CHAPTER 3
EFFECT OF SOY PROTEIN ISOLATE ADDED IN CA-ALGINATE BEADS ON STABILITY OF ENTRAPPED *vB_salP-p*YM BACTERIOPHAGE

3.1 Abstract

Bacteriophages (phages) have emerged as a promising alternative for controlling bacterial pathogens. However, their susceptibility to environmental factors such as low pH and high temperatures limits their effectiveness in oral administration. This study evaluates the impact of soy protein isolate (SPI) on the stability of *vB_salP-p*YM bacteriophage entrapped in Ca-alginate beads. Phage entrapment efficiency, stability under varying pH, temperatures, bile salts, simulated gastric fluid (SGF), release of phages from simulated intestinal fluid (SIF) and long-term storage at 4°C were investigated. The results showed that phage entrapment efficiency (EE) was 97–98% in both SPI-modified and unmodified Ca-alginate beads. The addition of 0.3% (w/v) SPI significantly enhanced phage stability under acidic conditions (pH 2.5) with a survival number of 84.90% and improved thermal stability, resulting in a survival number of 76.92% at 50 °C. SPI also extended added Ca-alginate beads to phage viability in SGF for 2 hours and facilitated the release of SIF more than unmodified microbeads. Furthermore, SPI-modified beads better preserved phage viability during six months of storage at 4°C. These findings indicated that the potential of SPI incorporation in Ca-alginate beads could enhance phage stability during stressful environments, especially in the gastrointestinal tract during oral administration for phage therapy.

Keywords: Soy protein isolate, Ca-alginate beads, Salmonella bacteriophage, Entrapment.

3.2 Introduction

Bacteriophages (phages) are ubiquitous and the most abundant in the ecosystem. Phages are an alternative biological agent to control bacteria pathogens because of their killing capability (Pulit et al., 2015; Kimminau et al., 2020). However, phage therapy had limitations due to the sensitivity of phages to stressful

environmental factors. Phages were destroyed at high temperatures and low pH (Ranveer et al., 2024). The oral administration of phages targeting core zoonotic bacteria in the animal digestive tract is challenging due to the difficulty in controlling the survival rate of phages because of the loss of viability throughout the digestive tract (Ranveer et al., 2024). Therefore, encapsulation was useful technique for protecting phages.

Ca-alginate beads have been generally used for encapsulation or entrapment of biological agents such as microorganisms and enzymes (Weng et al., 2023). The main problem with using Ca-alginate beads was their instability under to acidic conditions or high temperature for a long time (Puscaselu, Lobiuc, Dimian and Covasa, 2020). Colom et al., (2017) reported that the phage cocktail was encapsulated within alginate/$CaCO_3$ microcapsule resulting in phage survival under gastric juice in chickens, while all free phages were inactivated. Śliwka et al., 2019) entrapped bacteriophage T4 inside dry mannitol-alginate beads and evaluated the survival of the encapsulated bacteriophage during exposure to simulated gastric fluid (SGF; pH 2.5) for 60 min. The beads were then transferred to simulated intestinal fluid (SIF; pH 7.5) and then incubated for 120 min, and the final bacteriophage titers were measured. The results showed that the bacteriophage T4 in mannitol-alginate beads had a survival of 6.22 log10 pfu/ml, while no survival was observed for free phages (initial titer 9.767 log10 pfu/ml). These results demonstrated that the addition of 0.3 M mannitol enhanced the protection of bacteriophage against gastrointestinal conditions resulting in remaining viability of T4 phage.

