Microencapsulation of *Lactobacillus plantarum* 299v incorporated with oligofructose in chitosan coated-alginate beads and its storage stability in ambarella juice

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ABSTRACT

Aims: Microencapsulation has been used to protect the viability of probiotics in harsh environments such as gastrointestinal conditions and food composition. The present study aimed to optimize the microencapsulation of *Lactobacillus plantarum* 299v (Lp299v) using co-extrusion by varying two parameters (calcium chloride ($CaCl_2$) and oligofructose (FOS) concentrations) and storage stability of the beads produced in ambarella juice at refrigerated and room temperature.

Methodology and results: Chitosan coated-alginate microcapsule prepared with 4.0% (w/v) FOS and 2.5% (w/v) $CaCl_2$ showed highest microencapsulation efficiency (93%). The microcapsules were subjected to gastrointestinal treatment and storage test in ambarella juice. Both encapsulated Lp299v with and without FOS showed higher viabilities compared with free cells after incubated in simulated gastric juice (SGJ) and simulated intestinal juice (SIJ). After 5 h of incubation in SIJ, the viabilities of both encapsulated probiotic with and without FOS were more than 10^7 CFU/mL. The Lp299v were stored in ambarella juice under refrigerated (4 °C) and room temperature (25 °C) for 4 weeks. At 25 °C, all forms of Lp299v lost their viabilities after one week. On the other hand, at 4 °C, viable cells count of both encapsulated Lp299v with and without FOS were reported to be more than 10^7 CFU/mL after 4 weeks of storage.

Conclusion, significance and impact of study: Microencapsulation with FOS was able to improve Lp299v's viability during storage in low pH fruit juices compared to those without FOS. The microencapsulated probiotics could be applied in ambarella juice for the development of functional food.

Keywords: Ambarella, encapsulation, gastrointestinal condition, *Lactobacillus plantarum*, oligofructose

INTRODUCTION

Sufficient amount of probiotics in the food products must remain viable at the end of shelf life in order to confer its health benefits to the host (Ying *et al.,* 2016; Damodharan *et al.*, 2017). The viability of probiotics in free cell form would be highly compromised when they are subjected to harsh environment such as low pH of gastric juice and food compositions (Phoem, 2015; Calabuig-Jiménez *et al.*, 2019). Therefore, microencapsulation of probiotic has been introduced to overcome this problem by preserving their viabilities and at the same time to facilitate controlled release of the cells across the intestinal tract (Kailasapathy, 2014). Conventional microencapsulation techniques include emulsion, extrusion, and spray-drying. Co-extrusion is a promising technology in microencapsulation that consists of a concentric nozzle equipped with a vibrating device. This process allows the production of uniformly sized microcapsules at high production rate and low size dispersion (Homar *et al.*, 2007; Chew and Nyam, 2016).

The commonly used wall materials for microencapsulation are chitosan, alginate, whey proteins, pectin, gelatin and starch (Loh and Ting, 2015). These polymers are obtained from natural sources, inexpensive, biocompatible and non-toxic (Nazzaro *et al.*, 2012; Dragostin *et al.*, 2017). Alginate is an anionic linear polysaccharide that forms a three-dimensional structure that entrapped the active ingredients with the presence of divalent ions (Martin *et al.*, 2015). Chitosan is a cationic polysaccharide which is obtained through chitin's deacetylation (Ruiz and Corrales, 2017). Chitosan is a preferred coating material for alginate beads as it can improve both physical and chemical stability by forming a polyelectrolyte complex (Chew *et al.*, 2015). This can help

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to overcome the porous structure of alginate beads that leads to degradation of core materials.

Probiotics are widely incorporated into dairy products such as yogurt, fermented milks, ice cream, and cheese. (Kumar *et al.*, 2015; Shori, 2016). However, there is an increasing demand for dairy-free probiotic functional foods due to the increase in popularity of vegetarian diet or vegetarianism recently (Ranadheera *et al.*, 2010). In addition, lactose intolerance and the cholesterol content are two major concerns associated to the consumption of dairy products (Yoon *et al.*, 2006; Kumar *et al.*, 2015). Fruit juices contain many nutrients (i.e. minerals, vitamins, antioxidants, and fibre) and they do not contain dairy allergens such as lactose and casein. Hence, they have been suggested as an ideal medium for probiotic bacteria (Luckow and Delahunty, 2004; Phoem *et al.*, 2015). Fruit juices such as apple, mandarin, pomegranate, cranberry and pineapple juices had been studied previously by different authors (Nualkaekul *et al.*, 2013; Phoem *et al.*, 2015; Gandomi *et al.*, 2016; Calabuig-Jiménez *et al.*, 2019). These studies had revealed that fruit juices incorporated with encapsulated probiotic could serve as the ideal medium for delivery of probiotics as their viabilities were retained at high levels the end of storage.

