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Microencapsulation and Delivery of *Pediococcus acidilactici* 3G3 in Citrate-crosslinked Chitosan/Polyaniline Composite

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ABSTRACT

With the emergence of consciousness to the benefits of probiotic microorganisms, their use has created an enormous market worldwide. Numerous methods and strategies are developing, such as microencapsulation, that can protect probiotic bacteria against harsh conditions in the stomach and thus favor their survival in the gut. This study determined the potential microencapsulation and delivery of *Pediococcus acidilactici* 3G3 in chitosan/polyaniline (CS/PANI) composite, wherein ionic gelation through extrusion method of microencapsulation was used. The optimal CS/PANI ratio that could encapsulate *Pediococcus acidilactici* 3G3 is 3% CS / 0.5% PANI in 1% sodium citrate. The number of probiotic cells that can be entrapped per microbead is 6.21±0.08 log CFU. The cell release of entrapped probiotics in simulated gastric fluid due to the immediate swelling of the CS/PANI microbeads has a cumulative range of 10^5-10^6 CFU, which is considerably higher than expected. At the end of the exposure to the simulated gastric and intestinal fluid (3 h), the entrapped 10^6 to 10^7 CFU probiotics were released but only 10^5 CFU remained viable. It was found that the cell viability of microencapsulated (46.23%±0.02%) probiotics is significantly lower compared to the free cells (69.64%±0.04%) after 30 days of storage at 4 ºC. On the contrary, cell viability on the 30th day of storage at room temperature was significantly higher ($p<0.05$) for microencapsulated (33.10%±0.13%) than free cells (25.92%±0.16%). In conclusion, microencapsulation of *Pediococcus acidilactici* 3G3 can be a considerable tool to achieve higher cell viability both during gastrointestinal delivery and storing at room temperature.

INTRODUCTION

When present in sufficient amounts in the digestive tract of the host, probiotics are viable bacteria that may provide health benefits (Shori, 2017). They can be introduced to human or animal through foods as mono- or mixed culture of live microorganisms. Primarily, they beneficially affect the host by improving the properties of indigenous microflora in the intestine (Espitia *et al*., 2016). These beneficial effects include competing against pathogenic microorganisms that can inhabit the intestine, lactose utilization improvement, managing food-induced allergy, controlling serum cholesterol level, and lowering the risk of colon cancer (Markowiak & Ślizewska, 2017). In line with this, numerous methods and strategies are being developed, such as microencapsulation of probiotic microorganisms in foods and drinks, to improve digestion and health.

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Microencapsulation is a process of incorporating functional materials (e.g., probiotics) into the encapsulating materials to form microcapsules (Espitia *et al*., 2016). This technique was designed to employ protection in functional compounds or materials such as live microorganisms or therapeutic live cells as they are introduced in the stomach's high acidity and churning movement and deliver them in adequate amounts in the colon (Ariful *et al*., 2010). Generally, the encapsulation materials to use are labeled as safe when mixed in foods. Hence, food-grade carbohydrate polymers such as starch, alginate, chitosan, gelatin, pectin, cellulose, carrageenan, and xanthan gum are widely utilized to form microcapsules (Shori, 2017).

Chitosan (CS) is a polycationic carbohydrate polymer derived from the partial alkaline deacetylation of chitin, one of the most abundant carbohydrate polymers in nature. It is the biomolecule mainly composed of the exoskeleton of crustaceans such as crabs and shrimps. (Prabaharan, 2008). CS is broadly known for its ability to form films, biocompatibility, chemical inertness, and high mechanical strength. Additionally, it can be obtained at a low cost, which makes it a promising natural carbohydrate polymer to be used variously (Yavuz *et al*., 2009). Unlike any other polysaccharides that are neutral or anionic at low pH, CS is distinctive because it possessed a positive charge when dissolved in an acidic environment that enables it to form a neutral complex that has a multilayer structure when reacted to anionic natural or synthetic polymers (Cheung *et al*., 2015). A considerable amount of research has been conducted to use CS as a drug delivery vehicle, especially for the treatment of various diseases associated with the colon like ulcerative colitis and Crohn's disease (Iglesias *et al*., 2019), and improve high fat/high-cholesterol diet-induced oxidative stress, lipid profile, and insulin sensitivity (Bahijri *et al*., 2017). Modifying CS to enhance its ability to interact with other polymers and intensify its capability to load functional materials and its release is of great interest nowadays (Prabaharan, 2008). One of the chemical modifications in CS is forming composite, hydrogel, or film with polyaniline to enhance its encapsulation and delivery activity.

Polyaniline (PANI), on the other hand, is a conductive polymer that is known to have lightweight and readily to synthesize. It is a type of polymer that exhibits high conductivity and low operational voltage. (Yavuz *et al*., 2009). The most interesting ability of PANI is its rapid pH switching capability. This enables it to be protonated and deprotonated upon the addition of acid or base, respectively. However, like other conductive polymers, it is insoluble in common solvents and exhibits infusibility (because it decomposes before melting). Other polymers with known considerable mechanical (e.g., good compactness) and physical properties are grafted with PANI to overcome these limitations. The PANI salts are highly soluble in some mixtures between organic common polar solvents and water. Uses in biosensors, switchable membranes, rechargeable batteries, electronic devices, and anticorrosive coatings are its potential applications. (Thanpitcha *et al*., 2006). PANI is a non-cytotoxic and biocompatible polymer that enables various applications in the biomedical field (Ivanov *et al*., 2002). Accordingly, it has been used in skeletal and cardiac muscle regeneration, glucose biosensors for bioimplanted devices, and engineering of never tissues.

Nonetheless, further studies must be conducted to explain the fate and behavior of PANI inside the human body to design some novel materials that can contribute in controlling diseases (Shahadat *et al*., 2017). Also, PANI is used in tandem with bacterial cellulose (BC), and some studies have successfully formed bacterial and fungal biofilms. The composite that can be formed from PANI and BC or fungal biofilm can be used as potential chemical sensors, biosensors, separation membranes, display devices, anti-corrosion coatings, and biomedical devices (Mikušová *et al*., 2017). Although many studies used PANI and bacterial parts in combination, no reported studies explore the direct use of PANI with probiotics, which this study aims to explore.

In recent years, numerous researches have proven that CS and PANI, when used separately, are two polymers that have effectively and efficiently contributed to the advancement of medicine in where they can be appropriately utilized. In line with this, this study combined CS and PANI and tested their potential microencapsulation and delivery of *Pediococcus acidilactici* 3G3, a bacteriocin-producing (Moghadam & Elegado, 2017) lactic acid bacteria (LAB) considered to have probiotic properties because of its strong adherence in the duodenum and middle colon and exhibited several potential biomedical applications such as preventing hypercholesterolemia (B. Banaay *et al*., 2013) and reducing body weight of diet-induced obese female Swiss mice (Parungao et al., 2009). Thus, when included in drinks, would potentially be taken as a food supplement in pill form.

