



Microencapsulation of *Lactobacillus plantarum* 299v and its storage in kuini juice

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ABSTRACT

Aims: Probiotics are living microorganism, when administrated in sufficient quantity can exert beneficial effect to the host. This study focused on the microencapsulation by co-extrusion to increase the viability of *Lactobacillus plantarum* 299v (Lp299v) in gastrointestinal conditions, and its storage stability in kuini juice at refrigerated (4 °C) and ambient temperature (25 °C).

Methodology and results: Lp299v was encapsulated with 1.5% w/v sodium alginate and chitosan coating (0.1% w/v) and yielded a microencapsulation efficiency of 97.71%. The Lp299v microbeads produced were spherical in shape and exhibited a mean microbeads size of $618.75 \pm 25.85 \mu\text{m}$. Acid and bile tolerance of both free and encapsulated Lp299v were tested in simulated gastric juice (SGJ) for 2 h and in simulated intestinal juice (SIJ) for 4 h, respectively. The encapsulated Lp299v maintained above 10^8 CFU/mL after exposure to artificial gastrointestinal juice, whereas a significant loss of viability was observed in the free cells. The storage stability of encapsulated Lp299v in kuini juice was determined during 4 weeks of storage at 4 °C and 25 °C. Results showed that encapsulated Lp299v was capable to remain viable (10^7 CFU/mL) for at least 4 weeks in a refrigerated condition. However, free Lp299v did not survived under both refrigerated and ambient temperature as the storage period extended.

Conclusion, significance and impact of study: Lp299v entrapped in chitosan-coated alginate microbeads produced by co-extrusion method is able to enhance the viability of Lp299v above the minimum recommended level in harsh environment (gastrointestinal conditions and low pH of kuini juice).

Keywords: Lp299v, microencapsulation, simulated gastrointestinal juices, storage stability

INTRODUCTION

Recent years have witnessed a growing demand for probiotics products as the public have raised their consciousness on diet-related issue. Probiotics are reported to have extensive beneficial effects to human guts such as reducing constipation, travelers' diarrhea and irritable bowel syndrome (Kechagia *et al.*, 2013). Other health functions include inhibiting the growth of pathogenic bacteria; promoting the synthesis of vitamin B; reducing blood ammonia levels, cholesterol absorption, and tumour formation; and improving calcium absorption (Shi *et al.*, 2013). Hence, based on these advantages related to the probiotics, they have been widely incorporated into many foods and beverages in the modern market (Shah *et al.*, 2016).

In present market, *Lactobacillus* genera is commonly used for commercial probiotic foods or supplements (Homayouni *et al.*, 2008). *Lactobacillus plantarum* 299v (Lp299v) is known for its high adhesion rate to the intestinal mucosa (Cremonini *et al.*, 2002). It is able to withstand and grow in harsh environment of the

gastrointestinal tracts and is considered as a generally regarded as safe (GRAS) strain. Thus, Lp299v is an edible probiotic which can be applied for food industry (de Vries *et al.*, 2006). Nowadays, this strain has been commercially embodied in many food products such as yogurt and fruit juice (Naruszewicz *et al.*, 2002).

Probiotic products, especially probiotics dairy beverages, have been introduced into the market for many years, are getting more and more popular among lactose non-persistent consumers and vegetarian (Pereira *et al.*, 2011; Shah *et al.*, 2016). In recent years, fruit juice has been reported as an ideal medium for probiotics due to its high contents of such as antioxidant, vitamins, minerals and dietary fibers (Nagpal *et al.*, 2012; Perricone *et al.*, 2015; Ng *et al.*, 2019). In addition, it is exclusive for the growth of probiotics, which means no other starter cultures would compete for the consumption of fruit nutrients (Ding and Shah, 2008).

Mangifera odorata which is also known as kuini, is a mango fruit full of valuable compounds such as polyphenolic components, antioxidants and fibre (Ötles and Ozgoz, 2014; Ariffin *et al.*, 2015). However, factors

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such as extreme pH environment and storage temperature are the main challenges to maintain the viable cell counts of probiotics in juices (Sheehan *et al.*, 2007). In order to unleash beneficial results to the host, the total amount of live probiotics bacteria should retain at least 10^7 CFU/mL throughout the shelf life (Nualkaekul *et al.*, 2013).

Microencapsulation is an effective method to protect probiotics from adverse environments (e.g., gastrointestinal juice and storage condition), and to control the release of probiotics into the end product (Sathyabama *et al.*, 2014; Chew *et al.*, 2019). In addition, microencapsulation techniques are reported to enhance the survivability of probiotics in gastrointestinal ingestion (Brinques and Ayub, 2011; Nazzaro *et al.*, 2012). To date, the co-extrusion microencapsulation of probiotics has yet to be studied extensively.