Prebiotics are non-digestible dietary components that beneficially affects the host by selectively stimulating the growth and/or activity of certain bacterial species in the host's colon (Pandey *et al.*, 2015). Recently, prebiotics have been suggested as part of the coating materials in the microencapsulation of probiotics as they are able to improve the probiotics' survivability in the upper GI tract. This allows the probiotic cells to adhere and colonise the intestine, and exert health benefits (Etchepare *et al.*, 2016; Silva and Sato, 2017). Fructooligosaccharide (FOS) is a type of prebiotic that consists of chains of fructose monomer with a terminal glucose that can be found naturally in chicory, Jerusalem artichoke and onion. The health benefits of FOS include lowering blood pressure, reducing cholesterol levels and better absorption of calcium (Sridevi *et al.*, 2014).

Ambarella (*Spondias cytherea* Sonnerat or *Spondias dulcis*) fruit is also known as golden apple or Otaheite apple that belongs to the Anacardiaceae family (Franquin *et al.*, 2005). The fruits are typically oval or pear shape (Ishak *et al.*, 2005). The soft fibrous flesh of the matured-green ambarella is crisp and juicy with sweet and sour flavour (Mohammed *et al.*, 2011). It is traditionally use for treating itchiness, sore throat and internal ulceration. It is rich in phenolic contents that exhibits antioxidant activity, cytotoxic, and thrombolytic activity (Islam *et al.*, 2013).

However, the incorporation of probiotic in fruit juice is challenging due to the acidic pH and the presence of phenolic compounds that may reduce the viability of the probiotic (Ding and Shah, 2008; Perricone *et al.*, 2015). Hence, the aim of this study is to optimise the microencapsulation parameters (calcium chloride and prebiotic concentration) on the probiotic *Lactobacillus plantarum* 299v (Lp299v). The viability of Lp299v beads after exposure to simulated gastrointestinal juices as well

as storage in ambarella juice for 4 weeks at 4 and 25 °C were also evaluated.

MATERIALS AND METHODS

Preparation of probiotics

Lactobacillus plantarum 299v (Lp299v, BiO-LiFE) cells were cultivated in 100 mL MRS (de Man, Rogosa, Sharpe) broth (Merck, Germany) and incubated at 37 °C for 16 h. The cells were harvested by centrifugation (MIKRO 220R, Hettich Zentrifugen, Germany) at 1088 × g for 15 min at 4 °C. The pellets were washed with phosphate buffer saline (PBS) and centrifuged at 1088 × g for 15 min at 4 °C. The pellets were then suspended in PBS to obtain a final cell count approximately 10^{10} CFU/mL.

Microencapsulation of Lp299v by co-extrusion and optimization of parameters

Microencapsulation of Lp299v was carried out using co-extrusion (Büchi Encapsulator B-390) technique described by Chew *et al.* (2015) with some modifications. During the microencapsulation process, the core fluid (oligofructose (Sensus, Nehterlands) and Lp299v mixture) and the shell fluid (1.5% (w/w) sodium alginate (R&M Chemicals, UK)) were simultaneously pumped into the inner (150 µm) and outer nozzles (300 µm). The air pressure (600 mbar), vibration frequency (300 Hz) and voltage (1.5 kV) were fixed for each encapsulation. The beads formed were hardened in sterile 0.1% (w/v) chitosan (R&M Chemicals, UK) solution for 20 min. The chitosan solution was prepared by dissolving 1 g of chitosan in 900 mL ultra-pure water acidified with 10 mL glacial acetic acid (Friendemann Schmidt, Australia). Different $CaCl_2$ (R&M Chemicals) concentration and 0.1% (w/v) Tween 80 (R&M Chemicals) were added to the mixture and the pH was adjusted to 5.0 using 0.1M NaOH (Merck KgaA, Germany). The final volume of $CaCl_2$-chitosan solution was adjusted to 1000 mL and pasteurized at 72 °C for 30 sec prior to microencapsulation. The alginate-chitosan beads were then collected using a sieve and washed thoroughly with sterile distilled water. The beads were then dried using filter papers. Different concentrations of $CaCl_2$ (1.0, 1.5, 2.0, 2.5 and 3.0% w/v) and FOS (1, 2, 3, 4 and 5% w/v) were studied. The concentration of $CaCl_2$ was determined first before the determining the FOS concentrations. The optimal process parameters were determined by microencapsulation efficiency (MEE) and mean diameter size. The mean diameter of the beads (20 beads, randomly selected) were measured using an optical microscope (Model: CX23, Olympus, Japan) and stage micrometer at a magnification of 100× while MEE was calculated using the following equation:

Microencapsulation efficiency (MEE) = (Log_{10} N/Log_{10} N_0) × 100%

whereby N is the number of entrapped bacterial cells loaded inside the bead and N_0 is the amount of free bacterial cells in the culture.