MATERIALS AND METHODS

Materials

The materials and reagents used in this study were the following: chitosan low molecular weight (Merck, Germany), polyaniline emeraldine base (Merck, Germany), acetic acid, calcium chloride, sodium citrate, sodium chloride, phosphate buffered saline, de Man-Rogasa-Sharpe (MRS) broth and agar, peptone water, normal saline solution (NSS), pepsin from porcine gastric mucosa (Merck, Germany), pancreatin from porcine pancreas (Merck, Germany), α – amylase, trehalose, and gram staining solutions (crystal violet, safranine, and ethanol).

Preparation of hydrocolloid mixture

An amount of 0.30 g of chitosan (low molecular weight [LMW; 50–190 kDa]) was dissolved in 7 ml 1% acetic acid (standard solvent), pH of 4.5 (suited for the growth of *P. acidilactici* 3G3, by agitating to obtain a 3% solution. Since CS is a polycationic polymer, 0.5 M sodium hydroxide (NaOH) and deionized water were added to adjust the solution to the desired pH and bring it to the final volume. The solution was stirred in a magnetic stirrer for 15 min until chitosan has been fully dissolved.

The emeraldine base form of PANI with weights 0.05 g, 0.1 g, and 0.2 g was dissolved in 3 ml of 1% acetic acid, pH of 4.5 (suited for the growth of *P. acidilactici* 3G3), to obtain 0.5%, 1.0%, and 2% (w/w) solutions, respectively. The solution was stirred in a magnetic stirrer for 10 mins until PANI have been fully dissolved.

Optimization of microbeads formation

$CaCl_2$ as the crosslinking solution

Different concentrations of CS (3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, and 6.5%) hydrocolloid mixture with the same volume (7 ml) were prepared. In each concentration of CS, 3 ml of PANI was added and agitated continuously for 1 hr to allow ionic interaction. Consequently, each of different concentrations of CS with PANI mixture was allowed to free-fall using a manually operated syringe with a 0.7 mm cannula (flow rate 1.0 ml/min) to form microbeads in 50 ml of 0.05 M, 0.5 M, and 1.0 M $CaCl_2$ solution (gelling solution) and left to harden for 30 min.

Sodium citrate as the crosslinking solution

Three ml of PANI solution was added dropwise to 7 ml of CS using a syringe and a pump. The CS solution was agitated continuously for 1hr to allow ionic interaction. The mixture was then allowed to free-fall using a manually operated syringe with

a 0.7 mm cannula (flow rate 1.0 ml/min) to form microbeads in 50 ml of 0.5% and 1.0% sodium citrate (gelling solution) independently and left to harden for 30 min. The microbeads were then decanted, washed twice with ultrapure water, and then freeze-dried for 24 h. The size of the wet microbeads was measured using a vernier caliper, and scanning electron microscopy, SEM (Hitachi, SUI5IO, Tokyo Japan) was used to observe the surface morphology of freeze-dried microbeads at 32x, 50x, 1000x, 3000x, and 4,500x magnification.

Measurement of the degree of swelling

The degree of swelling of the unloaded microbeads was measured to determine if the sodium citrate gelling solution used has crosslinked the CS and PANI. The swelling of 10 freeze-dried microbeads in physiological media was determined by submerging them in 0.5% w/v NaCl and adjusting the pH to 2, 3, 4, and 5 with 1 M HCl, a suitable pH range for mimicking the conditions in the nonfasted stomach. The weight of the microbeads was measured every 30 min for 180 min. After allowing for swelling, beads were washed twice with deionized (DI) water, blotted in sterile tissue, and weighed accurately. The lower the degree of swelling, the higher the crosslinking of the two polymers. The degree of swelling was calculated using the following formula:

$$\mathrm{SD} = \frac{Wt}{Wo}$$

Where:

Wo = weight of microcapsules before swelling
Wt = weight of microcapsules after swelling

Microencapsulation of *Pediococcus acidilactici* 3G3

Inoculation

One milliliter of *P. acidilactici* 3G3 cells was inoculated into 100 ml de Man-Rogosa-Sharpe (MRS) broth. The composition of MRS broth includes the following: dextrose 20 g L^{-1}, proteose peptone 10 g L^{-1}, beef extract 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium acetate 5g L^{-1}, ammonium citrate 2 g L^{-1}, disodium phosphate 2 g L^{-1}, tween 80 1.0 g L^{-1}, magnesium sulfate 0.10 g L^{-1}, manganese sulfate 0.050 g L^{-1} with pH 6.5 ± 0.2 at 25ºC (Kunmani *et al.,* 2011). Then, the inoculum was incubated at room temperature in a candle jar for 24 h. Actively growing cells were recovered from MRS broth by centrifuging (HERMLE, Z326K, Germany) at 4,152.96 x g (6000 rpm) at 15 ºC for 15 min. The cells were recovered by adding 1 ml of 32% trehalose solution and then added to 1 ml of 5% gelled starch solution.

Formation of loaded microbeads

The extrusion method was developed using the method described by Liliana & Vladimir (2013) with some modifications including the use of cryoprotectant (Tymczyszyn et al., 2007) and sodium citrate (Shu et al., 2001) as crosslinking agent. Briefly, the *P. acidilactici* 3G3 cell suspension was dropwise mixed with a CS/PANI mixture. The mixture was gently stirred continuously for 1 h using a magnetic stirrer at 200 rpm. The mixture was then allowed to free-fall using a manually operated syringe with a 0.7 mm cannula (flow rate 1.0 ml/min) to form microbeads in 50 ml of 1% sodium citrate (gelling solution) and left to harden for 30 min. The microbeads were then decanted, washed twice with ultrapure water, placed in a sterile petri dish, and blotted with sterile tissue to remove excess water. The wet microbeads were set at -20 ºC for 2 h and freeze-dried (CHRIST, Germany) at 37 ºC for 24 h. The microbeads would contain the minimum required probiotic concentration (10^6-10^7 CFU) to have a therapeutic effect**.** Hence, the number of viable cells encapsulated in the microbeads was quantified in log CFU ml^{-1} by individually homogenizing ten microbeads in 1 ml of peptone water using a homogenizer to disintegrate the CS/PANI matrixes resulting in cell liberation. Then, 100 µl of peptone containing the homogenized microbeads was taken and diluted from 10^{-1} – 10^{-5}, 10µl of each dilution was taken to enumerate the viable cells using the drop plate method**.** The plates were prepared in duplicates and incubated for 48 h in a candle jar at room temperature.