In view of the above, the first objective of this study was to investigate the efficacy of co-extrusion microencapsulation on the viability of Lp299v after the exposure to simulated gastrointestinal environment. The second objective was to determine the suitability of kuini juice as the ideal vehicle for Lp299v by evaluating the storage stability of free and encapsulated Lp299v in the juice that stored under refrigeration and at room temperature for 4 weeks.

MATERIALS AND METHODS

Media preparation

De-Man, Rogosa and Sharpe (MRS) agar (Merck, Germany) was prepared by dissolving 68.20 g of MRS agar powder in 1 L of distilled water. The powder was dissolved well and autoclaved (HV-110, Hirayama, Japan) for 15 mins at 121 °C. After autoclaving, molten agar was cooled off in water bath set at 55 °C. After that, a layer of MRS agar was poured on the surface of Petri dishes inside a sterilized laminar flow station. The plates were then left open to avoid the formation of water vapor on the surface of lids. Once the agar became solid, all the plates were stacked up on top of the other and sealed properly with parafilm.

MRS broth (Merck, Germany) was made by dissolving 5.22 g of MRS broth powder in 100 mL of distilled water. The mixture was mixed well to ensure the dissolution of the powder, followed by autoclaving at 121 °C for 15 min. After autoclaving, MRS broth was cooled down to room temperature before the addition of probiotic *L. plantarum* 299v (Lp299v).

Phosphate buffer saline (PBS) is a balanced salt solution containing 0.20 g/L of KCl (J-Kollin, UK), 1.44 g/L of Na_2HPO_4 (Merck, Germany), 0.24 g/L of KH_2PO_4 (Merck, Germany) and 8.00 g/L of NaCl (R&M Chemicals, UK) (Zneimer *et al.*, 2017). The mixture was regulated to pH 7.4 with 1 M HCl (Merck, Germany) or 1 M NaOH (Merck, Germany) by a calibrated pH meter and autoclaved. The autoclaved PBS was used for washing cells after centrifugation and microencapsulation process.

Preparation of simulated gastrointestinal juices (SGJ) and simulated intestinal juice (SIJ)

Simulated gastric juice (SGJ) was prepared according to Chia *et al.* (2015) with slight adjustment. SGJ was prepared by mixing 9.0 g of NaCl and 3.0 g of pepsin in 1 L of distilled water. The SGJ was adjusted to pH 2.0 with HCl using a calibrated pH meter.

Simulated intestinal juice (SIJ) was prepared as described by Chia *et al.* (2015) with slight adjustment. SIJ was prepared by dissolving 3.4 g of KH_2PO_4 in 95 mL of NaOH solution and then topped up to 500 mL with distilled water. After that, 10.0 g of bile salt was added subsequently, and pH was adjusted to 7.2.

Preparation of probiotics

The preparation of Lp299v culture (BiO-LiFE, Malaysia) was adapted from Albadran *et al.* (2015) with modifications. Lp299v was cultivated in 100 mL of autoclaved MRS broth and incubated at 37 °C for 24 h. The cells were then collected by centrifugation (Allegera™ X-22R, Beckman Coulter Life Sciences, USA) for 15 min with 3200 rpm at 4 °C. After centrifugation, the supernatant was discarded, and the pellets were washed twice with PBS. The pellets were then re-suspended in 100 mL PBS. Lp299v was mixed thoroughly by vortex mixer (VTX-3000 L, LMS, Japan) for 30 s and then was used for microencapsulation.

Cell enumeration

The dissolution of encapsulated Lp299v cells was carried out according to Tootoonchi *et al.* (2015) with modification prior to the cell enumeration. The first dilution was prepared by adding 1 g of chitosan-coated alginate microbeads in 9 mL of trisodium citrate solution (Bendosen, Germany). The sample was then sent to stomacher machine (BagMixer® 400 W) for 2 min at normal speed to ensure the capsules were fully decomposed. After the decomposition of the microbeads, cells enumeration was carried out according to Shinde *et al.* (2013) with adaptations to determine the viability of Lp299v. Sequential dilution was conducted by serially diluting 1 mL of the mixture with 9 mL of peptone (Merck, Germany) water (0.1% w/v) until 10⁻⁸ fold dilution.

After the completion of serial dilution, the samples of each dilution were mixed well by using vortex mixer before adding 0.1 mL of aliquot onto the MRS agar Petri dishes. The Petri dishes were placed in incubator at 37 °C for 48 h. After 48 h of incubation, the amount of colonies on the MRS agar plates were counted. The viable cell counts of bacteria in the initial sample was calculated as formula below (Siang *et al.*, 2019).

