ORIGINAL ARTICLE

Microencapsulation of *Lactiplantibacillus plantarum* with inulin and evaluation of survival in simulated gastrointestinal conditions and roselle juice

Microencapsulação de Lactiplantibacillus plantarum com inulina e avaliação da sobrevida em condições gastrointestinais simuladas e suco de rosélia

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Abstract

This study aimed to evaluate the survivability of *Lactiplantibacillus plantarum* 299v encapsulated in chitosan-coated calcium alginate beads with inulin as prebiotic in simulated gastrointestinal conditions and roselle juice. The concentration of calcium chloride and inulin for *L. plantarum* 299v microencapsulation was optimised and the survivability of free and microencapsulated *L. plantarum* was assessed under simulated gastrointestinal conditions. Storage stability of the optimised encapsulated *L. plantarum* 299v-inulin was determined throughout four (4) weeks of storage in roselle juice at 4 °C and 25 °C. The optimized formula for *L. plantarum* 299v was 2.0% (w/v) of calcium chloride and 3.0% (w/v) of inulin. Optimized calcium alginate-chitosan *L. plantarum* 299v microbeads with inulin did not affect ($p > 0.05$) the bead diameter, with a mean diameter of 685.27 µm, and microencapsulation efficiency of 95%. Encapsulated *L. plantarum* 299v with inulin showed higher survivability ($>10^7$ CFU/mL) than free cells and encapsulated *L. plantarum* 299v without inulin under simulated gastrointestinal conditions and after four (4) weeks of storage in roselle juice at 4 °C. The results indicate that co-extrusion encapsulation and addition of inulin had improved the viability of *L. plantarum* 299v in roselle juice by protecting probiotic against unfavourable gastrointestinal conditions and prolonged storage.

Keywords: Co-extrusion; Probiotic; Storage; Prebiotic; Gastrointestinal digestion; Optimization.

Resumo

O objetivo deste estudo foi avaliar a capacidade de sobrevivência de *Lactiplantibacillus plantarum* 299v encapsulado em esferas de alginato de cálcio revestidos com quitosana, com inulina como prebiótico, em condições gastrointestinais simuladas e suco de rosélia. A concentração de cloreto de cálcio e inulina para a microencapsulação

de *L. plantarum* 299v foi otimizada e a sobrevivência de *L. plantarum* livre e microencapsulado foi avaliada em condições gastrointestinais simuladas. A estabilidade do encapsulado otimizado *L. plantarum* 299v-inulina durante armazenamento foi determinada ao longo de quatro semanas em suco de rosélia a 4 °C e 25 °C. A fórmula otimizada para *L. plantarum* 299v foi de 2,0% (w/v) de cloreto de cálcio e 3,0% (w/ v) de inulina. Microesferas otimizadas de alginato de cálcio-quitosana contendo *L. plantarum* 299v com inulina apresentaram diâmetro médio de 685,27 μm sem diferença significativa ($p > 0,05$) em relação às partículas sem inulina, e eficiência de microencapsulação de 95%. *L. plantarum* 299v encapsulado com inulina mostrou maior capacidade de sobrevivência ($> 10^7$ CFU/mL) do que células livres e *L. plantarum* 299v encapsulado sem inulina em condições gastrointestinais simuladas e após quatro semanas de armazenamento em suco de rosélia a 4 °C. Os resultados indicam que a a encapsulação por coextrusão e a adição de inulina melhoraram a viabilidade de *L. plantarum* 299v em suco de rosélia, protegendo o probiótico contra condições gastrointestinais desfavoráveis e prolongando o armazenamento.

Palavras-chave: Coextrusão; Probiótico; Armazenamento; Prebiótico; Digestão gastrointestinal; Otimização.

1 Introduction

Probiotic is defined as a viable non-pathogenic microorganism that can provide health benefits to consumers when consumed in sufficient amounts (Hill et al., 2014). The consumption of probiotics can assist in promoting health such as improving lactose intolerance and digestive system, modulating the immune system and reducing serum cholesterol (Garcia-Castillo et al., 2019; Tsai et al., 2014; Vonk et al., 2012). However, the viability of probiotics is being influenced by several parameters such as pH, temperature and water activity (Aw) (Patel, 2017). Therefore, technologies such as microencapsulation were used to enhance the survivability of probiotic cells during storage and delivery into the human body (Cook et al., 2012). Microencapsulation provides effective protection to probiotic by separating them from the external environment using wall materials, hence maintaining the viability of probiotic (Chew et al., 2019; Siang et al., 2019).

