for the isolate to colonize the intestine. Therefore, the viability of *L. casei* 97/L3 to withstand the GI tract condition was evaluated by measuring the survival rate of the isolate in simulated gastric and intestinal fluid. On the other hand, *L. delbrueckii* has been commonly applied with *Streptococcus thermophilus* as starter culture for yogurt [14]. The effect of microencapsulation on *L. delbrueckii* 94/L4 to be applied as starter culture for yogurt through measurement of cell viability was conducted. Subsequently, organoleptic assessments from the microencapsulated culture were evaluated to determine the quality of the dairy yogurt made.

Materials and Methods

Phylogenetic tree

Lactobacillus casei 97/L3 and *Lactobacillus delbrueckii* 94/L4 previously obtained conventionally from extracted dairy milk in Bogor, Indonesia were employed for this study [10]. The 16S rRNA gene nucleotide sequences GenBank entry are MH298536 for *L. casei* 97/L3, and MH298535 for *L. delbrueckii* 94/L4. The evolutionary history was inferred by using the Maximum Likelihood phylogenetic tree after MUSCLE alignment for isolates verification based on 16S rRNA gene sequences showing the position of strain *L. delbrueckii* 94/L4 and *L. casei* 97/L3 among related *Lactobacillus* species. Bootstrap values at the branches are based on 1000 replicates. The sequence data used were obtained from GenBank (accession numbers are given in parentheses). Evolutionary analyses were conducted in MEGA.X. *Bifidobacterium brevis* strain was used as outgroup strain.

Acid and bile tolerance assessment

Both *Lactobacillus* isolates were grown overnight in MRS broth (Merck, Germany) under microaerophilic conditions. The culture was centrifuged at 2400 ×*g* for 3 min and bacterial cells were collected. The cells were washed with phosphate-buffered saline (PBS; pH 7.4) and re-suspended in 3 ml of PBS. One hundred microliters or PBS. An aliquot of 100 µl of the culture was inoculated into 5 ml MRS broth adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 with 1 M HCl which was then incubated at 37℃ for 6 h under microaerophilic condition. Optical density of each isolates was analysed through optical density at wavelength 600 nm (OD_{600}) reading using UV/Vis spectrophotometer (BioDrop, UK) and compared to initial OD_{600} reading (T_0) before incubation. Resultant culture was also plated on MRS agar (Merck) and incubated at 37℃, overnight under microaerophilic condition. The isolates ability to resist bile salts were analysed as well with MRS broth supplemented with bile salt (Oxoid, UK) with concentrations of 0.0%, 0.3%, 2.0%, and 10.0%.

$$\text{Coefficient of Inhibition} = ((\Delta T_t - T_0 \text{ Control}) - (\Delta T_t - T_0 \text{ Treatment}))/(\Delta T_t - T_0 \text{ Control})$$

where, $\Delta T_t - T_0$ represents the difference in absorbance at time zero (T_0) and after tested hours (T_t). Lesser Coefficient of Inhibition signifies similarity to isolates growing in MRS only.

Microencapsulation and viability assessment

L. delbrueckii 94/L4 and *L. casei* 97/L3 were encapsulated within sodium alginate-chitosan using an extrusion-ionic gelation method. Sodium alginate solution 4% was made, autoclaved, and cooled at room temperature. The sodium alginate-cell suspension solution was prepared aseptically by mixing sterile alginate solution with approximately 10^{11} CFU/ml of suspended isolate (10:1; w/v) [15]. The mixture was agitated to distribute the cells, and syringe pump was used to aliquot the mixture into 0.4 M $CaCl_2$ mixed with 0.75% (w/v) of aqueous chitosan solution. Chitosan solution was made by dissolving chitosan in 1% (v/v) glacial acetic acid. After 1 h of gelation, fresh capsules formed were collected by filtration, then rinsed with deionized water and re-filtered. The encapsulation yield of the fresh capsules was immediately enumerated. Encapsulation yield (%) = (number of cells encapsulated/number of viable cells in suspension) × 100%. Remaining microcapsules were then freeze-dried.

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Freeze-dry and viability assessment

Before freeze-drying, the fresh capsules were initially frozen in a deep-freezer (-30℃) for 24 h. Freeze-drying was performed using Martin Christ Alpha 1–2 LD Plus (John Morris, Germany) at the following conditions: 48 h; 0.63–0.47 mbar; 15–20℃. Freeze-dried capsules were then weighed, stored at -30℃, and cell survivability was enumerated after the second and fourth week of storage. Cell survival (%) = (number of encapsulated cells in freeze-dried capsules / number of cells in fresh capsules) × 100% [16].