Enumeration of free and encapsulated Lp299v

The microcapsule containing probiotic bacteria were released prior to enumeration. One gram of beads was added to 9 mL sodium citrate solution and homogenised using a stomacher (Interscience, France) for 5 min. The free cell suspension was first harvested by centrifugation at 1088 × g for 15 min at 4 °C. PBS was then added into the pellets to a total volume of 10 mL. Aliquots (1 mL) were serially diluted to achieve countable cells numbers for both free and encapsulated probiotic. Lp299v were then enumerated by spread plating method in MRS agar (Merck, Germany) at 37 °C under aerobic conditions for 48 h of incubation (Valero-Cases and Frutos, 2015). The viable cell counts were calculated using the following formula and expressed in colony forming unit per millimetre (CFU/mL) and converted into log CFU/mL.

Viable cell count =

$$\frac{\text{Average of colonies formed from triplicate plates}}{\text{Dilution factor} \times \text{volume plated (0.1mL)}}$$

Viability of free, Lp299v beads with and without FOS under simulated gastrointestinal conditions

The free and encapsulated Lp299v (with and without prebiotics) may influenced the viability in gastrointestinal environment. Thus, the survival of all three forms of Lp299v were evaluated under *in vitro* simulated gastrointestinal conditions (Chia *et al.*, 2015). One millilitre or 1 g of beads from each formulation (with and without FOS) were added to 9 mL of sterile simulated gastric juice (SGJ: HCl (Merck KgaA, Germany) 7 mL/L, NaCl 2.0 g/L, pepsin (HmbG Chemicals, Germany) 3.2 g/L, pH 2.0) for 2 h incubation at 37 °C under constant agitation (150 rpm). After 2 h of incubation, they were then transferred into 9 mL sterile simulated intestinal juice (SIJ: NaOH solution 190 mL/L, KH$_2$PO$_4$ (Bendosen, Germany) 6.8 g/L, bile salt (R&M Chemicals, UK) 6.0g/L) for further incubation for 5 hours at 37 °C under constant agitation (150 rpm). After each interval of incubation (SGJ: 0, 1 and 2 h; SIJ: 0, 1, 2, 3, 4 and 5 h), the free and encapsulated cell (with and without FOS) were enumerated by spread plate method in MRS agar incubated at 37 °C for 48 h.

Survival of free and Lp299v beads with and without FOS during storage in ambarella juice

The ambarella fruits were washed, peeled and cut into small pieces. The fruit (150 g) was then blended with 100 mL of pre-boiled water and 20 g of sugar. The brix value of the juice was kept at 11 Brix. After that, the juice was sieved to remove the fibrous pulp before storage test.

Table 1: Microencapsulation efficiency and average diameter of chitosan coated-alginate Lp299v beads hardened with different CaCl$_2$ concentrations.

CaCl$_2$ concentration (% w/v)	Microencapsulation efficiency (%)	Diameter of beads (μm)
1.0	85.98 ± 0.98[ab]	648.34 ± 139.07[a]
1.5	82.37 ± 1.05[a]	701.67 ± 63.64[a]
2.0	87.41 ± 0.40[ab]	663.44 ± 33.14[a]
2.5	91.58 ± 3.09[b]	790.00 ± 61.28[a]
3.0	90.88 ± 2.66[b]	733.34 ± 18.86[a]

Values are expressed as mean ± standard deviation of three independent replicates (n=3), followed by different small letters (a-b) indicate significant difference ($p < 0.05$) within the same column.

The viability of free, encapsulated Lp299v with and without FOS during storage in ambarella juice were evaluated as descried by Nualkaekul *et al.* (2012). One gram of beads (with and without prebiotic) and 1 mL of free Lp299v cell were added into 9 mL of ambarella juice. The juices were stored at both room temperature (25 °C) and refrigerated temperature (4 °C) for 4 weeks. For monitoring of probiotic viability, enumeration of the cells was performed weekly over the period of 4 weeks using spread plate method in MRS agar incubated at 37 °C for 48 h.