Wall materials such as alginate have been incorporated during the microencapsulation process in order to protect the core material. Alginate is a linear anionic polysaccharide derived from different types of algae and it is commonly used as shell wall material (Ng et al., 2019). It is made up of two structural units which are the L-guluronic acid and D-mannuronic acid that are joined through the glycosidic bond (Heise et al., 2005). On the other hand, chitosan also functions as an additional coating to alginate beads. Chitosan is a linear cationic polysaccharide which can be obtained through the deacetylation of chitin that is found in the shell of the shrimp, crustaceans or the cuticles of insect (Hamed et al., 2016). The chitosan-coated alginate beads was claimed to show better resistance towards the deteriorative effect of calcium chelating and denser in structure (Mortazavian et al., 2007b). Thus, it provides better protection towards probiotics during storage and under gastrointestinal conditions (Chávarri et al., 2010).

Apart from wall materials, the addition of prebiotic also assists in improving the survivability of probiotic during the exposure to gastrointestinal conditions as well as storage (Gandomi et al., 2016). Prebiotics are short-chain carbohydrates that are non-digestible by humans, however, they can function as food for probiotics (Al-Sheraji et al., 2013). In addition, prebiotic has been gaining popularity due to its nutrition and health-relevant properties. Furthermore, prebiotics offer health benefits to the host by optimising the colonic function and metabolism, modulate the immune system, assist in lipid metabolism and mineral absorption (Fernández et al. 2016; García-Vieyra et al., 2014; Williams et al., 2016). Examples of prebiotics incorporated into foods are inulin-type-fructans and fructooligosaccharides (FOS) (Al-Sheraji et al., 2013).

Nowadays, probiotics are commonly incorporated into dairy-based products such as yoghurt and milk. However, this limits the choice for people who are lactose intolerant (Panghal et al., 2018). Hence, there is an urge to develop a non-dairy probiotic beverage in order to provide an alternative choice for those who cannot consume the dairy product (Mohan et al., 2013). Non-dairy beverages such as fruit juice, tea, flower and herbal extracts could act as an ideal medium for the delivery of probiotics due to their high essential

nutrient content and potential functional properties (Acevedo-Martínez et al., 2018; Chaikham, 2015; López de Lacey et al., 2014; Lai et al., 2020a, 2020b; Yee et al., 2019).

Roselle, a bushy and sub-shrub with thick, fleshy and red inflated edible calyces, is a flower that has gained its popularity due to its high commercial value, high anthocyanin and ascorbic acid content (Alaga et al., 2014). A recent study has suggested roselle as a good source of dietary fibre and polyphenol with antioxidant property (Mercado-Mercado et al., 2015). Since roselle juice has high demand among the food industry and consumers, incorporation of probiotic into roselle juice could further confer health benefits to the consumers. However, as Roselle juice has a low pH ranging from 3.13-3.25 (Bolade et al., 2009), probiotics should be microencapsulated prior to incorporating into Roselle juice. In this study, the inclusion of inulin and chitosan on the viability of *L. plantarum* 299v microencapsulated with sodium alginate using co-extrusion technique against simulated gastro-intestinal conditions and storage in roselle juice were evaluated.

2 Materials and methods

2.1 Materials

L. plantarum 299v was purchased from BIO-LIFE, Malaysia. Inulin was purchased from Sensus (Roosendaal, Netherlands) and used as prebiotic. Sodium alginate, chitosan and calcium chloride were purchased from R&M Chemicals, UK to produce microparticles. Pepsin (HmbG chemicals, Germany) and bile salt (R&M Chemicals, UK) were used to prepare simulated gastric and intestinal juices. The deMan, Rogosa and Sharpe (MRS) agar and MRS broth were purchased from Merck KGaA (Darmstadt, Germany) and used for microbiological analysis. Roselle (*Hibiscus sabdariffa* L.) was purchased from a local market in Johor, Malaysia and the juice was used for storage studies.

2.2 Preparation of culture

L. plantarum 299v cells were transferred into De Man Rogosa Sharpe (MRS) broth and incubated at 37 °C for 24 h. The cells were collected by centrifugation (MIKRO 220R, Germany) at 3200 rpm at 4 °C for 15 min and were rinsed two times with phosphate buffer saline (PBS).

2.3 Microencapsulation process

Microencapsulation of *L. plantarum* 299v was conducted through co-extrusion method using Büchi Encapsulator B-390 (Büchi Labortechnik AG, Flawil, Switzerland) in accordance with Chew et al. (2015) with slight modification. During encapsulation, core fluid (*L. plantarum* 299v with inulin or *L. plantarum* 299v cell suspension) and wall material (1.5% (w/v) sodium alginate solution) flowed through the inner (150 μm) and shell nozzle (300 μm) using 600 mbar of air pressure. The nozzle's parameters were set at 300 Hz for vibration frequency, 3 for amplitude and 1.5 kV for voltage.