Survival of *L. casei* 97/L3 in simulated GI tract condition

The determination of cell viability after exposure to simulated GI tract condition was prepared for simulated gastric fluid (SGF) [17] and for simulated intestinal juice (SIJ) [15]. Freeze dried capsules weighing 0.1 g was added to 9.9 ml of SGF/SIJ; and 1 ml of free cell suspension was added to 9 ml of SGF/SIJ. SGF consisted of 0.084 mol/l HCl, 2 mg/ml NaCl, 6 mg/ml pepsin, adjusted to pH 2.0 with 1 M NaOH, pre-warmed to 37℃ before use. SIJ consisted of 6.5 g/l NaCl, 0.835 g/l KCl, 0.22 g/l $CaCl_2$, and 1.386 g/l $NaHCO_3$ adjusted to pH 7.5. The mixtures were incubated with gentle agitation at 37℃. Surviving bacteria were enumerated by spread plate counts in MRS agar incubated overnight under microaerophilic condition. Viable cells were enumerated in log CFU/g at time interval of 30, 60 and 120 min.

Yogurt making and sensory attribute assessment

Both microencapsulated and suspended *L. delbrueckii* 94/L4 and *S. thermophilus* 24/S1 were used to ferment full-cream milk. Yogurt with suspended starter cultures (S1L4), yogurt with microencapsulated starter cultures (ES1L4), and commercial yogurt (Chr Hansen) were made to compare their sensory attributes. The viability (CFU/ml) of both cultures were controlled by assessing the starter cultures in liquid media through optical density (OD_{600}) and plated on MRSA to achieve 10^7–10^9 CFU/ml. Each isolate was then added into the milk after obtaining the cell pellet by centrifugation. Fermentation process began in an enclosed container by incubating mixture at 37℃ for 4 h and 42℃ for the remaining 5.5 h. Fermentation container was then taken out of the incubator and left at room temperature for at least 2 h and stored at 4℃, while maintaining the quality of the yogurt by ensuring the acidity to fall between pH 4.0–4.6 after fermentation [18]. The sensory assessment was performed by consumer acceptance test based on the appearance, texture, flavor, aroma, and overall impression of the product, using a 7-point hedonic scale (1 – extremely disliked; 7 – extremely liked) [19].

Statistics

The data were analyzed using by Minitab 15 (Minitab Inc., USA) software. All values were stated as the mean ± SD at and statistical significance of the result obtained at p value ≤ 0.05.

Results and Discussion

Phylogenetic tree

A phylogenetic construction based on the isolates 16S rRNA gene is reflected in Fig. 1. The two isolates were compared to some related strains and the tree was built using Maximum Likelihood method with bootstrap consensus tree inferred from 1000 replicates after alignment with MUSCLE. Branches with partitions reproduced lesser than 50% bootstrap replicates were collapsed. The evolutionary data was made using MEGA.X software. Phylogenetic tree revealed that the genetic of *L. casei* 97/L3 isolate is closer to other *Lactobacillus* species than that of *L. delbrueckii* 94/L4.

Acid and bile tolerance test

Unlike gastric, intestine provides a stable condition with pH ranging from 6.0 to 8.0 depending on the site of intestine allowing probiotics to perform their effects [20]. However, despite its normal physiological function, bile is highly toxic for microorganisms that have not adapted to the intestinal conditions. The bile tolerance of the isolates is indicative of their possible survival in small intestine. Bile applies deleterious effect by causing lipid destruction and eventually cell death. To date, genes and proteins involved in such mechanisms have been found in *Bifidobcateria* and *Lactobacillus* [21, 22]. It was reported that different strains of *Lactobacillus* exhibit different endurance when exposed to low pH and bile salts [11].