Viable cell count (\log_{10} CFU/mL) = \log [Average number of colonies from triplicate plates/ (Dilution factor × volume plated)]

The survivability for both encapsulated and non-encapsulated Lp299v was calculated as formula below:

$$\text{Survivability (\%)} = [(\log N / \log N_0)] \times 100\%$$

where log N indicates the number of viable cell count (CFU/mL) at a specific time and log N₀ indicates the initial viable cell count (CFU/mL) at the beginning (Savedboworn *et al.*, 2017).

Microencapsulation of Lp299v by using co-extrusion technique

Microencapsulation of Lp299v was carried out using co-extrusion method. The shell solution (1.5% w/v) sodium alginate was prepared by dissolving 6.0 g of sodium alginate powder (R&M Chemicals, UK) in 394 mL ultra-pure water. The solution was then homogenized by using T25 digital Ultra-Trax homogenizer (IKA, Malaysia) at 12000 rpm for 2 min. The homogenized solution was pasteurized using double boiler technique at 85 °C for 30 min (Shinde *et al.*, 2013). The sterile sodium alginate solution was then stored in a sterilized Schott bottle at 4 °C overnight for further usage (Tootoonchi *et al.*, 2015).

The preparation of chitosan solution (0.1% w/v) was according to Ahonkhai *et al.* (2006) with minor modification that 1 g of chitosan powder (R&M Chemicals, UK) was dissolved in 900 mL ultra-pure water which had been acidified with 10 mL glacial acetic acid (Friendemann Schmidt, Australia). After that, 30 g of CaCl₂ (R&M Chemicals, UK) and 1 g of Tween 80 (R&M Chemicals, UK) were dissolved in the chitosan solution, then the pH of the solution was adjusted to 5 with NaOH (Nazzaro *et al.*, 2012). The chitosan solution was pasteurized at 72 °C for 30 sec and then cooled down into room temperature prior microencapsulation.

Encapsulation of Lp299v was conducted by using the Büchi Encapsulater B-390 equipped with inner and outer nozzle. During encapsulation, the core substance (Lp299v) and the shell material (sodium alginate solution) were pumped through the outer (300 µm) and inner (150 µm) nozzles simultaneously by the 600 mbar air pressure to form a core-shell fluid stream. The microbeads were sprayed out through the nozzle under a vibration frequency of 300 Hz, and at the same time a 1.5 kV voltage was used (Chew *et al.*, 2015).

The microbeads produced became firm after immersing in sterile chitosan solution for 30 min. The microbeads were then collected through a sieve and rinsed thoroughly with sterilized PBS. Subsequently the microcapsules were dried by tissue papers before transferring to bottle wrapped with aluminum foil and stored at 4 °C.

Encapsulation yield

The mean diameter of the microbeads was measured by using optical microscope (CX31, Olympus, Japan) and stage micrometer. The encapsulation yield was a comprehensive evaluation of entrapment efficiency and

cell survivability during the microencapsulation process (Zanjani *et al.*, 2014). Encapsulation yield was calculated as follows:

$$\text{Encapsulation yield (\%)} = [(\text{Number of viable cells released from microbeads} / \text{number of viable cell in bacterial culture})] \times 100\%$$

Acid tolerance test

The acid tolerance test was designed according to Pimentel-González *et al.* (2009) with slight adjustments. To examine the survivability of encapsulated Lp299v under simulated gastric condition, 1 g of microbeads and 1 mL of free cells were separately added into 9 mL of sterilized SGJ for 0, 1 and 2 h at 37 °C. After each interval, the microbeads and free cells were rinsed twice with PBS and centrifuged (3200 rpm for 15 min at 4 °C) to remove the excess SGJ. The supernatant was discarded, and the microbeads was suspended in 9 mL of trisodium citrate solution for dissolution. The microbeads were solubilized completely in a stomacher machine for 2 min to release the probiotic cells. The solution was then serially diluted by using peptone water (0.1% w/v). Aliquots (0.1 mL) was plated onto MRS agar plates and the viability for free cells and the microbeads were represented as colony forming unit per milliliter (CFU/mL).

Bile tolerance test

Bile tolerance test was adapted from Su *et al.* (2011) with modification. In order to evaluate the survival of free and encapsulated Lp299v in the presence of bile salts, 1 g of the microbeads or 1 mL of free cells which had been incubated in SGJ after 2 h were suspended in 9 mL of sterile SIJ. The viable cell counts were then tested at time interval of every 0, 1, 2, 3 and 4 h at 37 °C. Both microbeads and free cells were rinsed twice with PBS and centrifuged (3200 rpm for 15 min at 4 °C) to remove the excess SIJ. The microbeads was suspended in 9 mL of trisodium citrate solution for dissolution. The microbeads were dissolved completely in stomacher machine for 2 min to release the probiotic cells from the microbeads. After that, each suspension was serially diluted in peptone water (0.1% w/v). Then, 0.1 mL of samples from each time interval were inoculated on MRS agar plates and incubated at 37 °C for 48 h to determine the viability.