Statistical analysis

All experiments were carried out in triplicates and the results were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) and paired T-test were carried out, where the average values were compared with Tukey's post hoc test for one-way ANOVA using Minitab v17.3.0 (Minitab Statistical Software, Minitab Inc., USA). Results were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Optimisation of parameters

Microencapsulation process was optimised by two parameters: (1) concentration of calcium chloride (CaCl$_2$), hardening solution for microcapsules, and concentration of oligofructose (FOS) based on the microencapsulation efficiency (MEE) and beads diameter. CaCl$_2$ concentration was the first parameter to be optimised as it would affect the diffusion characteristics of the beads as well as the gel spheres formation which in turn affect the MEE and beads size (Lin, 2012; Teanpaisan *et al.*, 2015). From Table 1, it was observed that the MEE was increased at higher CaCl$_2$ concentration (2.5% (w/v)) with more than 9% as compared with lower concentration (1.5% (w/v)) of CaCl$_2$. Similar trend was also observed in the study conducted by Zam *et al.* (2014) when concentration CaCl$_2$ concentration were used for optimization the loading efficiency of pomegranate peel's

polyphenols in alginate beads. Woraharn *et al.* (2010) reported that beads produced at higher CaCl$_2$ concentration had higher mechanical strength and lower gelation rate that prevent the dissolution of beads in simulated gsatric juice. This was also in agreement with Homayouni *et al.* (2007) that the MEE was increased when higher ratio CaCl$_2$: algiante solution.

Table 2: Microencapsulation efficiency and average diameter of chitosan coated-alginate Lp299v beads hardened at 2.5% (w/v) incorporated with different FOS concentrations.

FOS concentration (% w/v)	Microencapsulation efficiency (%)	Diameter of beads (µm)
1.0	89.99 ± 0.88[ab]	736.67 ± 4.72[a]
2.0	90.62 ± 1.29[ab]	745.00 ± 2.36[a]
3.0	87.82 ± 1.49[a]	765.00 ± 25.92[a]
4.0	93.90 ± 2.47[b]	776.77 ± 14.28[a]
5.0	90.14 ± 1.46[ab]	745.00 ± 16.50[a]

Values are expressed as mean ± standard deviation of three independent replicates (n=3), followed by different small letters (a-b) indicate significant difference ($p < 0.05$) within the same column.

Table 3: Microencapsulation efficiency and average diameter of chitosan-coated alginate beads with and without FOS.

Parameter	Without FOS	With FOS
Diameter of beads (µm)	556.80 ± 10.40[a]	748.20 ± 16.60[b]
Microencapsulation efficiency (%)	97.36 ± 0.60[a]	93.46 ± 0.19[b]

Values are expressed as mean ± standard deviation of three independent replicates (n=3), followed by different small letters (a-b) indicate significant difference ($p < 0.05$) within the same row.

Jyothi *et al.* (2010) proposed that the difference in encapsulation efficiency may probably due to the concentration and type of polymer used for encapsulation. The degree of cross-linking between calcium ions and guluronic units of alginate molecules is dependent on the intramolecular distribution and proportion of the two monomers residues in alginate molecules (Lotfipour *et al.*, 2012; Solanki *et al.*, 2013). Hence, alginate molecules with higher proportion of guluronic acid to mannuronic acid would be more dependent on concentration of CaCl$_2$ and hardening time in the production of alginate beads (Lotfipour *et al.*, 2012). At higher CaCl$_2$ concentration, more Ca^{2+} ions are available to cross-linking between the guluronic acid residues and ensure the entrapment of the core material (Lin, 2012).

Besides that, the mean diameter of the encapsulated Lp299v beads produced by different CaCl$_2$ concentrations was in the range of 648–790 µm. The concentrations of CaCl$_2$ had no significant ($p \geq 0.05$) effect on the mean diameter of the beads and this was in agreement with the findings reported by Lotfipour *et al.* (2012). The size

range of beads produced by co-extrusion was smaller than those produced by extrusion (Krasaekoopt and Watcharapoka, 2014). The diameter range of Lp299v microcapsules were similar to the range of kernel seed oil beads produced by co-extrusion (Chew *et al.*, 2015).

Fructooligosaccharide (FOS) is prebiotic which regulates the intestinal microbiota and to increase the stability and viability of probiotic cultures during refrigerated storage as well as survival in simulated gastrointestinal conditions (Rajam and Anandharamakrishnan, 2015; Silva and Sato, 2017). Table 2 shows the MEE and mean diameter size of Lp299v beads using different FOS concentrations. The mean diameter of the chitosan coated-alginate probiotic beads incorporated with varying concentrations of FOS ranged from 736 to 776 µm. It was observed that FOS concentrations had no significant ($p \geq 0.05$) effect on the microcapsules' mean diameter. The result was in agreement with the study conducted by Haghshenas *et al.* (2015), who reported that there was no significant difference ($p \geq 0.05$) in average diameters of *Enterococcus durans* 39C entrapped in alginate-psyllium beads with different concentrations of inulin.