When exposed to bile, isolates demonstrated lower coefficient inhibition value at all concentrations tested in 6 h and increased coefficient inhibition value at 24 h. *L.*

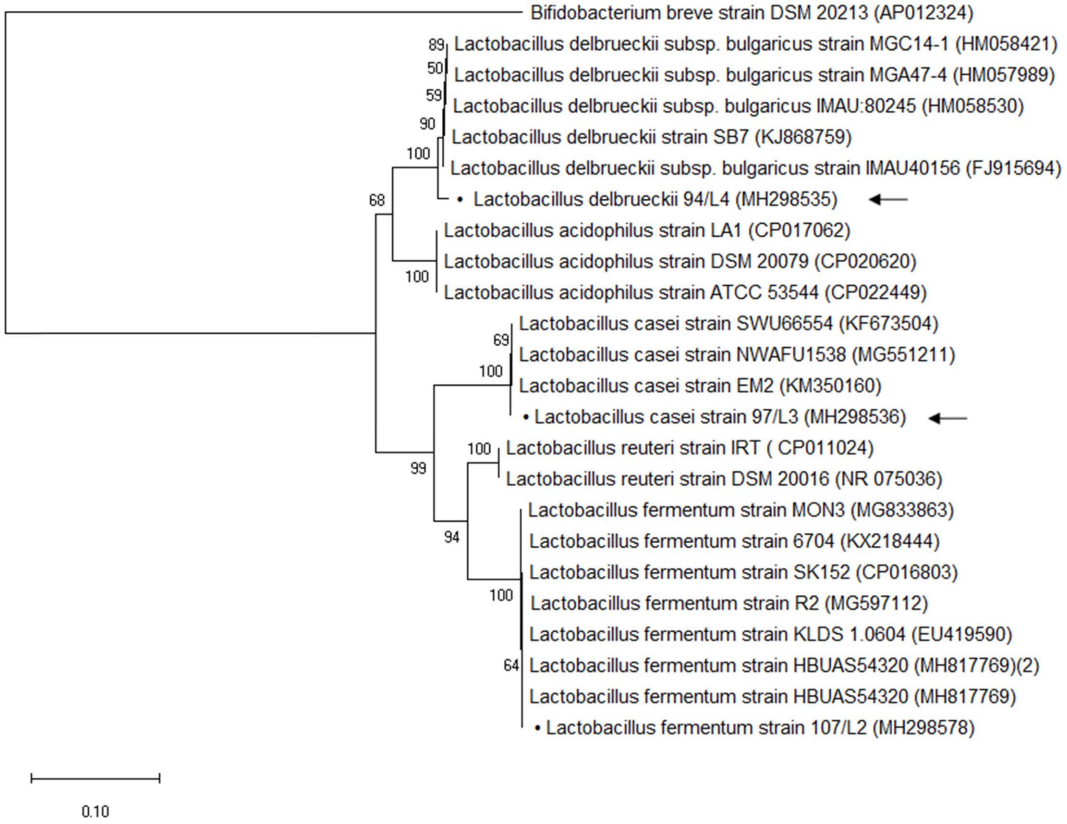

Fig. 1. The evolutionary history was inferred by using the Maximum Likelihood phylogenetic tree based on 16S rRNA gene sequences showing the position of strain *L. delbrueckii* 94/L4 and *L. casei* 97/L3 among related *Lactobacillus* species. Bootstrap values at the branches are based on 1000 replicates. The sequence data used were obtained from GenBank (accession numbers are given in parentheses). Bar, 0.10 sequence divergence. Evolutionary analyses were conducted in MEGA X.

delbrueckii 94/L4 was shown to exhibit more tolerance against bile than *L. casei* 97/L3. Unlike *L. casei* 97/L3, *L. delbrueckii* 94/L4, was shown to grow on plates after incubation under extreme bile concentration at 10% for 6 h. *L. delbrueckii* subsp. *bulgaricus* was highly resistant to bile salts and extreme acidity [23]. Despite the capacity to withstand bile, the response was denoted as part of the species stress response affecting the glycolytic pathway. Nevertheless, it was reported that cell adhesion related biomolecules were significantly affected when *L. delbrueckii* was grown in the presence of bile salts [24]. To add on, the response against bile was denoted as part of the species stress response affecting the glycolytic pathway [24].

The major challenge on the application of probiotic is the extreme pH imparted by the gastric juice. The pH of gastric varies, an empty stomach will have an average of pH lower than 4 [25–27]. Reports showed that strain isolates of *Lactobacillus* genus: *L. plantarum* (ST194BZ, ST414BZ, and ST664BZ), *L. rhamnosus* (ST461BZ, ST462BZ), and *L. paracasei* (ST242BZ, ST284BZ) isolated from fermented drink Boza from the Balkan Peninsula; *L. plantarum* 423 isolated from sorghum drink; *L. plantarum* 241 isolated from pig ileum; *L. curvatus* DF38 isolated from salami; *L. fermentum* (TCUESC01) extracted from fermenting cocoa; and *Lactococcus lactis* ssp. *lactis* HV219 isolated from human vaginal secretions showed stable growth in approximately pH 5.0 to pH 7.0 [28].