Using the formula, $CFU/ml = (no.\ of\ colonies)\ (100)(dilution\ factor)$ the number of probiotic cells entrapped inside a single microbead was quantified. The average CFU ml^{-1} of the three trials was computed concurrently with the standard deviation.

Microscopic examination of the bacterial cells from the representative colony grown from the homogenized microbeads confirms the identity of the encapsulated bacterial cells. This confirmatory procedure was carried out by subjecting the representative colony grown from the homogenized microbeads to gram-staining and viewed under the microscope (Zeiss, Primo Star Microscope, Germany) with 100x magnification.

The size of the wet microbeads was measured using a vernier caliper and scanning electron microscopy (SEM) (Hitachi, SIU5IO, Tokyo, Japan) was used to observe the surface morphology of freeze-dried microbeads at 32x, 50x, 1000x, 3000x, and 4,500x magnification.

Cell release

Figure 3.2 illustrates the different mechanisms of how entrapped probiotic cells will be released from the microbeads. The method chosen was based on the study of Kanmani *et al*., (2011) with slight modifications. The cell release time-course of the loaded microbeads was tested in prewarmed simulated gastric fluid (SGF), which was prepared by lowering the pH of 0.5% NaCl solution to pH 2 and adding pepsin at a ratio of 10 U/ml and in simulated intestinal fluid (SIF), composed of 1x PBS containing NaCl 8 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 1.44 g L^{-1}, KH_2PO_4 0.245 g L^{-1} with pH adjusted to 6.8 and sterilized by autoclaving at 120 ºC for 2 h, 3 mg/ml pancreatin and 16U/ml of α -amylase. Since pancreatin contains three different enzymes – lipase, protease, and amylase (Kuhn et al., 2007), to ensure the adequacy of α- amylase that enhances the digestion of chitosan (Wu, 2011), an additional 16 U/ml of α- amylase was introduced. Figure 3.3 shows the cell release experiment procedure. The cell release was evaluated by subjecting 1 ml of wet microbeads in 9 ml of SGF and SIF at 0.315 x g (150 rpm), 37 ºC, in a shaking incubator (Witeg, WIS-20, Korea) for 60 min in SGF and 120 min in SIF, which mimics the normal stomach transit of healthy human adults (Cheow, and Hadinoto, 2013). One hundred µl aliquot of SGF/SIF solution, where the microbeads were subjected, was taken every 30 min and diluted to 10^{-5}. Then, 10 µl of each dilution was taken to enumerate the viable cells using the drop plate method**.** The exact SGF and SIF solution was added after each sample was taken to avoid errors in calculations. The procedure was done in 3 trials, and each trial has 3 replicates. The plates were prepared in duplicates and incubated for 48 h in a candle jar at room temperature.

Another set-up for SGIF was carried out. After 60 min of subjecting the 1 ml microbeads, SGF was removed and replaced by 9 ml of SIF and incubated in a shaking incubator using the same condition for 120 min. For this set-up, 100 µl aliquot was taken and diluted from 10^{-1} – 10^{-5}, 10µl of each dilution was taken to enumerate the viable cells using the drop plate method**.** The exact amount was added after each sample was taken to avoid errors in calculations. The procedure was done in 3 trials, and each trial has 3 replicates. The plates were prepared in duplicates and incubated for 48 h in a candle jar at room temperature.

For comparison, free *P. acidilactici* 3G3 (non-encapsulated) suspended in normal saline solution and freeze-dried *P. acidilactici* 3G3 with 32% trehalose were examined by subjecting 1 ml of *P. acidilactici* 3G3 suspended in normal saline solution and 1 g of freeze-dried *P.acidilactici* 3G3 in 9 ml of SGF and SIF solution using the same procedure.

Shelf-life determination

The cell viability for long-term storage was determine using the method described by Kanmani *et al*., (2011) and Strasser *et al*., (2009) with some modification. Firstly, five grams of freeze-dried loaded microcapsules were placed in well-sealed vials and subjected to controlled temperatures, 4 ºC, and 30 ºC. Bacterial viability was determined on the 15th and 30th days after microencapsulation by homogenizing ten freeze-dried microbeads, individually, in 1 ml of peptone water using a homogenizer to disintegrate the CS/PANI matrixes resulting in cell liberation. After which, 100 µl of peptone containing the homogenized microbeads was taken and diluted to 10^{-5}, 10 µl of each dilution was taken to enumerate the viable cells using the drop plate method**.** The plates were prepared in duplicate and incubated for 48 h in a candle jar at room temperature. The percent cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{Log\left(\frac{CFU}{ml}\right) after\ drying}{Log\left(\frac{CFU}{ml}\right) before\ drying} x\ 100\%$$

The freeze-dried *P. acidilactici* 3G3 with cryoprotectant (trehalose) was used as a control. According to Nag and Das (2013), cryoprotective compounds during freeze-drying have been found helpful in maintaining the better viability of cells during prolonged storage. The storage viability, however, is highly related to the temperature and decreases at elevated storage temperatures.

RESULTS AND DISCUSSION

Optimization of microbeads formation

Calcium Chloride ($CaCl_2$) as a crosslinking solution

Initial attempts in forming microbeads used the same concentration of PANI with varying concentrations of CS and $CaCl_2$. The microbeads formed with increasing concentration of CS. However, with a higher concentration of CS, extruding the CS/PANI hydrocolloid in a small cannula was challenging and a bigger cannula was necessary. The bigger cannula formed bigger beads that defied the range considered "micro," as shown in Figure 1 (A). Moreover, with higher CS concentration, the viscosity of the CS/PANI mixture also increased, which made the mixture challenging to be homogenized. To improve the homogeneity of the mixture, the concentration of $CaCl_2$, the crosslinking solution, was also optimized. The CS concentration to form preeminent beads is 4.5% extruded in 1.0 M $CaCl_2$.

However, the beads formed were not on the microscale and not rigid enough. This could be due to the lack of crosslinking and repelling charges of CS, PANI, and Ca^{2+} in the crosslinking solution. Since CS and PANI in acidic media are protonated and the $CaCl_2$ dissociates in the solution as Ca^{2+} and Cl^-, only the chloride anion making the positive charges neutral shown in figure 1 (B). Hence, the rigidity of microbeads will only be achieved if the concentration of $CaCl_2$ is further increased but does not guarantee rigidity to the formed microbeads.