In contrast, Table 2 shows that the microencapsulation efficiency of chitosan coated-alginate Lp299v beads hardened at 2.5% (w/v) CaCl$_2$ with varying concentrations of FOS was in the range of 87.8–93.9%. The MEE of beads incorporated with 4% (w/v) FOS was approximately 6% greater at lower FOS concentration (3% (w/v)), and no significant ($p \geq 0.05$) effect was observed at 5% (w/v) FOS. However, Krasaekoopt and Watcharapoka (2014) reported that encapsulation yield was in the range of 0.3-79.4% when using different concentrations of galactooligosaccharides and inulin. They suggested that the effectiveness of microencapsulation process was not dependent on the presence of prebiotics (types and concentration). This probably due to the compatibility of the prebiotics to the specific probiotics strains as prebiotic only stimulates the certain the growth of certain probiotics (Pandey *et al.*, 2015).

Microcapsules prepared by 2.5% (w/v) CaCl$_2$ without prebiotic (served as control) and the beads incorporated with 4% (w/v) oligofructose were then further subjected for encapsulation efficiency, physical characteristics examination, survival in gastrointestinal treatment and storage test in ambarella juice.

Comparison of beads with and without FOS in mean size and MEE

Morphology and size of microencapsulated Lp299v beads examined under optical microscope was shown in Figure 1. The mean diameter size and MEE of the beads with and without FOS were presented in Table 3. The beads produced were spherical in shape and had a smooth surface. This is essential as the sphericity of the beads may prevent the problem of cell overgrowth in encapsulated beads (Fan *et al.*, 1990, McMaster *et al.*, 2005). However, beads with broken surface normally

results in protrusion of cells and eventually lower the entrapped cells' viability (Krasaekoopt *et al.*, 2004). Co-extrusion was reported to be effective in producing uniform micron size capsules (de Prisco *et al.*, 2015).
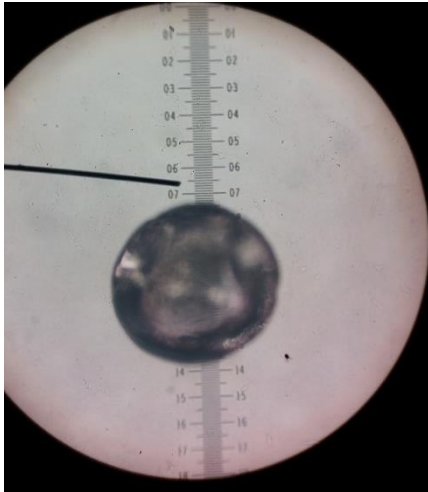
Figure 1: Morphology and size of Lp299v beads with FOS examined under optical microscope.

Table 3 shows that the encapsulation yield of the Lp299v with FOS was 93% and was significantly ($p <$ 0.05) lower than Lp299v beads without FOS. Such difference in MEE was also reported by Gandomi *et al.* (2016), whereby the MEE of the bacterial cells without inulin and with inulin were reported to be 52.6% and 49.3%, respectively. The low values of encapsulation efficiency might be explained by the increase the beads' mass due to the addition of prebiotics, thus resulting in lesser number of entrapped cells in the microcapsules. The relatively high MEE can be attributed to the gentle process in co-extrusion technique, which can protect the survivability of probiotic cells and exclude certain detrimental procedures such as the use of high shear force (Homar *et al.*, 2007; Nag, 2011; Chávarri *et al.*, 2012). In addition, the high initial cell concentration of Lp299v may probably results in high MEE values in this study.

The mean beads size of encapsulated Lp299v with FOS was 34% greater than the beads without prebiotic. Chávarri *et al.* (2010) also reported that the addition of quercetin (prebiotic) during encapsulation of *Bifidobacterium bifidum* and *Lactobacillus gasseri* had actually increased the size of the beads produced by approximately 50%. These findings were similar to the results reported by Krasaekoopt and Watcharapoka (2014), such that the mean diameter of beads incorporated with prebiotics was larger compared to the beads without prebiotics. The addition of FOS in probiotic microencapsulation increased the mass of the beads, resulting less number of probiotic bacteria to be entrapped in the microcapsules. Therefore, the

microencapsulation efficiency is lower compared to the beads without prebiotic.

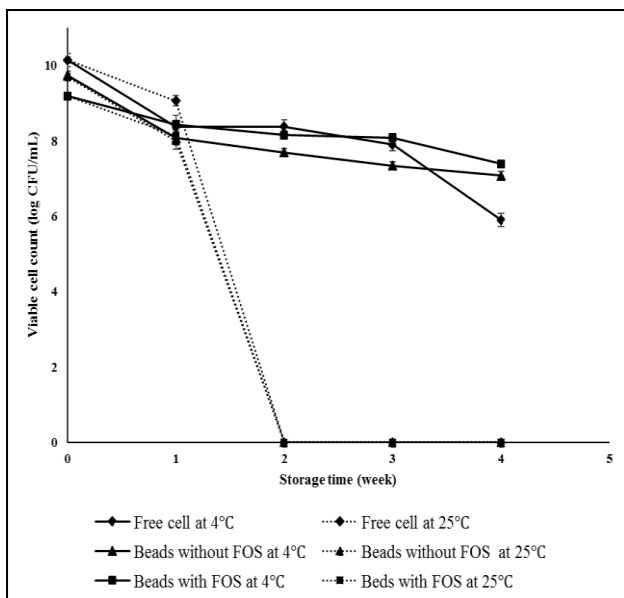## Viability of free, Lp299v beads without and with FOS under simulated gastrointestinal conditions