2.4 Optimisation of concentration of calcium chloride and inulin

To optimise the concentration of calcium chloride, the concentration of inulin and alginate was fixed at 3% and 1.5% (w/v), respectively. Different concentration of calcium chloride ranging from 0.5 to 3.0% (w/v) was used for the microencapsulation process.

To optimise the concentration of inulin, the calcium chloride concentration was fixed based on the optimum concentration chosen previously while the alginate concentration used was 1.5% (w/v). Then, the microencapsulation process was conducted with different inulin concentration ranging from 0 to 5.0% (w/v).

The optimal concentration of calcium chloride and inulin were determined based on bead diameter and microencapsulation efficiency.

2.5 Coating of microcapsules with chitosan

The preparation of chitosan aqueous solution was adapted from Ng et al. (2019). To prepare the chitosan solution (0.1% w/v), 1 g of chitosan powder was dissolved in 900 mL of distilled water acidified with 10 mL of glacial acetic acid. The mixture was adjusted to pH 5.0 with sodium hydroxide (NaOH) solution. The chitosan solution was pasteurised at 72 °C for 30 s and mixed with calcium chloride solution.

The microcapsules formed by co-extrusion encapsulation were hardened in chitosan solution for 30 min. The alginate-chitosan beads were collected using a nylon sieve and rinsed with Phosphate Buffer Saline (PBS). The microcapsules were then dried using filter paper before transferring them to a sterile collector.

2.6 Morphology and size of microcapsules

Fifty *L. plantarum* 299v microbeads were picked randomly. The morphology and diameter of beads were observed using an optical microscope (CX23, Olympus, Japan) with 100x magnification. The diameter of the beads was then recorded (Yee et al., 2019).

2.7 Release of entrapped bacteria

Prior to cell enumeration, the decomposition of the encapsulated *L. plantarum* 299v was adapted from Chia et al. (2015) with modification. One gram of beads was dissolved in 9 mL of sodium citrate and homogenised in the stomacher machine (BagMixer® 400 W, Interscience, France) for 5 min. The decomposed beads and 1 mL of free bacteria were serially diluted with PBS and plated onto MRS agar to be incubated at 37 °C for 48 h.

The viable number of bacteria was calculated and expressed in colony-forming unit per millilitre (CFU/mL) using Equation 1. The viable cell counts were then further converted into log CFU/mL. Microencapsulation efficiency was calculated using Equation 2.

$$Colony\text{-}for \min g \ unit \ (CFU/mL) = Average \ number \ of \ colonies / (Dilution \ factors \times volume \ plated) \qquad (1)$$

$$Microencapsulation \ efficiency(\%) = (N/N_0) \times 100 \qquad (2)$$

N is the number of entrapped cell counts (CFU/g) released from the microcapsules and N_0 is the number of free cells (CFU/mL) in culture.

2.8 Sequential digestion of free cell and encapsulated bacteria with or without inulin

The formulation of Simulated Gastric Juice (SGJ) and Simulated Intestinal Juice (SIJ) was adapted from Siang et al. (2019) with modification. The SGJ was formulated by adding 3.5 mL of hydrochloric acid (HCl) and 1 g of sodium chloride into 500 mL of distilled water. The solution was adjusted to pH 2.0 with HCl and sterilised at 121 °C for 15 min, then, 1.6 g of pepsin was added. Then, the SIJ was formulated by incorporating 3.4 g of potassium dihydrogen phosphate into 125 mL of distilled water. The solution was added with 95 mL of NaOH before filling up to 500 mL with distilled water. The mixture was adjusted to pH 7.5, sterilised at 121 °C for 15 min prior to adding 3 g of bile salt.

To determine the survivability of *L. plantarum* 299v under sequential digestion, 1 g of beads (encapsulated *L. plantarum* 299v with inulin and encapsulated *L. plantarum* 299v) or 1 mL of free cells were added into 9 mL of sterile pH 2.0 SGJ for 1 and 2 h, then centrifuged for resuspension in 9 mL of SIJ for 5 h. The microbeads or free cells were removed by filtration and centrifugation at 3200 rpm for 15 min respectively. The filtered

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microcapsules were washed with sterile PBS solution and then homogenised in stomacher to perform viable cell count. The viability for free cells and encapsulated cells were stated as CFU/mL and CFU/g, respectively.

2.9 Storage stability

2.9.1 Preparation of roselle juice

Roselle juice was prepared as stated by Bolade et al. (2009) with modification. Fresh roselle was added into warm water (50 °C) in the ratio of 1:2 and cooked for 30 min. The roselle juice was then sieved and sugar was added until 13 ºbrix. Pasteurisation was conducted at 82.5 °C for 20 min using the double boiler technique.