Cells survival during storage in kuini juice

The determination of viability for both free and encapsulated Lp299v cells during storage in kuini fruit juice was adapted from Teanpaisan *et al.* (2015) with modification. Well matured kuini fruit was washed with tap water, and then it was peeled and well blended by a juicer. The fibrous pulp of the fruit juice was filtered via a sieve. After went through pasteurization at 80 °C, it was stored at 4 °C for further usage.

To evaluate the viability of encapsulated Lp299v in kuini juice under different storage temperatures, 10 mL of

free cell or 10 g of encapsulated Lp299v were added into 100 mL of pasteurized kuini juice separately. The juice was stored for 4 weeks at 4 °C and 25 °C. The cultivation of probiotics was conducted by incubating at 37 °C for 48 h, and the enumeration of the probiotic cells was carried out on a weekly basis over a period of 4 weeks using colony counting on MRS agar.

Statistical analysis

All tests were conducted in triplicates. The results were expressed as average mean \pm standard deviation. One way analysis of variance (ANOVA) and paired T-test were analyzed by using IBM SPSS Statistics 22. The average values were compared with Tukey's post hoc test for one way ANOVA. The differences were considered significantly at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Encapsulation yield

In this study, *L. plantarum* 299v (Lp299v) was entrapped with sodium alginate through co-extrusion method and coated with chitosan. The mean microbeads size, encapsulation efficiency and the physical properties of the microbeads were evaluated after the production of microbeads. Figure 1 shows that the morphology of Lp299v microbead were spherical in shape with smooth round surface. Table 1 shows that the mean Lp299v microbeads size was $618.75 \pm 25.85 \mu\text{m}$. Similar microbeads size range (530-624 μm) was also reported by Yee *et al.* (2019) in the locust bean gum coated-alginate microbeads entrapping *Lactobacillus acidophilus* NCFM which produced by co-extrusion.



Figure 1: The morphology of Lp299v microbead under microscope.

Encapsulation yield refers to the efficiency of the probiotic entrapment and survival rates of viable cells after microencapsulation process. It is crucial to ensure co-extrusion is applicable and efficient to protect Lp299v from stress condition. The encapsulation yield of Lp299v was $97.71 \pm 0.18\%$. The viable cell counts of Lp299v in

the bacterial culture (before encapsulation) was $11.15\text{-}11.31 \log_{10}$ CFU/mL while, a high entrapment efficiency was obtained after encapsulation, with a viability of $10.93\text{-}11.01 \log_{10}$ CFU/mL. The results indicated that no considerable viability loss of Lp299v was observed after the co-extrusion encapsulation process.

Table 1: Mean microbeads size and encapsulation yield (%) of Lp299v.

Mean of microbeads size (μm)	Viable cell count in the bacteria culture (log CFU/mL)	Viable cell count released from the microbeads (log CFU/mL)	Encapsulation yield (%)
618.75 ± 25.85	11.23 ± 0.08	10.97 ± 0.04	97.71 ± 0.18

*Results are expressed as mean \pm standard deviation (SD).

In this study, an encapsulation yields above 97% was obtained via microencapsulation method. The results were in agreement with de Prisco *et al.* (2015) who have reported that *Lactobacillus reuteri* DSM 17938 entrapped in chitosan-alginate matrix was able to achieve high encapsulation yield (97%). In addition, Haghshenas *et al.* (2015) also reported that a high encapsulation yield (> 98%) was obtained though microencapsulation of probiotic bacteria *L. plantarum* 15HN by using alginate as wall material.

The entire co-extrusion process did not include any harmful factors such as high heat treatment or high shear stress, therefore resulted in a high encapsulation efficiency (Nag, 2011; Siang *et al.*, 2019). Besides that, the careful selection of wall materials and the prudent operations throughout the whole microencapsulation process also led to a high entrapment efficiency (Mortazavian *et al.*, 2007). The formation of polyelectrolyte complex between the amino groups (chitosan) and the carboxyl groups (alginate) which stabilizes the entrapped active ingredient and enhanced the encapsulation efficiency (Chew *et al.*, 2015).