In this study, there was no significant reduction of growth ($p > 0.05$) of both isolates when exposed to pH 6.0. Only when isolates were exposed to pH 5.0, both isolates growths were reduced significantly ($p < 0.05$) and were further reduced at pH 4.0, growth. Optical density (OD_{600}) of isolates at pH 7.0 was measured as standard of growth. At pH 4, the growth of all isolates was still

Table 1. *Lactobacillus delbrueckii* 94/L4 and *Lactobacillus casei* 97/L3 acid and bile tolerance assessment.

Strain	Bile salt (%)	Time (h)	Coefficient of inhibition	Growth when plated	Working pH	Optimum pH
L. delbrueckii 94/L4	0.3	6	0.94	+	4-7	6-7
		24	0.59	NA		
	1	6	0.94	+		
		24	0.43	NA		
	2	6	0.92	+		
		24	0.34	NA		
	10	6	1.00	+		
		24	0.12	NA		
L. casei 97/L3	0.3	6	0.65	+	4-7	6-7
		24	0.34	NA		
	1	6	0.8	+		
		24	0.42	NA		
	2	6	0.86	+		
		24	0.45	NA		
	10	6	0.92	-		
		24	0.87	NA		

NA = Not Applicable
(+) = Colonies detected
(-) = no growth

apparent when plated, but no growth observed at pH ≤ 3 for all isolates. The viability of all three isolates was seen only up to pH 4 after 6 h incubation. This study verified that often isolates of interest are not readily resistant to pH ≤ 4.0. The protection of probiotics through microencapsulation has been proposed to ensure stability of the microorganism when exposed to pH as low as 2.5 [29]. Microencapsulation was intended to preserve the viability of microorganism to reach and colonize the intestine, keeping functional properties such as protecting the integrity of the intestinal lining and assisting in digestion through enzyme activities [8, 29]. Summary of bile and acid tolerance for both tested isolates were reflected in Table 1.

Microencapsulation affecting cell survivability

In this study, microcapsules were coated with sodium alginate and chitosan measuring a diameter of 1.5–3.0 mm (Fig. 2). Even though the cells were sucessfully entrapped, there was a significant difference ($p < 0.05$) in the yield after freeze-drying of *L. delbrueckii* 94/L4 and *L. casei* 97/L3, 0.71–2.70 log reduction. Attending to the issue, future incorporation of cryoprotectant such as the combination of trehalose, sodium ascorbate and skim milk can be employed to reduce the destructive impact imposed by freeze drying by up to approximately 80% [30].

Microencapsulated *L. delbrueckii* 94/L4 and *L. casei* 97/L3 on storage

Storing assessment was done on *L. delbrueckii* 94/L4 and *L. casei* 97/L3 to confer the effectiveness of the conventional encapsulation within sodium alginate-chitosan using an extrusion-ionic gelation method of protection. Cell survival upon 14 and 28 days of storage showed that there was no significant difference ($p > 0.05$) in log reduction between the number of encapsulated *L. delbrueckii* 94/L4 and *L. casei* 97/L3 when stored in 4℃ and room temperature. Meanwhile, the viability of suspended cells decreased significantly when stored in 4℃ and room temperature at day 14 and further decreased at day 28 (Fig. 3). The results confirmed that microencapsulation with sodium alginate-chitosan using an extrusion-ionic gelation method was suitable to

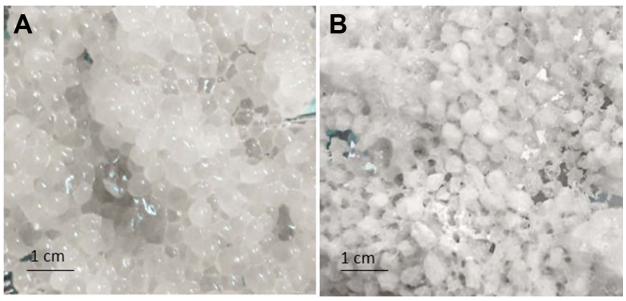

Fig. 2. Microcapsules (A) before and (B) after freeze-drying.

provide the living cells *L. delbrueckii* 94/L4 and *L. casei* 97/L3 physical barrier on storage against adverse impact brought by the low pH and temperature.