2.9.2 Storage stability of probiotics in roselle juice

The viability of free and encapsulated *L. plantarum* 299v during the storage in roselle juice was performed according to Teanpaisan et al. (2015) with modification. Beads with or without inulin (1 g) or 1 mL of free cells were added into 9 mL of pasteurised roselle juice. Roselle juice containing bead or free cells was stored in a refrigerator at 4 °C and room temperature at 25 °C for four (4) weeks. The viability of probiotics in roselle juice was determined each week over the storage of four (4) weeks.

2.10 Statistical analysis

All analyses were conducted in triplicate and presented as mean ± standard deviation. Data were analysed using Minitab 17 software (Minitab, Inc, Pennsylvania, USA). Significant differences between means were evaluated using Analysis of Variance (ANOVA) with Tukey's post hoc test at $p \leq 0.05$.

3 Results and discussion

3.1 Effect of calcium chloride and inulin concentrations on the microencapsulation efficiency, size, and morphology of beads

Different concentrations of calcium chloride on bead diameter and microencapsulation efficiency of chitosan-coated *L. plantarum* 299v microbeads were shown in Table 1. Throughout the optimisation process of calcium chloride concentration, alginate and inulin concentrations were fixed at 1.5% and 3.0% (w/v), respectively. From Table 1, the increment in calcium chloride concentration did not affect ($p > 0.05$) the bead size. A similar result was reported by Homayouni et al. (2007) and Lotfipour et al. (2012) who found that the bead size was not affected by the use of different calcium chloride concentrations in microencapsulating *L. casei* and *L. acidophilus*, respectively.

Table 1. Bead diameter and microencapsulation efficiency of chitosan-coated *L. plantarum* 299v microbeads with different calcium chloride and inulin concentrations.

Calcium chloride (% w/v)	Bead diameter (µm)	Microencapsulation efficiency (%)	Inulin (%, w/v)	Bead diameter (µm)	Microencapsulation efficiency (%)
0.5	680.00 ± 61.28[a]	77.16 ± 4.89[b]	0.0	663.34 ± 47.14[a]	97.69 ± 1.7[a]
1.0	650.00 ± 32.99[a]	79.13 ± 6.33[b]	1.0	711.67 ± 16.50[a]	88.67 ± 0.49[c]
1.5	616.67 ± 127.28[a]	85.11 ± 1.63[ab]	2.0	708.34 ± 2.35[a]	85.99 ± 10.60[cd]
2.0	683.33 ± 28.28[a]	92.88 ± 3.90[a]	3.0	711.67 ± 7.07[a]	93.40 ± 1.97[b]
2.5	705.00 ± 30.65[a]	86.86 ±1.74[ab]	4.0	728.17 ± 7.30 [a]	87.89 ± 0.21[c]
3.0	716.67 ± 23.57[a]	82.65 ± 0.35[ab]	5.0	710.00 ± 23.57[a]	82.86 ± 0.79[d]

[a-b] Means ± Standard Deviations (SD). Means within the same column followed by different letters are significantly different ($p \leq 0.05$) via Tukey's test.

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In Table 1, the microencapsulation efficiency of the bead using 2.0% (w/v) of calcium chloride was significantly higher as compared to other concentration. The result is in agreement with Teanpaisan et al. (2015) where the highest microencapsulation efficiency was reported on the encapsulation of *Lacticaseibacillus paracasei* SD1 with 2.0% (w/v) alginate. We observed that microencapsulation efficiency decreased when calcium chloride concentration higher than 2.0% (w/v) was used. The possible explanation for the probiotic loss during the microencapsulation process is associated to the increase in viscosity from high calcium chloride concentration that had interfered the cross-linking with sodium alginate (Nagpal et al., 2012). In addition, the cell electrolyte could be disrupted by the high calcium chloride concentration (2.5% and 3.0% w/v) which leads to the damage of cell membrane of probiotic bacteria (Cao et al., 2012). Hence, 2.0% (w/v) calcium chloride was chosen for the microencapsulation of *L. plantarum* 299v.

The bead diameter and microencapsulation efficiency of chitosan-coated microencapsulated *L. plantarum* 299v with different inulin concentrations were displayed in Table 1. According to Table 1, the increase in inulin concentration also had no impact ($p > 0.05$) on the size of the microbeads. A similar trend was observed by others where the diameter of the microbeads was not affected by different prebiotic concentrations (Chan & Pui, 2020; Haghshenas et al., 2015; Yong et al., 2020). Microencapsulation efficiency of beads using 3.0% (w/v) of inulin was higher as compared to other concentration except for the control. The control (without prebiotic) displayed the highest microencapsulation efficiency as higher probiotic cells were allowed to be encapsulated with the absent of prebiotic (Krasaekoopt & Watcharapoka, 2014). We also observed that further addition of prebiotic in *L. plantarum* 299v microencapsulation more than 3% (w/v) had significantly decreased the microencapsulation efficiency. This could be due to the prebiotic was overloaded during the microencapsulation which leads to damage of probiotic cell membrane from the friction between the probiotic cells and wall materials (Ann et al., 2007). Hence, 3.0% (w/v) of inulin was chosen as optimal concentration as it has the highest microencapsulation efficiency.