Sodium citrate as a crosslinking solution

Both 0.5% and 1% sodium citrate were used as a crosslinking solution, but the CS/PANI composite was successfully formed into microbeads in 1% sodium citrate, as shown in Figure 2 (A), and therefore the microbeads used for the succeeding analyses. The wet microbeads were rigidly round with black color, which corresponds to the physical appearance of PANI and has an average size of 1.92 mm ± 0.18mm. On the other hand, when the microbeads were freeze-dried, the grayish to the whitish color of CS is more visible. The microbeads have an average size of 1.54 mm ± 0.12mm.

The rigid structure of the microbead is attributed to the electrostatic interaction that exists between protonated CS and deprotonated citrate (Rana *et al.,* 2004), as well as the protonated PANI showed in figure 2 (B). Since CS is a polycationic polymer and can be protonated in an acidic medium, particularly acetic acid (Wu *et al*., 2014), theoretically, its protonated amino group is attracted to the deprotonated sodium citrate through electrostatic attraction (Rana *et al*., 2005). Moreover, PANI has a pH switching ability that enables it to be protonated in an acidic medium like acetic acid and deprotonated quickly upon adding a basic substance (Thanpitcha *et al*., 2006). Similarly, its protonated amino group is attracted to the deprotonated sodium citrate by electrostatic attraction. As shown in figure 4.4, the exposed protonated amino group of CS and PANI is electrostatically attracted to the deprotonated carboxylate group (COO-) of sodium citrate, forming ionic bonds. In addition, the possible existence of hydrogen bonds among the exposed nitrogen (N), oxygen (O), and hydrogen (H) are considered in the hypothesized structure.

The difference in the size of the chloride anion and citrate also contributed to the level of ionic interaction. Because chloride anion has a smaller size, it is adequate and incapable of forming crosslinking action between CS and PANI, while citrate apparently is a bigger anionic molecule, thus, enables it to form crosslinking effect in CS/PANI composite.

Swelling Degree

This experiment shows the swelling behavior of sodium citrate crosslinked CS/PANI microbead through simulation in different pH that it can encounter as it transits from the stomach to the intestine. Consequently, the effect of adjusting pH within the stomach on the microcapsules was demonstrated using a variety of pHs. Swelling characteristics would suggest that if there is a decrease in the degree of swelling, the microbead disintegrates and would release its load before dissolution. Along with disintegration, Cook *et al*., 2011 mentioned that diffusion via the pores in the polymer network is another putative release mechanism.

Figure 3 shows almost the same swelling for pH 4 and 5 while in pH 3, the microbeads showed continuous swelling until the end of the 180 min exposure. In pH 2, on the other hand, the microbeads showed drastic swelling after 30 mins of exposure and showed disintegration after that time point. As time progressed, the microbeads showed a decrease in swelling that resulted in total disintegration at 120 min. The decrease in swelling indicated disintegration of the citrate-crosslinked CS/PANI matrix. According to Rana *et al*., 2005, sodium citrate crosslinked chitosan produces very weak ionic bonds below pH 4.5 due to an increase in protonation and above pH 6.5 due to the deprotonation of sodium citrate. The swelling behavior of sodium citrate crosslinked CS/PANI composite implies that the microbeads are damaged at pH 2 beyond 60 min exposure, which might release the encapsulated probiotics prematurely. Hence, the microbeads should transit from the stomach down to the intestine. After 60 min it was subjected to pH 2 to ensure that the probiotics would be released in the intestine.

Microencapsulation of *Pediococcus acidilactici* 3G3

Enumeration of Entrapped probiotic cells

The successful formation of CS/PANI microbeads resulted in

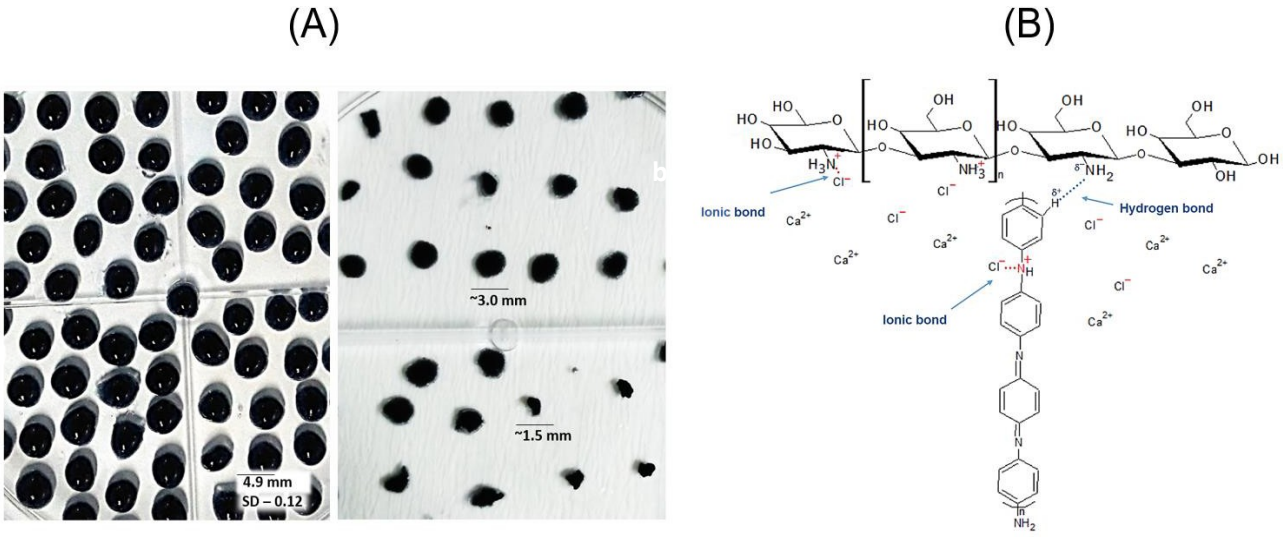

Figure 1: (A) 4.5% CS/0.5% PANI ratio: wet microbead (left) freeze dried (right). (B) Schematic representation of hypothesized ionic interaction of chloride ions in $CaCl_2$ and cationic moieties of CS/PANI composite.