In order to exert positive health benefits, probiotics must remain viable during gastrointestinal transition at high levels (at least $10^6 - 10^8$ CFU/g or mL) in the intestine (Marteau and Rambaud, 1993). The optimum pH of Lp299v was reported in the range of pH 4.0 to 8.0 (Hamon *et al.*, 2014). Therefore, probiotic encapsulation is proposed to improve the Lp299v survivability and tolerance to low pH in gastric conditions and high bile salt concentration in the intestinal region (Ansari *et al.*, 2017; Etchepare *et al.*, 2016). The wall materials selection and addition of prebiotic for encapsulation of Lp299v were vital to ensure sufficient protection to the cells (Gandomi *et al.*, 2016). The viability of encapsulated Lp299v (with and without FOS) and free cells under simulated gastric (SGJ, pH 2.0 for 2 h) and intestinal (SIJ, pH 7.5 for 5 h) conditions were evaluated and the results were presented in Figure 2.

Based on Figure 2, the viabilities of encapsulated Lp299v with and without FOS decreased gradually while the viability of free cell decrease steeply during the first hour of SGJ incubation. The reduction in the free cell viability was about 10% and 17% higher as compared to the beads without and with FOS, respectively. Moreover, the viability of both encapsulated beads was significantly ($p < 0.05$) higher by at least 11% as compared to free Lp299v cell at 1 and 2 h of gastric digestion. The viability of free Lp299v cells reduced more than 20% at the end of gastric digestion. This shows that free cells were easily damaged when exposed to low pH of SGJ (Mustafa *et al.*, 2016; Jamilah and Priyani, 2018). Zanjani *et al.* (2014) also proved that *Lactobacillus casei* and *Bifidobacterium bifidum* entrapped in chitosan coated-alginate-gelatinized starch beads showed higher survivability compared to free cells.

On the other hand, approximately 9% and 3% of viable cell reduction was observed for beads without and with FOS, respectively during the first hour of SGJ digestion. Since the viability of the entrapped Lp299v cells were greater than 90% after gastric digestion, this demonstrated the effectiveness of microencapsulation technique in protecting the probiotic viability at low pH in SGJ. The high viability in encapsulated probiotic during SGJ incubation were also reported by various studies (Chávarri *et al.*, 2010; Valero-Cases and Frutos, 2015; Solanki and Shah, 2016; Gandomi *et al.*, 2016). The coating of alginate beads with chitosan forming a polyelectrolyte complex by electrostatic interaction between the carboxylic acid of alginate with amine group of chitosan (Ren *et al.*, 2017). This improved the mechanical strength and permeability of the alginate beads that minimise the leakage of the entrapped materials (Luo and Wang, 2014; Chew *et al.*, 2015; Călinoiu *et al.*, 2019).
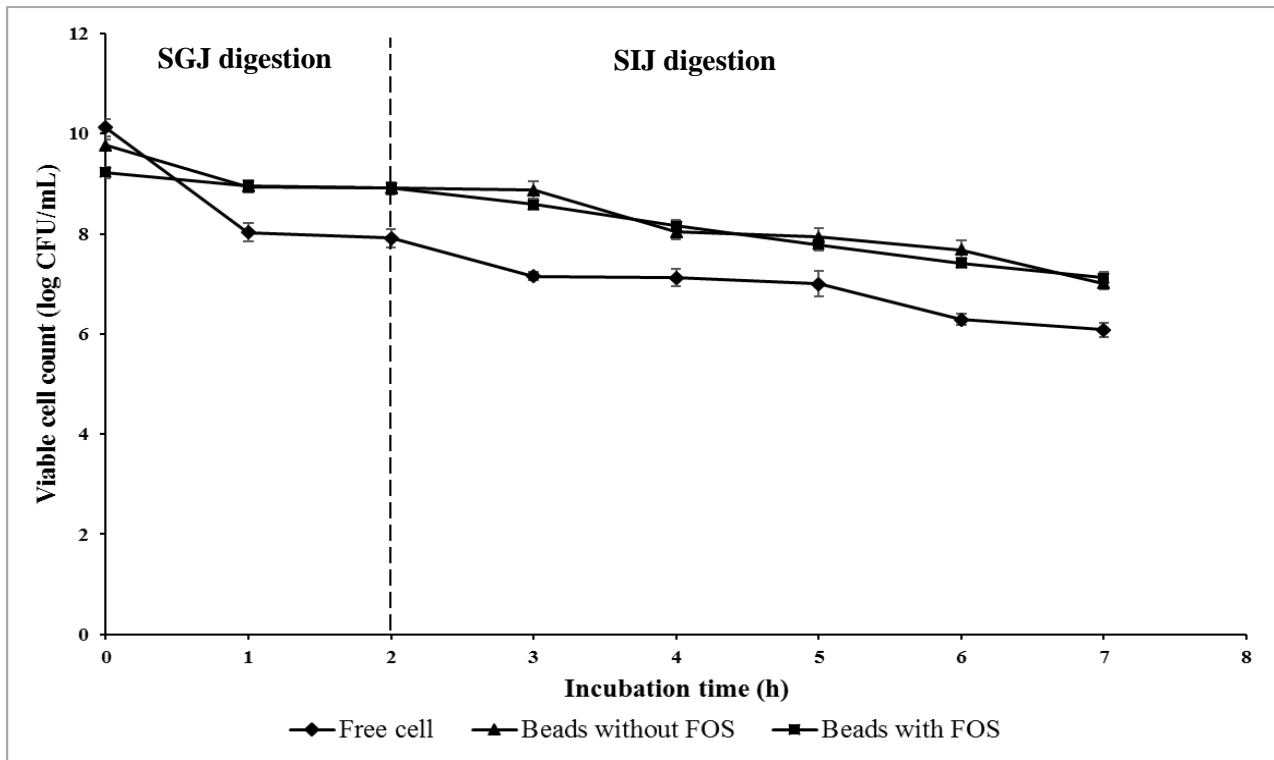
Figure 2: Total viable cell count of free, Lp299v beads with and without FOS when incubated in SGJ at pH 2.0 for 2 h and SIJ at pH 7.5 for 5 h. Error bars are represented as ± standard deviation of three independent replicates (n=3).