L. casei 97/L3 viability in simulated GI tract condition

L. casei was often consumed as probiotic to support the gastrointestinal health [31, 32]. Thus, in order to convey the impact, the isolate needs to maintain its viability to colonize the gut. The survivability of *L. casei* 97/L3 was evaluated in simulated GI tract condition, including simulated gastric juice (SGJ) and simulated intestine fluid (SIF). The number of free suspended and encapsulated *L. casei* 97/L3 was further decreased with longer incubation duration in low pH and bile salts (Fig. 4A & 4B). Although there was a rapid loss of cell viability after 30 min of exposure in SGJ, free suspended *L. casei* 97/L3 could maintain its viability at approximately 10^3 CFU/ml throughout the exposure up to 120 min. Meanwhile, free suspended *L. casei* 97/L3 showed no viability after 30 min of incubation in bile salts.

Overall, encapsulated cells showed improved survivability to suspended cells. Nevertheless, sodium alginate-chitosan using an extrusion-ionic gelation method might not be suitable for protection of probiotic microor-

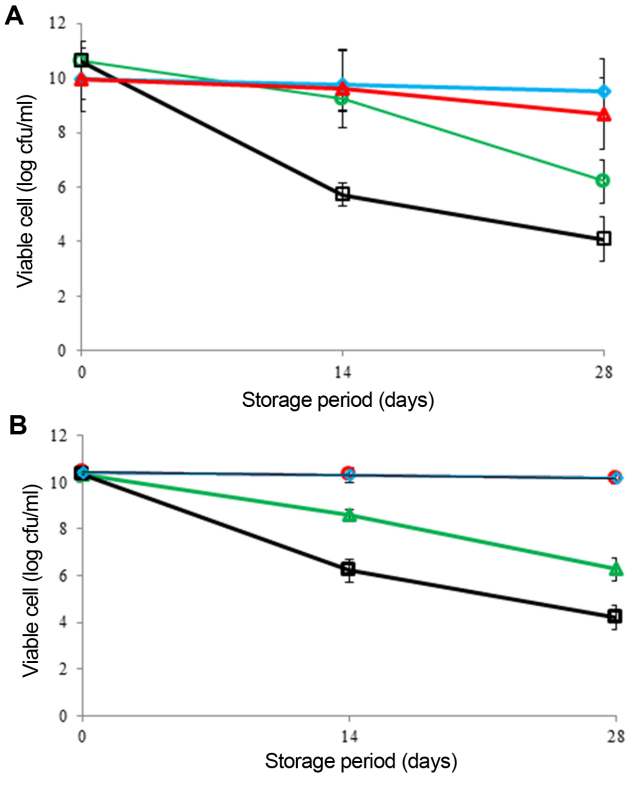

Fig. 3. Cell viability of free suspended and encapsulated (A) *L. casei* 97/L3 and (B) *L. delbrueckii* 94/L4. Incubation was observed over 28 days of storage at 4℃ and room temperature (RTP, 25 ± 2℃). Data are presented as mean ± SD (n = 4). —○—: isolate suspension stored at 4℃, —□—: isolate suspension stored at RTP, —◇—: microencapsulated isolate stored at 4℃, —△—: microencapsulated isolate stored at RTP.