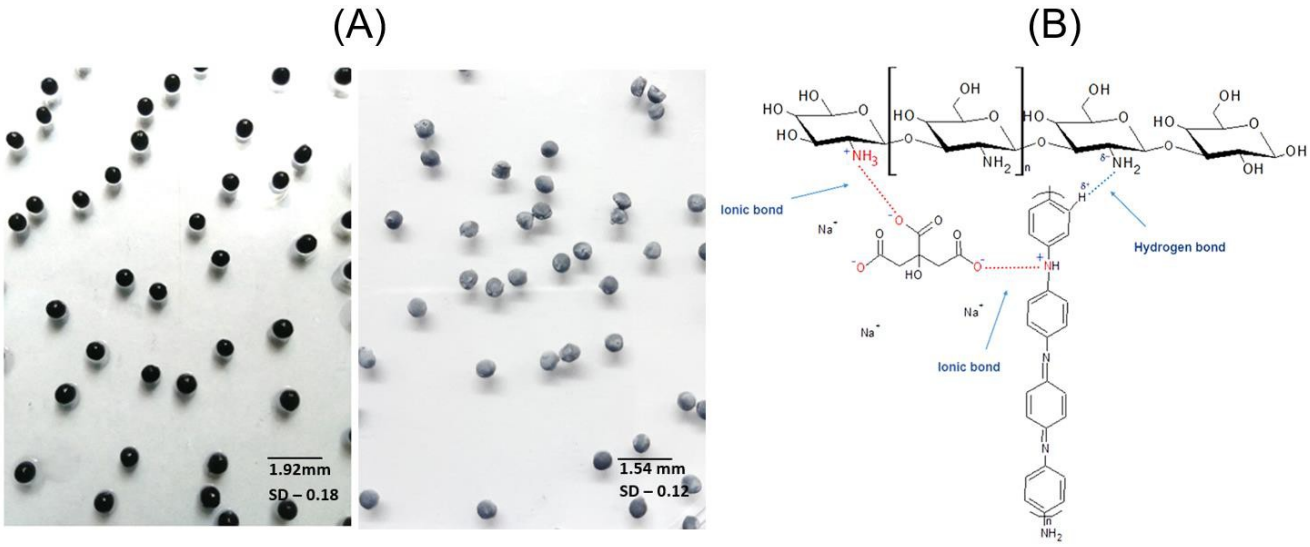

Figure 2: (A) 3% CS / 0.5% PANI in 1% sodium citrate: Optimized citrate- crosslinked CS/PANI microbeads; Wet microbeads (left) and freeze-dried microbeads (right). (B) Hypothesized structure of citrate-crosslinked CS/PANI composite.

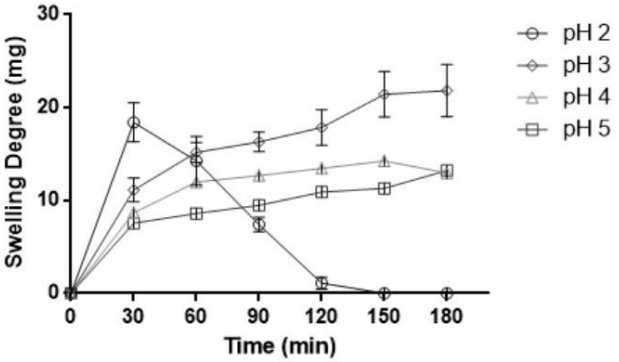

Figure 3: Swelling degree (net weight change) at different pH values. Data presented as mean±SD of 3 trials, n=3 per replicate.

the encapsulation of *P. acidilactici* 3G3, as illustrated in Figure 4. Every single microbead contained an average of 6.21±0.08 log CFU of entrapped probiotic cells. The identity of viable cells was verified through microscopic examination.

For probiotics to confer a therapeutic effect to humans, the recommended dose is 10^8 to 10^{11} CFU per serving per day (Raghuwanshi *et al*., 2018), while the probiotic concentration in products should have a minimum concentration of 10^6 CFU ml- or gram (Kechagia *et al*., 2013) as recommended by the international dairy federation (Halim *et al*., 2017). Thus, the number of encapsulated *P. acidilactici* 3G3 fits the required concentration for this probiotic to confer a positive effect on humans.

Surface morphology

A closer look at the CS/PANI freeze-dried microbead through a scanning electron microscope (SEM) reveals a spherical shape with a rough outer surface (Figure 4 (A)) (Cook *et al*., 2011). The morphology of the cracked freeze-dried microbead (Figure 4 (B)) shows that the inner surface of the microbead contains internal cavities (Figure 4 (C)) where the entrapped *P. acidilatici* 3G3 can be seen. The coccus-shaped *P. acidilactici* 3G3 cells were homogeneously embedded and trapped inside the CS/PANI microbead, as shown in the photomicrograph (Figure 4 (D)).

The SEM analyses revealed the spongy pores morphology of CS/PANI composite like the findings of H. Zhao *et al*., 2013 and Gicheva *et al*., 2012 that used CS to synthesize microspheres. The internal cavities throughout the microbeads are created by ice crystal formation in the interior of the CS hydrogel beads,