The beads produced were white in colour and enclosed by a thin membrane layer (Figure 1). Besides, the shape of the calcium alginate-chitosan microbeads observed was generally spherical, uniform and intact. *B. animalis* subsp. *lactis* encapsulated via co-extrusion technique, using calcium alginate as the wall material and chitosan as the coating material, displayed a smooth and uniform surface (Yong et al., 2020). This showed that the production of microbeads using the co-extrusion technique with calcium alginate and chitosan was able to provide a smooth and uniform texture (Shinde et al., 2014). The surface of calcium alginate microbeads remained intact after microencapsulation and after exposed to SGJ solution at pH 2.0 (Zanjani et al., 2014).
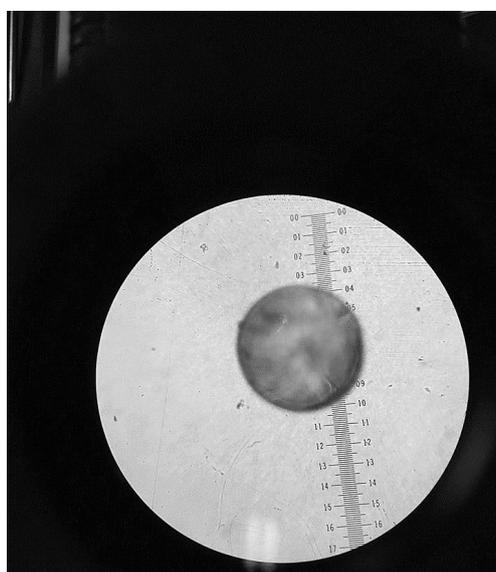

Figure 1. Shape and size of microcapsules measured with a scale micrometer.

In Table 2, the addition of inulin did not affect ($p > 0.05$) the diameter of beads. This is in agreement with Haghshenas et al. (2015) who reported that the diameter of bead was not influenced by the addition of inulin. The large diameter of microbeads (> 1000 μm) may affect the texture of the food or beverage products (Champagne & Fustier, 2007; Nag et al., 2011). However, at least 100 μm of the microbead diameter are recommended to protect the probiotics through gastrointestinal digestion (Hansen et al., 2002). Hence, the diameter of the probiotic beads reported in Table 2 was within the acceptable range of 100-1000 μm.

Table 2. Average bead diameter and microencapsulation efficiency of *L. plantarum* 299v with and without inulin.

Probiotic	Prebiotic	Wall and coating materials	Bead diameter (μm)	Microencapsulation efficiency (%)
L. plantarum 299v	-	Calcium-alginate-chitosan	684.67 ± 10.28[a]	98.16 ± 1.46[a]
L. plantarum 299v	Inulin	Calcium-alginate-chitosan	685.27 ± 14.22[a]	94.98 ± 0.63[b]

[a-b] Means ± Standard Deviations (SD). Means within the same column followed by different letters are significantly different ($p \leq 0.05$) via Tukey's test.

Microencapsulation efficiency of *L. plantarum* 299v without inulin was found higher than encapsulated *L. plantarum* 299v with inulin in this study. According to Krasaekoopt & Watcharapoka (2014), the addition of prebiotics might increase the mass of the microbeads which subsequently lowered the number of entrapped cell. Although the microencapsulation efficiency of the *L. plantarum* 299v with inulin was lower than without inulin, the microencapsulation efficiency was still above 95.0%. This reflects that only a low number of probiotic cells was lost from the microencapsulation process.

3.2 Survival of free and microencapsulated *L. plantarum* 299v under simulated gastrointestinal conditions

From Table 3, it was observed that the viability of all three forms of *L. plantarum* 299v (free cells, encapsulated bacteria with alginate-inulin-chitosan and encapsulated bacteria with alginate-chitosan) decreased as the time of incubation increased. After 2 h of incubation in SGJ, the viability of free cells decreased by 24.1%; while the viability of encapsulated bacteria without and with inulin decreased by 19.4% and 17.1%, respectively. The reduction of viable cell counts of all three forms of *L. plantarum* 299v was attributed by the acidic environment and digestive enzymes in gastric juice (Mokarram et al., 2009).