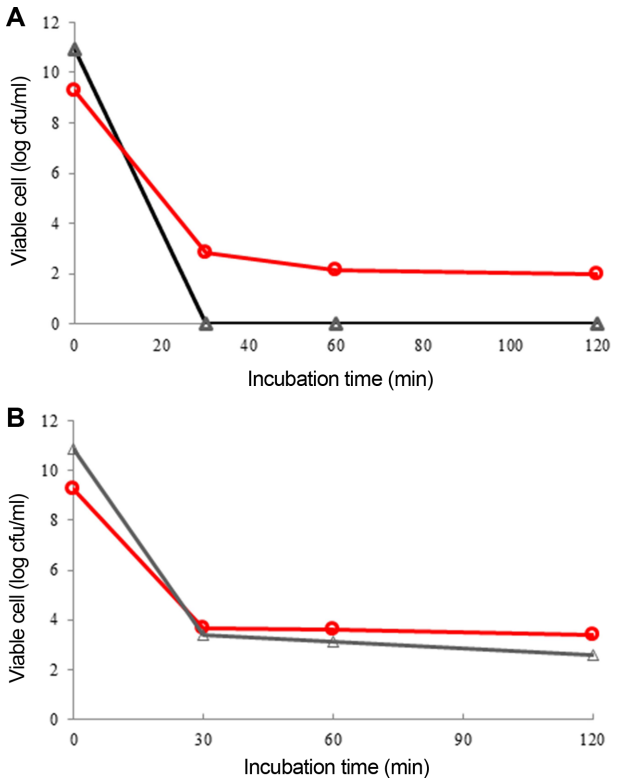

Fig. 4. Viable cells (log CFU/ml) of microencapsulated *L. casei* 97/L3 in (A) simulated gastric fluid with pH 2.0, and (B) simulated intestinal fluid with pH 6.0. Incubation was observed over 120 min at 37℃. —△—: suspended; —○—: freeze-dried microcapsules of *L. casei* 97/L3.

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ganism against the harsh condition of the GI tract. Other microencapsulation method such as plant materials-based microencapsulation using carrageenan-LB gum-coated milk and alginate-chitosan-carboxymethyl chitosan microcapsules might confer improved protection of the probiotic intended isolate [33, 34].

Organoleptic assessment

L. delbrueckii was often found as part of starter culture together with *S. thermophilus* [35]. The yogurt S1L4 was previously compared to a commercialized yogurt made of similar strains of *S. thermophilus* and *L. delbrueckii* with the addition of *L. acidophilus* and *Bifidobacterium* sp. BB-12. Organoleptic score on both commercialized yogurt and yogurt S1L4 showed to have no significant results ($p > 0.05$) [10].

Sensory profile was made through organoleptic assessment of yogurt made of the microencapsulated (ES1L4) and free suspended starter culture (S1L4). *S. thermophilus* 24/S1 was incorporated with *L. delbrueckii* 94/L4 as starter culture of the yogurt product. The viability of *S. thermophilus* 24/S1 before and after microencapsulation, along with its storage time after 28 days in 4℃ and room temperature displayed a similar result as *L. delbrueckii* 94/L4 (result not shown). Organoleptic assessment of both yogurts was done by evaluating appearance, aroma, flavor, and texture as shown in Fig. 5.

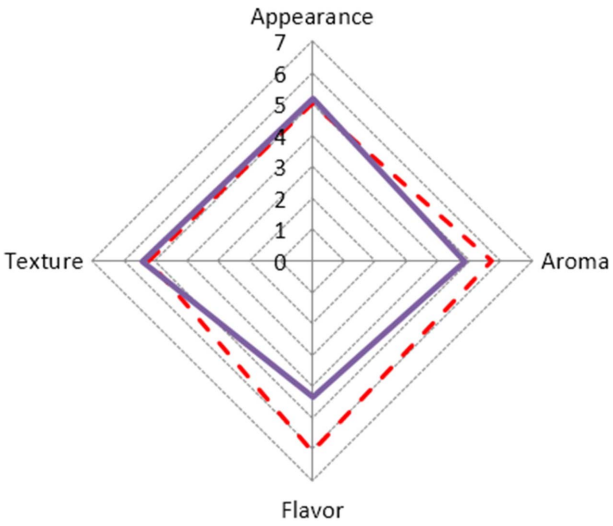

Fig. 5. The overall preferences of yogurt with ——: encapsulated starter cultures (ES1L4); and – –: non-encapsulated starter culture (S1L4) consists of appearance, aroma, flavor, texture, and overall liking.

The organoleptic score on appearance and texture of both yogurts showed to be non significantly affected ($p > 0.05$). The addition of alginate-chitosan to the yogurt product did not affect the process duration and the appearance of the yogurt made. However, the presence of alginate creates a more granular texture with gritty consistency compared to yogurt S1L4. In fact, the interaction between proteins in milk and alginate strengthened as the pH goes lower [36]. Proper agitation of the encapsulated starter cultures in milk prior to yogurt production is necessary to dissolve the alginate and produce yogurt with softer texture. Meanwhile, the aroma and flavor of both yogurts were shown to have significant difference ($p < 0.05$). The aroma and flavor of yogurt was mainly affected by the production of acetyldehyde, diacetyl, and acetic acid from starter cultures employed. The pH value of final product of yogurt S1L4 and encapsulated yogurt ES1L4 were 4.42 and 4.60, respectively. The panelist showed preference to yogurt that had milder and milky taste (ES1L4) as compared to the acidic yogurt made from non-encapsulated yogurt S1L4. Overall organoleptic preference was shown to be significantly improved ($p < 0.05$) in yogurt with microencapsulated starter cultures [37].

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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