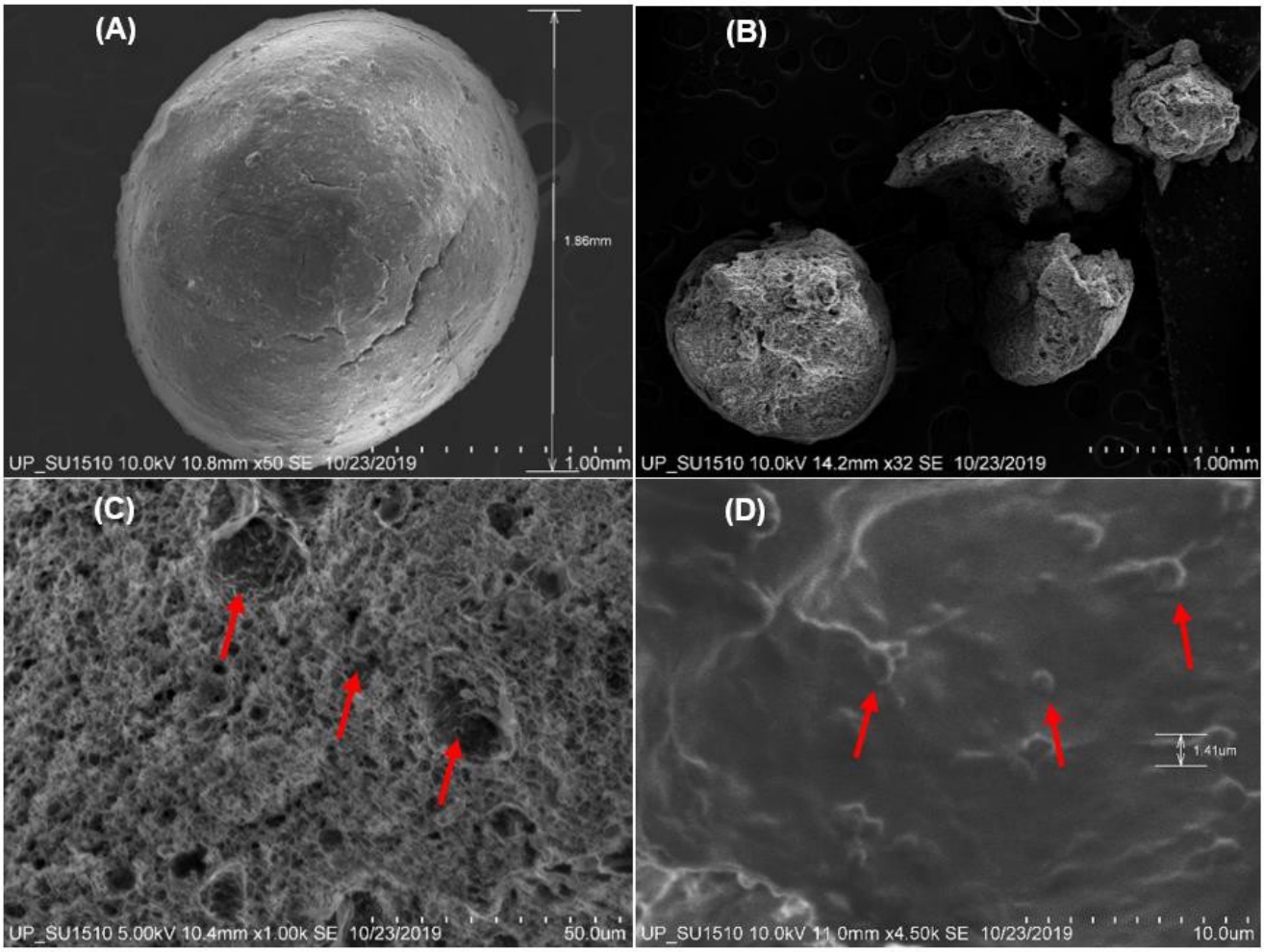

Figure 4: SEM images of (A) whole microbead at 50x (B) cracked microbead at 32x (C) inner surface showing internal cavities at 1000x (D) cavity surface where *P.acidilactici* 3G3 cells are embedded at 4,500x magnification.

which is induced by the highly one-dimensional character of the temperature gradients initiated during freezing (Ren *et al*., 2019). The freeze-drying method, however, is important to increase the shelf life (Silva *et al*., 2014) and preserve both the probiotics and microbeads.

Cell Release

The result of the cell release of microencapsulated *P. acilactici* 3G3 in terms of cell viability after 180 min exposure to both SGF and SIF was compared to the controls referred to in Figure 4.9 as "free cells". These free cells were non-encapsulated cells. There are two controls used for comparison, the free cells alone and freeze-dried free cells. The cell viability of freeze-dried free cells with added cryoprotectant – 32% trehalose, prior to lyophilization was also determined because the microbeads contained 1 ml of 32% trehalose. The trehalose was added to increase the cell viability of *P. acidilactici* 3G3 after freeze drying. The time that the controls were exposed to SGF and SIF were the same as microencapsulated. However, they are exposed independently.

The data in figure 5 regarding the cell release of microencapsulated probiotics show a significant sudden release of probiotic cells about 2.08%, equivalent to 10^5 CFU of probiotic concentration, in simulated gastric fluid (SGF) immediately after the microbeads were exposed to it. The observed relative cell release in the next incubation period has significantly increased to 40.16%, equivalent to 10^6 CFU. Hence, this indicates that the microbeads have already swelled, resulting in the release of the entrapped probiotic cells. As the microbeads continue to swell in SGF, the cell release increased to 43.21% at 60 min. When the SGF was replaced with simulated intestinal fluid (SIF), the gradual dissolution of the microbeads took place, resulting in a further cell released of 46.04% and constantly increase through time. At 180 min, the remaining entrapped probiotic cells were released as the microbeads have been fully dissolved, which suggests that 100% of the entrapped probiotic cells having a concentration of 10^7 CFU were released. However, data showed that 10^5 CFU were only viable at the end of the exposure.

While the survival of the probiotic-free cells in SGF dramatically decreased from 100% to 35.89% after 30 min exposure and dropped down to 0% after 60 min exposure. In SIF, however, the gradual decrease in percent survivability was observed from the start of the exposure until it reached 0% survivability at 150 min exposure.

On the other hand, the exposure of freeze-dried probiotic-free cells in SGF showed no survival after 30 min exposure. And a rapid decrease in survivability after 30 min exposure in SIF having 29.1%, which in turn dropped down to 0% after 90 min.

Although the immediate release of probiotic cells is undesirable at 30 min after subjecting the microbeads in SGF (pH 2), it has already been predicted by the result of the swelling behavior of sodium citrate crosslinked CS/PANI composite. In the test for the swelling degree, the microbeads showed disintegration after the 30 min time point at pH 2 and swelled continuously at pH 3, 4, and 5 over time. This can be attributed to the attenuated ionic bond below pH 4.5 due to the increase in protonation of sodium citrate that makes it electrically neutral and therefore unable to

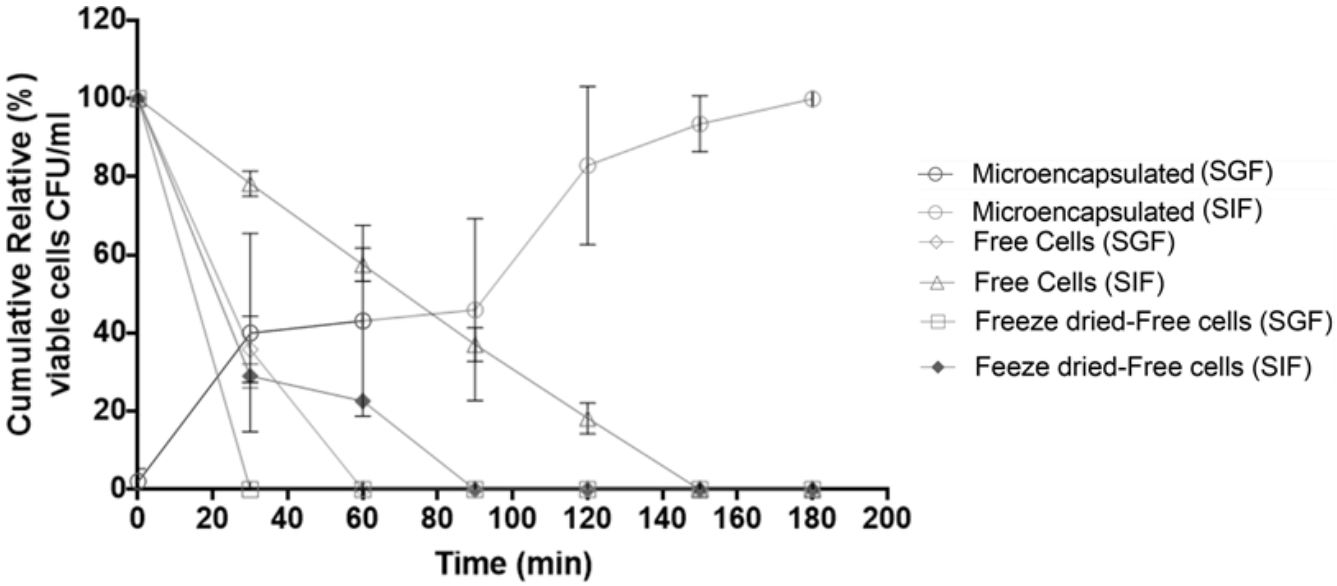

Figure 5: Cell release in terms of cell viability in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) of microencapsulated *P. acidilactici* 3G3 (with added 1ml of 32% trehalose) in the microbeads compared to the cell viability of free cells in normal saline solution (NSS) and freeze-dried free cells with 32% trehalose (cryoprotectant) as controls. Data presented as mean±SD of 3 trials, n=6 per time point of each trial.