Table 3. Average log CFU/mL and survivability of free cells, encapsulated *L. plantarum* with and without inulin under sequential digestion.

Sequential incubation	Time (h)	Average log CFU/mL		
		Free cells	Encapsulated *L. plantarum*	Encapsulated *L. plantarum* with inulin
SGJ (pH 2)	0	10.81 ± 0.02[a]	10.60 ± 0.76[a]	10.31 ± 0.01[a]
	1	9.02 ± 0.11[b]	9.13 ± 0.02[b]	9.13 ± 0.02[b]
	2	8.21 ± 0.12[c]	8.54 ± 0.04[c]	8.55 ± 0.03[c]
SIJ (pH 7.5)	3	8.15 ± 0.07[c]	8.43 ± 0.08[d]	8.55 ± 0.04[c]
	4	8.14 ± 0.11[cd]	8.28 ± 0.03[e]	8.40 ± 0.01[d]
	5	7.98 ± 0.08[d]	8.19 ± 0.06[e]	8.25 ± 0.03[e]
	6	7.30 ± 0.02[e]	8.08 ± 0.04[f]	8.16 ± 0.08[f]
	7	7.27 ± 0.07[f]	7.95 ± 0.02[g]	8.11 ± 0.04[f]

[a-f] Means ± Standard Deviations (SD). Means within the same column followed by different lowercase letters are significantly different ($p \leq 0.05$) via Tukey's test. [A-C] Means ± Standard Deviations (SD). Means within the same column followed by different uppercase letters are significantly different ($p \leq 0.05$) via Tukey's test. SGJ, Simulated Gastric Juice; SIJ, Simulated Intestinal Juice.

Moreover, the survivability of free bacteria after 2 h of incubation in SGJ was 75.92% which was lower than both encapsulated *L. plantarum* 299v with and without inulin (Figure 2). Higher survivability found in encapsulated bacteria depicted the protective effect of microencapsulation. The result is in agreement with Gandomi et al. (2016) on the survivability of *Lacticaseibacillus rhamnosus* GG encapsulated with alginate and chitosan was higher as compared to free cells.
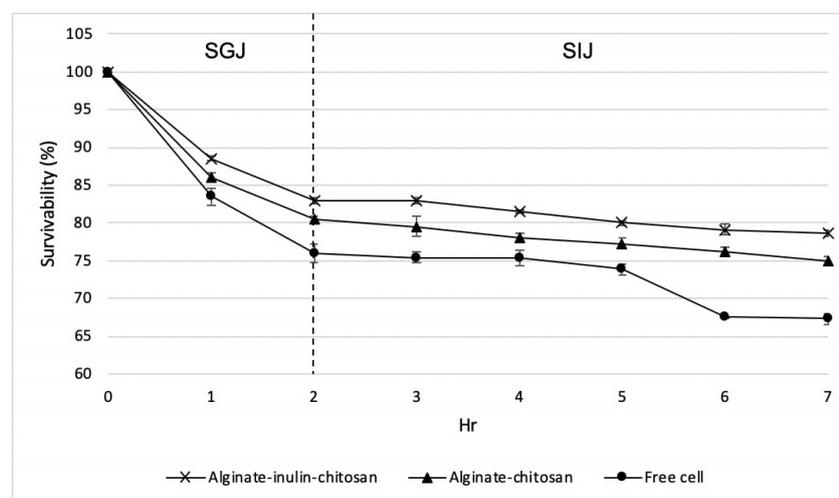
Figure 2. Survivability of free cells, encapsulated *L. plantarum* with and without inulin under sequential digestion. Error bars indicate the standard deviation of triplicate experiments. SGJ = Simulated Gastrointestinal Juice (pH 2.0); SIJ = Simulated Intestinal Juice (pH 7.5).

Furthermore, the survivability of encapsulated bacteria with inulin after 2 h of incubation in SGJ (82.93%) was higher than encapsulated bacteria without inulin (80.54%) (Figure 2). This indicates that the addition of inulin had a synergistic effect on the survivability of encapsulated *L. plantarum* 299v. Krasaekoopt & Watcharapoka (2014) showed a similar positive effect of inulin on the survival of alginate-chitosan encapsulated *Lactobacillus acidophilus* 5 and *Lacticaseibacillus casei* 01 during exposure to gastrointestinal conditions. Although free bacteria, encapsulated bacteria with and without inulin showed a decreasing trend in their viability during 2 h of incubation in SGJ, the number of cell count for three forms of *L. plantarum* 299v remained high at the level above 8.0 log CFU/mL.