Alginate is a widely used biopolymer; however, it possesses several limitations, including low mechanical strength and instability under heat treatment. These drawbacks can be mitigated by blending alginate with other biopolymers (Puscaselu et al., 2020). For instance, Tang, Huang, Baxi, Chambers, Sabour and Wang, (2013) investigated that alginate-whey protein microsphere of could be able for protect their viability of phages in simulated gastric fluid (SGF) at pH levels of 2.0 and 2.5 for 2 hours. Furthermore, the encapsulated phages were completely released from AWM within 3 hours in simulated intestinal fluid (SIF). These findings indicated that incorporating whey protein into alginate microspheres could enhances the protection of phages under gastrointestinal tract. Similarly, Kim et al., (2022) revealed that polylactic-co-glycolic acid (PLGA)/alginate composite microspheres could

prolong phage viability in tissue up to 28 days was detected for up to 28 days following administration, whereas free phages survived in vivo for only 3 to 5 days.

Puscaselu et al. (2020) reported that limitation of Ca-alginate microgel was pour heat resistance and mechanical properties. Thus, modification alginate with other biopolymers such as proteins, starch might enhance %EE of calcium alginate (Ca-alginate) for phage encapsulation. Babot, Martínez, Apella and Chaia, (2023) reported that the combination of alginate and SPI significantly enhanced the protection of probiotic bacteria in simulated gastric fluid (SGF) compared to alginate alone. In another study, Jin et al. (2023) investigated the encapsulation efficiency, swelling behavior, and in vitro digestion behavior of calcium alginate (CA) microgels and calcium alginate-soy protein isolate (CAS) microgels encapsulating beta-carotene. Their findings indicated that CAS microgels exhibited higher encapsulation efficiency and enhanced stability for beta-carotene compared to CA microgels. Notably, the incorporation of SPI into CAS microgels led to greater gel shrinkage in gastric fluid and reduced swelling in intestinal fluid relative to CA microgels. Furthermore, in vitro digestion experiments revealed that CAS microgels demonstrated superior resistance to simulated gastric fluid, thereby improving the controlled release of encapsulated compounds. Additionally, Praepanitchai, Noomhorm and Anal, (2019) evaluated that the survival of Lactobacillus plantarum, a probiotic encapsulated within alginate-SPI hydrogels, was high under acidic conditions (pH 2). After 3 hours other wish no survival was found in the free probiotic. Therefore, this was to evaluated the effect of SPI addition in Ca-alginate beads on phage stability.

3.3 Material and Methods

3.3.1 Bacteria and bacteriophage

The *Salmonella* Typhimurium ATCC 13311 was bought from American Type Collection Culture (Microbiology, Inc, U.S.A.). Bacteriophage *vB_salP-p*YM. (Phage) was isolated from pork meat that was bought from a local market in Nakhon Ratchasima.

3.3.2 *Salmonella* cultivation and enumeration

Salmonella Typhimurium (*S.* Typhimurium) was cultured on xylose lysine deoxycholate (XLD; HIMEDIA®, India) agar and incubated at 37 °C for 24 hours.

The *S.* Typhimurium was routinely sub-cultured by using tryptic soy broth (TSB; HIMEDIA®, India). Subsequently, the bacterial cultures were collected by centrifugal at 1,500 x g for 15 min at 4 °C. The pellet cells were washed twice and resuspended with salt magnesium sulphate buffer (SM buffer) composed of 200 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl and 0.01% gelatin per litter pH 7.4. The resuspension was measured the absorbance at OD 600 nm to obtain 0.2 as approximate as $1x10^8$ CFU/ml (Ye, Kostrzynska, Dunfield and Warriner, 2010). This bacterial concentration was used for lawn preparation.

The *S.* Typhimurium was counted by using the spread plate technique. The ten-time serial dilution was performed using phosphate buffer saline (PBS) that was prepared by 10 mM Na_2HPO_4 and 1.8 mM NaH_2PO_4 per litter pH 7.4 and then cultured on XLD agar. The plates were incubated at 37 °C for 24 h. The colony-forming unit (CFU/ml) was calculated according to the equation below.