participate in ionic bonding. On the other hand, when the pH is above 6.5, ionic bonding is also disrupted because the cationic moieties of the CS and PANI become deprotonated and electrically neutral. Hence, ionic bonding is unable to form.

However, the result of the cell release of microencapsulated probiotic cells in terms of cell viability is still significantly considerable compared to the free cells (non-encapsulated) subjected in SGF, which have zero survival at 60 min exposure, and free cells that have dried with trehalose, which have zero survival rate as well after 30 min exposure to the same medium. This result is supported by the study of *(Halim et al*., 2017), which showed that although *P. acidilactici is* resistant to an acidic environment, it cannot survive more prolonged exposure. Hence, microencapsulation is still needed to deliver substantial amounts of this probiotic in the intestine.

In the study of B. Banaay *et al*., (2013) though stated that *P. acidilactici* 3G3 showed strong adherence in the duodenum and middle colon, due to its capability to survive in extreme pH, temperature, and osmotic pressure ranges (Bhagat *et al*., 2020), the goal of encapsulation is to ensure the viability of probiotics in an adequate number of populations that can be delivered in the gastrointestinal (GI) tract during its transit to confer a therapeutic effect. Thus, microencapsulation provides stability and increases the number of viable *P. acidilactici* 3G3 cells during their transit in the GI tract (Papagianni & Anastasiadou, 2009).

Shelf-Life Determination

Upon microencapsulating probiotic bacteria to ensure better survival during transit in the gastrointestinal tract, it is essential to test how long they will be viable in the microbead. Encapsulated probiotics are freeze-dried to preserve both the probiotics and microbeads and increase their shelf life (Silva *et al*., 2014). However, the drying process results in extremely low to zero viability of probiotics due to ice crystal formation and high osmolality reflected in the freezing conditions (Halim *et al*., 2017). To address this hurdle, the use of cryoprotectant, trehalose, was considered. In the study made by (Nag & Das, 2013), it has been found that trehalose and lactose helped maintain better viability of cells after drying and during prolonged storage. While another study made by (De Giulio *et al*., 2005) concluded that the most efficient cryoprotectant in retaining bacterial viability was trehalose. That is by establishing hydrogen bonds around the polar and charged groups in phospholipid membranes and proteins of the bacterial cell upon drying. As a result, even in the absence of water, the probiotic original cell structure was preserved (Tymczyszyn *et al*., 2007).

The encapsulation process itself and homogenization of formed microbeads, although using a gentle approach to enumerate the entrapped probiotic cells, have resulted in a minimal loss of viability from 10^9 to 10^7 CFU ml$^-$ recovery concentration. While it has been observed that lyophilization has contributed a significant loss on the viability of the *P. acidilactici* 3G3 in both microencapsulated and free cells with trehalose after it has been subjected to freeze-drying, having recovery percent viability of 82.69%±0.18% for microencapsulated and 87.52%±0.03% for free cells with trehalose, results denoted that the viability of free cells is statistically higher ($p<0.05$) than microencapsulated cells. The survival of probiotics during storage is influenced by the composition of encapsulating materials and storage environment, particularly the storage temperature and the water activity (H. Zhao *et al*., 2013). Hence, during storage at 4 ºC, the mean of 3 independent trials presented in Figure 6 showed that on the 15th day, free cells have decreased to 76.40%±0.03%, while microencapsulated cells significantly decreased by 68.60%±0.04%. The viability values continuously dropped to 69.64%±0.04% (free cells) and 46.23%±0.02% (microencapsulated) on the 30th day. Thus, results showed that the percent cell viability of microencapsulated *P. acidiliactici* 3G3 decreased by almost 50% at day 30. On the contrary, the percent cell viability of free cells remained statistically higher.

On the other hand, the data at room temperature storage presented in Figure 6 depicts a significant decrease in the viability of both free cells and microencapsulated on the 15th day. The reduction in the percent viability of *P. acidilactici* 3G3 is more than half on day 0; from both more than 80%, the viability went down to 42.41%±0.04% for free cells and 40.71%±0.06% for microencapsulated. On the contrary, the 30th day at room temperature storage showed opposite findings for free cells (25.92%±0.16%) and microencapsulated (33.10%±0.13%).