Survival of free and encapsulated Lp299v in simulated gastric juice (SGJ)

Stomach acid creates an acidic environment in digestive tract which inhibit the proliferation of most of the microbes, hence, acid tolerance is a critical prerequisite for the selection of probiotic strains (Song *et al.*, 2015). The acid tolerant ability of free and encapsulated Lp299v were evaluated by determining their viability after 2 h incubations in SGJ which was presented in Figure 2. The initial viable cell counts of free and encapsulated Lp299v was 9.39 and $9.28 \log_{10}$ CFU/mL, respectively. The viability of free and encapsulated Lp299v decreased throughout the incubation in SGJ. Free Lp299v exhibited higher cell reduction (0.65 log reduction) as compared to the encapsulated Lp299v (0.33 log reduction) (Figure 2).

At the end of SGJ incubation, encapsulated Lp299v was 1.02-fold higher than the free Lp299v. This is due to the protection from the chitosan coated-alginate microbeads against low pH of gastric acids (Mustafa *et al.*, 2016).

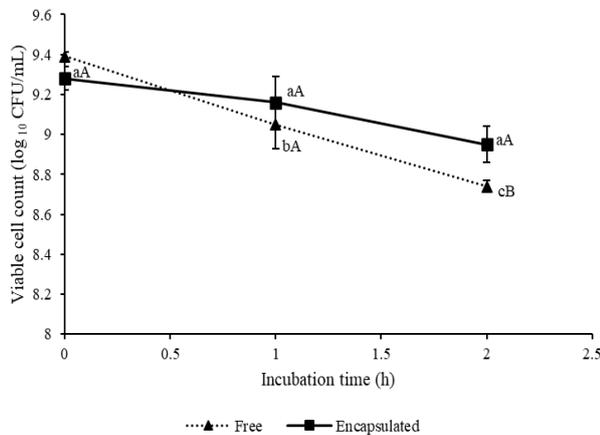


Figure 2: Total viable counts of free and encapsulated Lp299v in SGJ at pH 2.0 for 2 h. Error bars are represented as \pm standard deviation of three replicates ($n=3$). The letters a-c indicate the significant difference ($p < 0.05$) of Lp299v at different incubation time. The letters A-B indicate the significant difference ($p < 0.05$) of different forms of Lp299v on the same incubation time.

Although both free and encapsulated Lp299v showed a decrease in viability after 2 h of SGJ incubation, the number of viable Lp299v were above the minimum recommendation of 10^7 CFU/mL (Sabnis and Malavkar, 2016). The results also indicated that encapsulated Lp299v has a stronger ability to tolerate towards acidic environment as compared to free cells. The result was in agreement with Shi *et al.* (2013) such that the viable cell counts of encapsulated *Lactobacillus bulgaricus* is able to maintain at least $8.00 \log_{10}$ CFU/mL after 2 h incubation in SGJ at pH 2. Nualkaekul *et al.* (2012) reported that chitosan coating can improve the survivability of *L. plantarum* up to an amount of 0.50–1.00 \log_{10} CFU/mL at the end of gastric digestion.

Encapsulated Lp299v showed a high survivability (approximately 96%) after 2 h incubation in SGJ (Figure 2). The results demonstrated that co-extrusion microencapsulation method had significantly improved probiotics viability in acidic gastric condition at pH 2. Similar trend of results was observed by Sathyabama *et al.* (2014) that the encapsulated probiotics showed high survivability (88.75-98.75%) after simulated gastric digestion at pH 2 to 3 for 24 h.

The higher survival rate of encapsulated Lp299v can be attributed to the effective protection provided by the wall material (Mustafa *et al.*, 2016). The buffering nature of polysaccharides enhanced the protection against gastric medium, therefore avoided Lp299v from directly contacting with gastric acid. The electrostatic interaction had generated a strong binding between chitosan and

alginate, therefore improved the probiotics survivability remarkably (Etchepare *et al.*, 2015).

Survival of free and encapsulated Lp99v in simulated intestinal juice (SIJ)

After passing the stomach and right before reaching the colon, probiotics need to pass through an organ called small intestine. The small intestine contains high concentration of bile salts which are not bacteria-friendly and can inhibit the growth of bacteria (Cook *et al.*, 2012). To be considered as a good probiotics strain, the bacteria needs to not only withstand extreme environment such as low pH and high bile salts concentration, but also confer health effects to the host. Hence, bile tolerance is a vital criterion in selecting probiotics strain (Hamon *et al.*, 2011). In this study, the bile salt tolerance capacity of free and encapsulated Lp99v was evaluated to determine their viability during SIJ digestion. Free and encapsulated Lp99v were subjected to SGJ digestion for 2 h, followed by a further incubation in SIJ with 2% w/v bile salt for 0, 1, 2, 3, and 4 h. The results are depicted in Figure 3.