From Table 3, the viability of free cells was found to be reduced by 11.4%; while the viability of encapsulated bacteria without and with inulin had decreased by 6.9% and 5.1%, respectively after 5 h of incubation in SIJ. Bile salt could be toxic against the cell membrane of probiotics (Lai et al., 2020a). Hence, the decreasing trend of free cells, encapsulated bacteria with and without inulin were attributed by the bile salt present in SIJ (Ruiz et al., 2013).

The survivability of free bacteria after 5 h of SIJ incubation was shown to be lower than encapsulated bacteria with and without inulin (Figure 2). Higher survivability found in encapsulated bacteria has demonstrated the positive effect of microencapsulation with alginate and chitosan coatings towards bile salt. The result is in agreement with Trabelsi et al. (2013) where the encapsulation of *L. plantarum* TN8 with calcium alginate and chitosan had improved their viability in bile salt condition as compared free bacteria were significant.

On the other hand, the survivability of encapsulated bacteria with inulin (78.66%) was found to be higher than encapsulated bacteria without inulin (74.97%) after 5 h of incubation in SIJ (Figure 2). This indicates that the addition of inulin had a positive impact ($p \leq 0.05$) on the viability of encapsulated *L. plantarum* 299v. Similar results were claimed by And & Kailasapathy (2005) that the encapsulation of *L. acidophilus* CSCC 2400 with prebiotic had enhanced their survivability under bile salt condition.

Furthermore, the viability of free cells, encapsulated *L. plantarum* 299v with and without inulin showed higher reduction during gastric treatment than intestinal treatment (Table 3). The result could agree with Sahadeva et al. (2011) who had reported better survivability of bacteria under bile condition than acidic condition. The study explained that the probiotic cells may be adapted to the stress adaptation mechanism after exposed to the acidic stress prior to bile stress. Hence, minimising the relative stress in the intestinal environment as compared to the gastric environment.

Free cells, encapsulated bacteria with and without inulin all remained high viability after sequential digestion. They had met the minimum requirement cell count of more than 10^7 CFU/g at the end of digestion (Siang et al., 2019). Similarly, *L. plantarum* 299v was claimed to be able to survive under physiological stressful environments such as acidic and high bile salt (Melgar-Lalanne et al., 2014). This had shown that *L. plantarum* 299v is an acid and bile salt tolerance strain.

3.3 Storage stability of free cells and microencapsulated *L. plantarum* 299v in roselle juice

After four (4) weeks of storage under room temperature, free cells in Roselle juice demonstrated turbid appearance with gas and changes in odour similar to alcohol as compared to the encapsulated probiotic. This showed that cell fermentation could have occurred in Roselle juice. Similar observations were reported by Peerajan et al. (2016) after incorporated free *L. paracasei* into *Phyllanthus emblica* fruit juice for fermentation. On the other hand, the encapsulated *L. plantarum* 299v with and without inulin did not display any changes in smell and turbidity after four (4) weeks of storage under room and refrigerator temperature. This suggests that encapsulation of probiotics had prevented cell fermentation in Roselle juice (Hernández-Barrueta et al., 2020).

Figure 3 presents the total viable counts of free bacteria, encapsulated *L. plantarum* 299v with and without inulin during the four (4) weeks storage in roselle juice at room (2 5 °C) and refrigerator (4 °C) temperatures. The declining trend in the viable cell count of three different *L. plantarum* 299v forms in roselle juice were observed over the four (4) weeks at both temperatures (Figure 3). The viability of free cells decreased by 57.3% from 10.63 log CFU/mL at week 0 to 4.54 log CFU/mL at week four (4) during storage at 25 °C. In weeks three (3) and four (4), the viable cell count of free cells was lower than the minimum requirement which is above 10^6 CFU/mL.
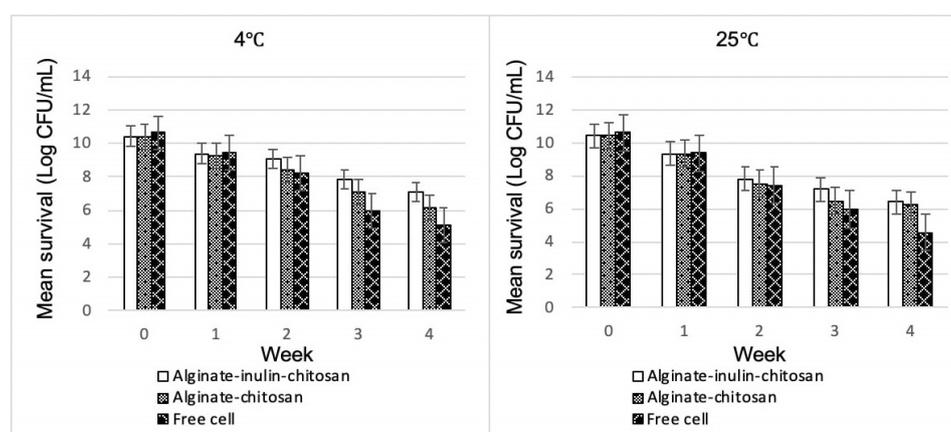
Figure 3. Survival of free and encapsulated *L. plantarum* 299v incorporated into roselle juice during storage at 4 °C and 25 °C. Error bars indicate the standard deviation of triplicate experiments.