Figure 3: Total viable cells count of free Lp299v beads with and without FOS in ambarella juice during 4 weeks storage at refrigerator (4 °C) and room temperature (25 °C). Error bars are represented as ± standard deviation of three independent replicates (n=3).

From the beginning (2 h) and the end (7 h) of intestinal digestion, the viability of free cells was significantly ($p < 0.05$) lower than both of the encapsulated cells as shown in Figure 2. The viable cell count was more than 10^7 CFU/mL of cells for both encapsulated Lp299v with and without FOS compared to free cells (6.08 log CFU/mL). It was also observed that the cell viabilities of beads with FOS decreased with less than 5% for each one-hour interval. The results indicated that Lp299v was more susceptible to bile salt in the intestinal conditions. The viability loss was 30.6% for free cell while 25.1 and 27.1% for microcapsules with and without FOS, respectively at the end of the SIJ incubations. Similar result was reported by Silva *et al.* (2018), who found that the survivability of *Lactobacillus acidophilus* La-5 was dropped to less than 50% after 4 h of SIJ incubation time.

Encapsulated probiotic cells experienced more reduction during intestinal digestion than gastric condition. It may be due to the fact that alginate is stable under low-pH solutions but it is usually swell in weak basic conditions (Annan *et al.*, 2008). Moreover, chitosan forms a semi-permeable membrane around the porous alginate matrix that allows intestinal basic condition to influence the capsules stability (Gbassi and Vandamme, 2012; Silva *et al.*, 2018). However, chitosan-coated alginate beads were still effective in protecting Lp299v and viability was maintained approximately 10^7 CFU/mL. The chitosan coating was able to provide protection in bile salt solution

as ion-exchange reaction would be taken place when the beads absorb bile salt (Li *et al.*, 2011; Moghtader *et al.*, 2017). Thus, this enhanced the mass transfer resistance and limits the penetration of bile salt into the beads (Obradović *et al.*, 2015). Similar findings were presented by Krasaekoopt *et al.* (2004) and Kamalian *et al.* (2014) whereby the survivability of *Lactobacillus casei*, *Bifidobacterium bifidum* and *B. pseudocatenulatum* G4 entrapped in chitosan-coated alginate beads were higher survivability compared with the uncoated alginate beads.

Although both encapsulated cells (with and without FOS) showed high survivability after simulated gastrointestinal digestion, however, no significant difference ($p \geq 0.05$) was found between the beads. This indicated that the addition of FOS did not improve the survivability of Lp299v in acidic gastric and high bile conditions and this was supported by Sathyabama *et al.* (2014). Etchepare *et al.* (2016) also reported that the freeze-dried encapsulated *Lactobacillus acidophilus* La-14 with Hi-Maize starch did not preserved the probiotic viability up to the minimum level of 10^6 CFU/mL after simulated gastrointestinal digestions.