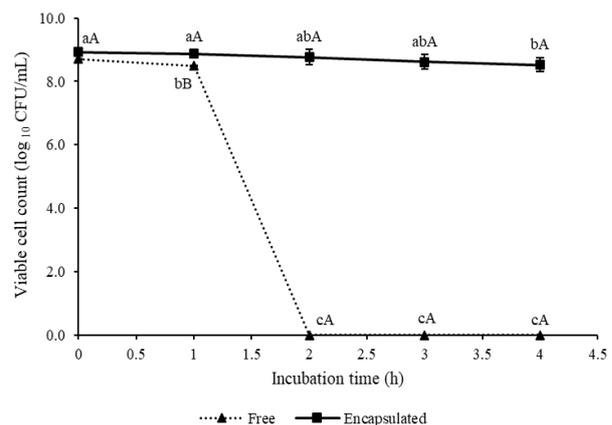


Figure 3: Total viable counts of free and encapsulated Lp299v in SIJ incubated at pH 7.2 for 4 h. Error bars are represented as \pm standard deviation of three replicates ($n=3$). The letters a-c indicate the significant difference ($p < 0.05$) of Lp299v at different incubation time. The letters A-B indicate the significant difference ($p < 0.05$) of different forms of Lp299v on the same incubation time.

Probiotic bacteria must able to survive in intestinal juice with relatively high concentration of bile salts (Sahadeva *et al.*, 2011). Figure 3 shows that the viability of free and encapsulated Lp299v decreases throughout the SIJ digestion with least pronounced in encapsulated Lp299v. Free Lp299v did not survived after 2 h of incubation in SIJ while encapsulated Lp299v experienced a 0.41 log reduction at the end of SIJ digestion (Figure 3). This indicated that free Lp299v is vulnerable to bile salts as the toxic effect of bile salts can destroy the microbial membrane (Solheim *et al.*, 2007). The results were in agreement with Chávarri *et al.* (2010) who reported that

the number of *Lactobacillus gasseri* survived after 60 min of incubation (SIJ with 3% w/v bile salt) was 4.48 log₁₀ CFU/mL and the viability was dropped to less than 1 log₁₀ CFU/mL after 120 min.

Controversially, the viability of encapsulated Lp299v (8.52 log₁₀ CFU/mL) was greater than the minimum recommended dosage of 10⁷ CFU/mL during prolonged digestion in SIJ. Annan *et al.* (2008) reported that the viability of encapsulated *Bifidobacterium adolescentis* was more than 7 log₁₀ CFU/g after 3 h incubation in SIJ at pH 7.4.

Encapsulated Lp299v showed a better survival (0.16 log reduction in cell viability, Figure 3) as compared to *L. bulgaricus* (2.10 log reduction in cell viability) after 2 h exposure in 2% w/v bile salt solution (Shi *et al.*, 2013). In addition, Hansen *et al.* (2002) also found that viability of encapsulated *B. adolescentis* dropped by about 2 log₁₀ CFU/mL after 2 h of incubation in 0.5% w/v bile at 37 °C. This is due to the coating of chitosan provided probiotics a better protection in SIJ. However, Siang *et al.* (2019) reported that *Lactobacillus rhamnosus* GG entrapped in poly-L-lysine coated-alginate microbeads was unable to preserve the probiotic viability throughout the SIJ incubation.

In the simulated sequential digestive process of SGJ (2 h) and SIJ (4 h), higher numbers of Lp299v survived in the chitosan-alginate microbeads (10⁸ CFU/mL) than the free Lp299v. Moreover, a strong bile tolerance was observed in encapsulated Lp299v with a high survivability (95.41%) after 4 h of incubation in SIJ (Figure 3). de Prisco *et al.* (2015) also declared that microencapsulation by chitosan-alginate matrix could improve tolerance of *L. reuteri* towards SIJ due to the formation of insoluble complex between chitosan and the bile salt. Therefore, the penetration of bile salt into the microcapsule can be mitigated, and the shell material can protect probiotics cells from exposing to toxic environment (Sabnis and Malavkar, 2016).

Survival of free and encapsulated Lp299v in kuini juice during 4 weeks of storage

Maintaining the viability and the activity of probiotics in fruit juice to the end of product shelf life are the two important criterions need to be fulfilled for probiotics fruit juices (Perricone *et al.*, 2015). However, it is challenging in food product because of the intrinsic factors of food matrices, processing methods, and different storage conditions (low pH environment and storage temperature) (Ozcan *et al.*, 2015). It is crucial to test the effectiveness of encapsulated Lp299v produced by co-extrusion in protecting the probiotics from the adverse environment including acidic environment in kuini juice and storage temperature.

In this study, both forms of Lp299v (free and encapsulated) were added into kuini juice (pH 3.5–3.6) and their storage stability were evaluated during 4 weeks of storage at 25 °C and 4 °C. The viabilities of free and encapsulated Lp299v that stored at 25 °C and 4 °C are presented in Figures 4 and 5, respectively.