On the other hand, the total viable cell counts of encapsulated *L. plantarum* 299v without inulin reduced by 40.2% from 10.42 log CFU/g at week 0 to 6.23 log CFU/g at week four (4); while the cell number of encapsulated *L. plantarum* 299v with inulin has reduced by 38.5% from 10.43 log CFU/g at week 0 to 6.41 log CFU/g at week four (4). Both encapsulated *L. plantarum* 299v with and without inulin showed a higher viable cell count as

compared to free cells. They achieved the minimum viable cell count requirement (>10^6 CFU/mL). This high viability was contributed by the microcapsule structure in encapsulated *L. plantarum* 299v which acted as a physical barrier protecting it from the acidic condition in the flower juice (Nami et al., 2020).

Furthermore, the viable cell count of encapsulated *L. plantarum* 299v with inulin was higher as compared to encapsulated *L. plantarum* 299v without inulin regarding weeks three (3) and four (4). The positive effect of inulin on the survivability of probiotics might be due to its role as a food source for probiotic during the storage period (Krasaekoopt & Watcharapoka, 2014). Gandomi et al. (2016) also demonstrated a similar result where inulin had a positive effect on *L. rhamnosus* GG during the 90 days storage in apple juice.

In Figure 3, results displayed that the viable cell count of three different forms of *L. plantarum* 299v in roselle juice decreased over the four (4) weeks. Encapsulated *L. plantarum* 299v with inulin had the highest average viable cell count at week four (4) which was 7.10 log CFU/g, followed by encapsulated *L. plantarum* 299v without inulin and free cells, which were 6.13 log CFU/g and 5.15 log CFU/mL, respectively. The result showed that the viable cell counts of encapsulated *L. plantarum* 299v with and without inulin in roselle juice were higher ($p \leq 0.05$) than free cells at 4 °C. This depicted the protective effect of microencapsulation towards the acidic environment of flower juice (Chaikham, 2015). The result concurs with Teanpaisan et al. (2015) in which the viability of probiotic in microencapsulated form was able to preserve at the level of 10^6 CFU/mL after eight (8) weeks of storage. Lai et al. (2020a) also found that encapsulated *L. plantarum* 299v remained at least 10^7 CFU/mL after four (4) weeks of storage in kuini juice at 4 °C; while free cells had decreased to 0 CFU/mL.

In addition, the total reduction of 32.3% was displayed for the viable cell counts of encapsulated bacteria with inulin at the end of week four (4), which was lesser than the encapsulated bacteria without inulin with a reduction of 41.2%. The higher viability found in encapsulated bacteria with inulin over the storage period showed the positive effect of inulin on *L. plantarum* 299v cells. Similar trends were also displayed by the viability of encapsulated *L. plantarum* 299v stored in roselle juice at 25 °C. However, encapsulated *L. plantarum* 299v with inulin and free cells were found to have lower viable cell count in room temperature as compared to refrigerator temperature. This might be due to warm temperature had increased probiotic's metabolic activity and maturation, hence their viability was reduced over time (Mortazavian et al., 2007a).

In the present study, the viability of *L. plantarum* 299v reduced throughout the storage at both temperatures. However, encapsulated bacteria with and without inulin were still able to maintain high viable cell counts ($\geq$ 10^6 CFU/mL) after 4 weeks of storage. This showed that co-extrusion encapsulation and the addition of inulin had successfully improved the viability of *L. plantarum* 299v in roselle juice, especially at refrigerator temperature.

4 Conclusion

*L. plantarum 299*v microbeads produced using 2% (w/v) of calcium chloride and 3% (w/v) of inulin by co-extrusion, were spherical in shape, with a mean diameter of 685.27 μm and microencapsulation efficiency of 95%. Furthermore, encapsulated *L. plantarum* 299v with inulin also displayed better survival rate (>10^7 CFU/mL) than free cells and encapsulated *L. plantarum* 299v without inulin under simulated gastrointestinal conditions, and after four weeks of storage in roselle juice at refrigerator temperature. This indicates that roselle juice is a potential probiotic carrier for the encapsulated *L. plantarum* 299v with calcium alginate, chitosan and inulin. Future studies could further investigate on the health benefits and sensory properties of the encapsulated *L. plantarum* 299v-inulin-roselle juice.

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