Survival of free, Lp299v beads with and without FOS in ambarella juice during storage at refrigerator (4 °C) and room temperature (25 °C)

Free probiotic cells did not survive well during storage in certain fruit juices, such as apple, orange, pomegranate and cranberry juice due to low pH of juice and high total phenolic contents that may exhibit antimicrobial activity (Ding and Shah, 2008; Nualkaekul and Charalampopoulos, 2011; Nualkaekul *et al.*, 2013; Perricone *et al.*, 2015). Therefore, it is essential to evaluate the effectiveness of co-extrusion microencapsulation technique and addition of prebiotics in protecting Lp299v cells during prolonged storage in low pH of ambarella juice at different temperatures. The storage temperature is vital as it would directly affect the probiotic survivability in fruit juice (Ozcan *et al.*, 2015).

In this study, Lp299v was added into ambarella juice as free cells, microcapsules with FOS and without FOS. Their viable cell count were evaluated weekly for 4 weeks of storage in the juice at refrigerated (4 °C) and room temperature (25 °C) as shown in Figure 3. All forms of Lp299v did not survive in ambarella juice after two weeks of storage in 25 °C. This indicated that encapsulation of Lp299v was not able to maintain its viability during prolong storage in ambarella juice at room temperature. Low pH of the ambarella juice and unfavourable storage temperature had caused the viable cells count to decline drastically and even killed the cells. Ambarella juice is high in total phenolic content and low in pH (pH < 3) which exerts destructive effect to probiotic cells (Perricone *et al.*, 2015; Rahman *et al.*, 2016).

According to Figure 3, it was observed that the viability of cell decreased within the first week of storage at refrigerated temperature for all forms of Lp299v. Free viable Lp299v decreased sharply during the first week of storage in ambarella juice at 4 °C. This may be due to the

exposure of probiotic cells to the injurious condition of the fruit juice, especially the low pH of the juice (Gandomi *et al.*, 2016). The viable cells count for free and Lp299v beads without FOS declined more than 17% after one week of refrigeration storage, while the viability of Lp299v beads with FOS was above 90%. The viable free cells count fell to 5.89 log CFU/mL at the end of storage in ambarella juice and did not meet the minimum limit for the development of probiotic functional food (Teanpaisan *et al.*, 2015).

On the other, the viable cells count of encapsulated Lp299v with FOS (7.39 log CFU/mL) and without FOS (7.08 log CFU/mL) were more than 10^7 CFU/mL at throughout the storage in 4 °C although Lp299v beads without FOS showed greater reduction (more than 27%) in viabilities as compared to the beads with FOS (approximately 20% of cell reduction). This result was in agreement with Krasaekoopt and Watcharapoka (2014), who reported that the numbers of survival cells with galactooligosaccharides (GOS) were higher than those of without GOS for *Lactobacillus casei* and *Lactobacillus acidophilus* in orange juice. The above findings reflected that the addition of prebiotic could enhance the survivability of encapsulated cells from the adverse conditions of fruit juices.

Moreover, FOS can provide the carbon and nitrogen source for microencapsulated probiotics during storage (Chen *et al.*, 2005; Saulnier *et al.*, 2008). From Figure 3, the viability of encapsulated Lp299v was higher than that of free Lp299v. Encapsulated Lp299v was able to maintain good stability in low pH ambarella juice since there was only little loss of viability compared to free cells during its storage at 4 °C. The results were in agreement with Ding and Shah (2008). Hossain *et al.* (2016) also reported that the lactic acid bacteria (*Lactobacillus acidophilus*, *L. bulgaricus*, *Lactococcus lactis* and *Bifidobacterium bifidum*) were higher than 10^6 CFU/mL after 5 weeks of storage in orange juice. Chitosan coating on alginate beads reduces effect of the adverse conditions on the encapsulated probiotic cells (Vandenberg and De La Noüe 2001; Nualkaekul *et al.*, 2012). The encapsulated Lp299v with and without FOS via co-extrusion technique under storage at 4 °C in ambarella juice were able to meet this requirement, whereby there was more than 10^7 CFU/mL of cells survived throughout the storage period.

CONCLUSION

The incorporation of FOS in the chitosan coated-alginate mirocapsules Lp299v produced by co-extrusion not only strengthening the protection to probiotics but also preserving the probiotic viability up to the minimum recommended level (10^7 CFU/mL) in adverse environment such as the simulated gastrointestinal conditions and storage in ambarella juices. FOS (4.0% (w/v)) provided the optimum microencapsulation efficiency and protects the probiotic viability during refrigerated storage in ambarella juice for 4 weeks. These

fruit juices are suitable for consumers with lactose intolerance and/or milk allergy.

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