Figure 4 shows that the viability of free and encapsulated Lp299v decreases throughout the storage after incorporation into kuini juice that stored at 25 °C. Free Lp299v experienced 1.18-2.11 log reduction in cell viability during the first 3 weeks of storage at room temperature. On the other hand, the loss of viability was less pronounced in encapsulated Lp299v. The encapsulated Lp299v shows higher in viability (by 25.54%) as compared to free Lp299v during 3 weeks of storage in kuini juice at 25 °C (Figure 4). This suggested that the Lp299v entrapped in chitosan-alginate matrix produced by co-extrusion was able to preserve the cell viability in adverse conditions (low pH and high storage temperature) (de Prisco *et al.*, 2015). This was due to the structure of microbeads that acts as a physical barrier separating probiotics from the acidic environment of fruit juices (Nualkaekul *et al.*, 2012; Perricone *et al.*, 2015).

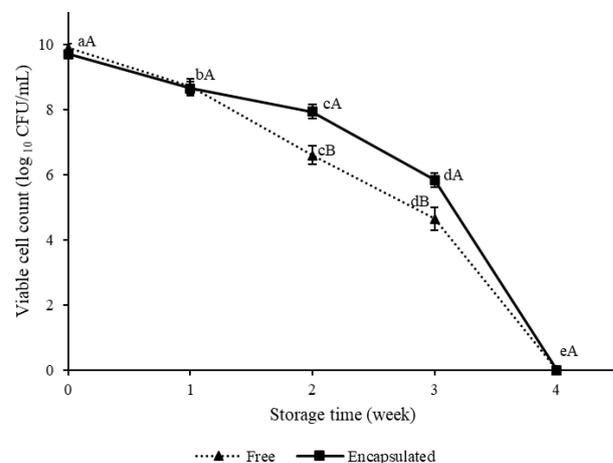


Figure 4: Total viable counts of free and encapsulated Lp299v in kuini juice during 4 weeks of storage at 25 °C. Error bars are represented as \pm standard deviation of three replicates (n=3). The letters a-e indicate the significant difference ($p < 0.05$) of Lp299v at different incubation time. The letters A-B indicate the significant difference ($p < 0.05$) of different forms of Lp299v on the same incubation time.

However, at 4th week of storage, the viable cell counts of free and encapsulated Lp299v declined to 0 log₁₀ CFU/mL. This was probably due to low pH and the polyphenolic content of kuini juice that has antimicrobial effect (Adnan *et al.*, 2018; Nualkaekul *et al.*, 2013; Perricone *et al.*, 2015). The low pH of the kuini juice is due to the presence of organic acids such as malic, tartaric and citric acids (Imran *et al.*, 2017). Since free Lp299v were expose directly to the organic acids and polyphenolic compounds, therefore, viability is lower as compared to encapsulated Lp299v. Phoem *et al.* (2015) also reported that the viability of free *Bifidobacterium longum* was significantly lower than the co-encapsulated form (alginate-*Eleutherine americana* extract) after 15 days of storage in pineapple juice.

The encapsulated Lp299v was only able to preserve the cell viability (above 10^7 CFU/mL) within 2 weeks of room temperature storage in kuini juice. This is possibly due to the diffusion of acids or polyphenolic compounds into the microbeads that are detrimental to the entrapped Lp299v (Moghtader *et al.*, 2017). In addition, the diffusion rate was higher at room temperature (Hess *et al.*, 2014). Therefore, drastic reduction in viability of encapsulated Lp299v was observed after 4 weeks of storage in kuini juice. Similar trend was observed when the free *L. acidophilus* NCFM was did not survived after two weeks of storage in mulberry leaf tea at 25 °C while the encapsulated form can survive after 4 weeks of storage (Yee *et al.*, 2019).

The results of this study were in agreement with Ng *et al.* (2019) who reported that the maintenance of the viability of probiotics above the minimum recommended concentration could be challenging especially when it is under room temperature. This is because the metabolic activity was higher at 25 °C as compared refrigeration temperature (Matejčková *et al.*, 2019). The viability of free Lp299v decline possibly due to the accumulation of toxic substances as a results of cell division during extended storage in fruit juice (Chaudhary, 2019). However, co-extrusion encapsulation of the Lp299v by using alginate-chitosan matrix was able to improve its viability to a large extent when the encapsulated Lp299v was incorporated in kuini juice that stored at refrigerated temperature (Figure 5).