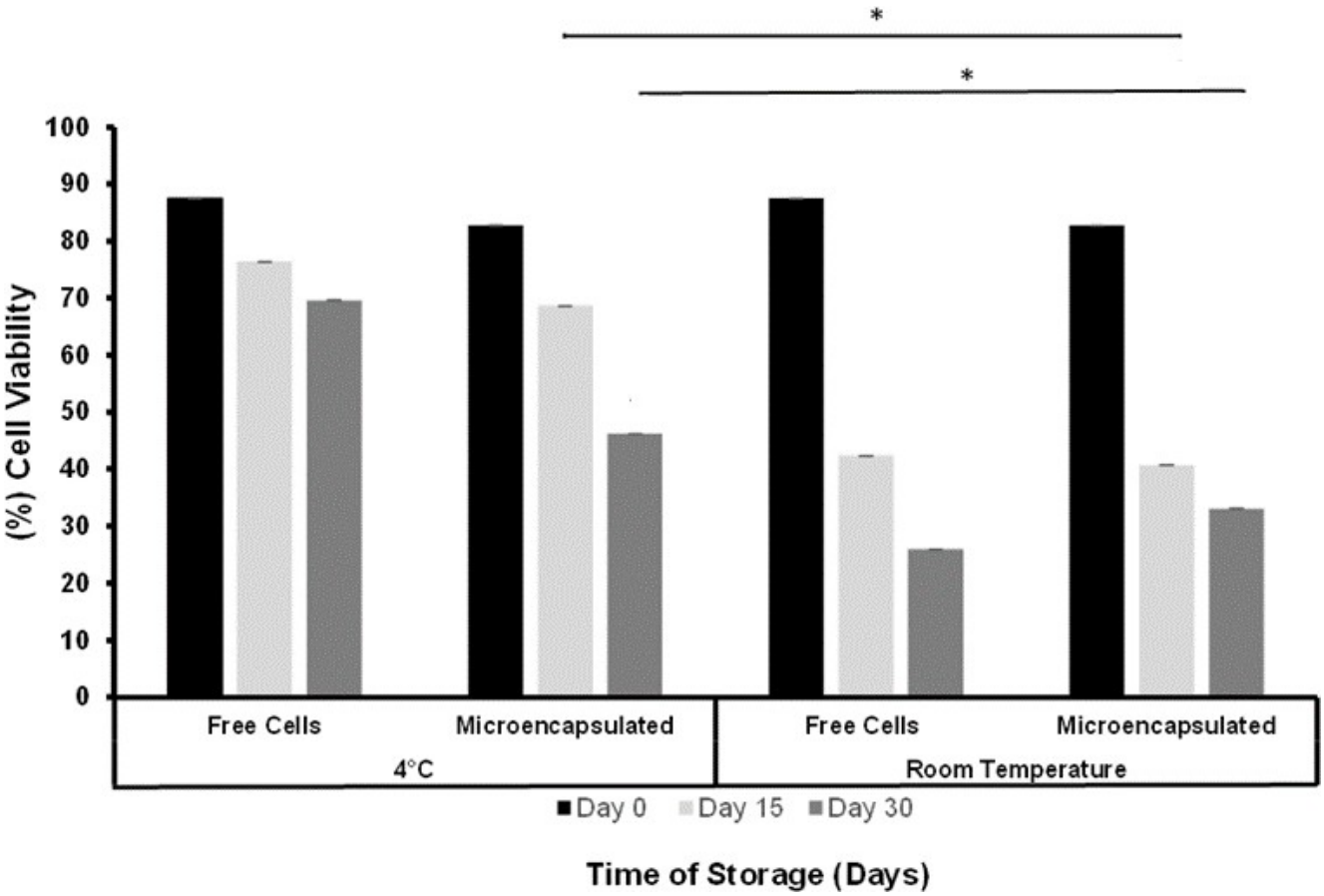

Figure 6: Shelf-life determination of microencapsulated *P. acidilactici* 3G3 with added 1ml of 32% trehalose in the microbeads and freeze-dried free *P. acidilactici* 3G3 cells with 32% trehalose (cryoprotectant) as a control. The cell viability was determined at 4ºC and room temperature (28°C) storage for 30 days. (*) represents statistical significance (p<0.05)—data presented in triplicate, n=3 per replicate.

The data also showed that the percent viability of microencapsulated *P. acidilactici* 3G3 on the 30th day at 4 ºC is significantly higher ($p<0.05$) than at room temperature. As stated by (Arepally *et al*., 2020) and (Dianawati *et al*., 2016), storage at 4 ºC has always been proven effective in prolonging the shelf life of probiotic bacteria by reducing the rate of detrimental chemical reactions. Moreover, the stability of probiotics decreases proportionally with the increasing storage temperature alone. When freeze-dried, the decrease of viable cell number during subsequent storage can be attributed to lipid oxidation (Savedboworn *et al*., 2019), and protein denaturation results in the destruction of probiotic cell macromolecules (Arepally *et al*., 2020).

Storage environment, particularly temperature, is a significant contributing factor in achieving and maintaining a high percentage of bacterial viability after the microencapsulation process. While the storage of probiotics at 4 ºC has proven effective in maintaining high viability, probiotic bacteria that are stored in the freezer necessitate significant handling and storage expenses, in addition to the increased danger associated with thawing (Dianawati *et al*., 2016). As a result, this increases the cost of transportation and storage. Aside from the convenience it brings, storage at room temperature reduces distribution and transportation costs (M. Zhao *et al*., 2018). However, probiotic life during storage at room temperature is affected by the type of coating materials used, water activity (a_w), humidity levels, and glass transition (Dianawati *et al*., 2016). The type of the coating materials dramatically affects the probiotics as it should protect the cells from environmental stresses (Halim *et al.,* 2017). Water activity, on the other hand, involves water accessibility for chemical reactions or the growth of microorganisms. Conversely, glass transitions strongly affect the rates of chemical reactions in reduced-moisture solid systems (Bell, 1995) or an amorphous material (Dianawati *et al*., 2016) like the CS/PANI microbeads. This glass transition happens when the hard, relatively brittle, and glassy state (caused by drying) of the material turned into a rubbery or viscous state due to the increase in temperature (Dyre, 2006). Hence, this may cause changes in the physical state (Dianawati *et al*., 2016) of the microbeads and negatively affects the viability of the probiotic cells in terms of compatibility.

CONCLUSIONS

The results obtained have demonstrated that CS/PANI composite was successfully formed into microcapsules and capable of probiotic cell entrapment. The optimal CS/PANI concentration ratio using 1 M $CaCl_2$ as crosslinking agent is 4.5%/0.5%. However, the 3.0% CS / 0.5% PANI with 1% sodium citrate as crosslinking agent is the optimal concentration that could encapsulate *Pediococcus acidilactici* 3G3. The number of probiotic cells entrapped by the microbead is 6.21±0.08 log CFU. The cell release of entrapped probiotics in the simulated gastric fluid (SGF) is considerably higher than expected, ranging from 10^5 to 10^6 CFU cumulative value due to the immediate swelling of the CS/PANI microbeads upon exposure to SGF. At the end of the exposure to simulated gastric and intestinal fluid (3 h), the cumulative cell release of 62±1.67 microbeads ranges from 10^6 to 10^7 CFU. The amount of viable probiotic cells at the end of the exposure was 10^5 CFU. Hence, the CS/PANI composite was able to deliver the probiotic cells in the simulated intestine. The cell viability of microencapsulated *P. acidilactici* 3G3 at 4 ºC is significantly higher compared to room temperature at days 15 and 30. The cell viability of microencapsulated (46.23%±0.02%) probiotics was significantly lower than the free cells (69.64%±0.04%) after 30 days of storage at 4 ºC. On the contrary, the results on the

30th day of room temperature storage were significantly higher for microencapsulated (33.10%±0.13%) than free cells (25.92%±0.16%).

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest of the manuscript material with any financial, personal, or other relationship to other people or organization.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Conceptualized and designed the experiments: JOA and LMD. Performed the experiments: JOA. Wrote and revised the intellectual content of the paper for publication: JOA and LMD.

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