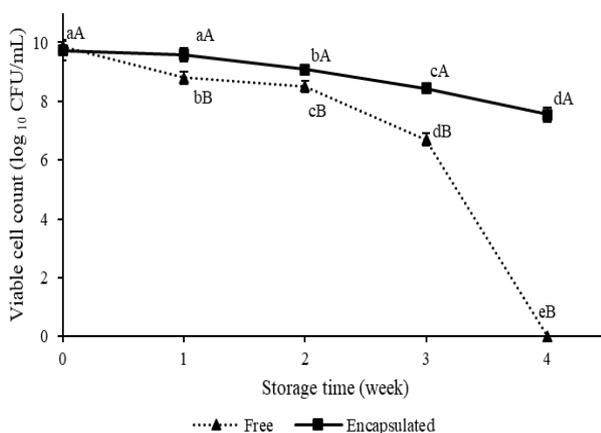


Figure 5: Total viable counts of free and encapsulated Lp299v in kuini juice during 4 weeks of storage at 4 °C. Error bars are represented as \pm standard deviation of three replicates ($n=3$). The letters a-e indicate the significant difference ($p<0.05$) of Lp299v at different incubation time. The letters A-B indicate the significant difference ($p<0.05$) of different forms of Lp299v on the same incubation time.

Figure 5 shows the viable cell counts of both free and encapsulated Lp299v at storage condition of 4 °C in kuini juice. A declining trend was observed in terms of the viability of Lp299v over the storage time with more

pronounced in the free Lp299v. The viability of counts of free Lp299v showed a gradual declination from 9.86 log₁₀ CFU/mL at week 0 to 8.51 log₁₀ CFU/mL at week 2. The viability of Lp299v experienced 1.82 log reduction at week 3 of refrigeration and did not survived at the end of storage in kuini juice (Figure 5).

This was in agreement with Nualkaekul *et al.* (2012) who studied the storage stability of *L. plantarum* NCIMB 8826 and *B. longum* NCIMB 8809 in pomegranate juice and both probiotic strains died after 4 weeks of storage at 4 °C. The drastic fall in the viability of free Lp299v in kuini juice was most likely associated with the low pH and the polyphenolic contents of the kuini juice (Nualkaekul *et al.*, 2013; Perricone *et al.*, 2015). On the other hand, a steady decreasing trend with a total of 2.18 log reduction in the viabilities was observed after 4 weeks of storage in kuini juice at 4 °C. The viable count of encapsulated Lp299v decreased slowly from 9.72 log₁₀ CFU/mL at week 0 to 7.54 log₁₀ CFU/mL at week 4 (Figure 5), while the viability dropped to 0 log₁₀ CFU/mL at room temperature (Figure 4). This suggests that the viability of Lp299v in fruit juice is affected by the storage temperature, and a lower storage temperature has a higher stability on the entrapped probiotics (Perricone *et al.*, 2015).

Ding and Shah (2008) reported that the encapsulated probiotics in orange juice and apple juice had a higher survivability than their respective free cells after 6 weeks of storage at 4 °C. Moreover, Teanpaisan *et al.* (2015) also reported that the viability of microencapsulated *Lactobacillus paracasei* SD1 was significantly higher than that of free cells in orange and aloe vera juices during 8 weeks of storage under refrigeration.

After 4 weeks of storage in kuini juice, only the encapsulated Lp299v stored at 4 °C exhibited viability higher than the minimum level (7 log₁₀ CFU/mL) to confer the health benefits to the host (Mansouripour *et al.*, 2013). Bruno and Shah (2003) revealed that the best method to protect the viability of probiotics in capsulated supplements is to store them in a cool temperature instead of room temperature. It is in accordance to our findings, in which the greater loss of free and encapsulated Lp299v was observed in room temperature instead of at 4 °C. The obtained results show that co-extrusion encapsulation as an emerging technology is able to improve viability of Lp299v (entrapped in chitosan coated-alginate microbeads) during gastrointestinal conditions and its storage stability in kuini juice under refrigeration.

CONCLUSION

The Lp299v entrapped in chitosan coated-alginate microbeads produced by co-extrusion were spherical in shape with a mean size of $618.75 \pm 25.85 \mu\text{m}$ and high encapsulation yield (97.71%). The encapsulated Lp299v was able to preserve the its viability in the adverse environment such as during sequential simulated gastrointestinal digestion and refrigerated storage in kuini juice for 4 weeks with viability above the minimum recommended level (10^7 CFU/mL). However, the free

Lp299v did not survive after 2 h of incubation in SIJ and at the end of storage in kuini juice at refrigerated and room temperature. This is due to the protective effect of the chitosan coating on the alginate microbeads that protect the entrapped Lp299v against low pH environment (during gastric digestion and fruit juice) and the presence of bile. This study shows that kuini juice is an ideal medium for the delivery of encapsulated Lp299v. The kuini juice containing encapsulated Lp299v is suitable for vegans and consumers with lactose intolerance.

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