CFU/ml = number of colonies / (volume of sample x dilution factor)

3.3.3 Propagation and enumeration of bacteriophage titer

The 500 ul of mixture of phage and bacterial lawn as described as above were added into 5 ml of molten TSB containing 0.6% agar and poured on the basal TSB containing 1.5% agar. The plates were incubated at 37 °C for 24 hours (Batalha et al., 2021). Five milliliters of SM buffers were added to the plate and then placed on a shaker incubator at 100 rpm, 4°C for 24 h. The phage suspension was centrifuged at 4,500 x g for 15 min at 4°C and the supernatant was filtered using a 0.2 µm syringe filter and stored at 4°C.

Phage titers were determined by using the agar overlay assay (Artawinata, Lorraine and Waturangi 2023). Briefly, phage suspension was diluted with ten-time serial dilution by using SM buffer. The 10 µl of diluted phage was dropped on the lawn as described on phage propagation without phage. The titers of phage were determined as the mean of three independent counts, as plaque-forming units (PFU/ml) (Batalha et al., 2021). The PFU/ml was calculated according to the equation below.

PFU/ml= Average number of plaque x Dilution factor/ Volume of phage

3.3.4 Entrapments efficiency of bacteriophage

The phage suspension was entrapped into sodium alginate (HIMEDIA®, Nashik, India) with or without soy protein isolate (SPI; Krungthepchemi, Bangkok, Thailand) by modified from method of Ma, Pacan, Wang, Sabour, Huang and Xu, (2012). The the Ca-alginate beads were composed of 3% (w/v), sodium alginate and added with 0.15, 0.3% w/v of SPI or CS, and then mixed with phage suspension (10^8 PFU/ml). The beads were extruded by using a syringe 20-gauge (G) needle and exposed in 4% (w/v) $CaCl_2$ that was stirrings at 100 rpm at room temperature on stirring plate with a magnetic bar. The Ca-alginate beads were left to solidify for 1 hour then washed the beads two times with distilled water and kept modified alginate beads in 50 ml SM buffer pH 7.4 at 4 °C during the experiment.

For enumeration of phage titers, 100 mg of beads were added into 5 ml of sterile PBS pH 7.4 and shaken by an orbital shaker at 300 rpm at room temperature for 1-2 hours, or until dissolved (Ma et al., 2012) and then were determined by using agar overlay assay. Entrapment efficiency (EE) of phage in Ca-alginate beads without/with SPI and CS were determined according to Batalha et al. (2021). The EE was calculated as the following equation below.

EE= (Phage entrapped (PFU)/Initial phage (PFU)) x 100

3.3.5 Scanning electron microscopy (SEM)

The SEM-EDS-JEOL/JSM-6010LV (InTouchScope™, Japan) at 3 kV was used to clarify the morphology of ca-alginate beads. Briefly, the samples were prepared by critical point drying (CPD). Ca-alginate beads were fixed using glutaraldehyde (1%–3%) or a mixture of 2.5% glutaraldehyde and 4% formaldehyde. Dehydration was carried out using a gradient series of ethanol including concentrations of 10%, 20%, and 100%. After that, the samples were placed in a critical point dryer, which requires connections to a CO_2 cylinder, hot and cold water supplies, a drain (Bray, 2000), and an exhaust tube and is coated with gold under a vacuum.

3.3.6 Lytic assay of entrapped salmonella phage

The lytic activity assay was used for investigating effiency of entrapment. The hypothesis was if it had free phage released from the entrapped salmonella phage, the amount of *Salmonella* was reduced or if not, the *Salmonella* was still growth. Free phage (100 µl, 10^7 PFU/ml) was added to 5 ml of *S.* Typhimurium suspension at a 10^7 PFU/ml concentration. The 100 mg of Ca-alginate beads were placed into 5 ml of *S.* Typhimurium cells (10^7 PFU/ml) and then incubated at 41 °C with agitation at 120 rpm for 6 hours. Five hundred microliters of sample were collected interval every 2 hours (Zhou et al., 2022). The number of *Salmonella* was counted using the drop plate method and incubated at 37 °C for 24 hours (Reed and Reed, 1948). The colony-forming unit (CFU/ml) was calculated as described above.

3.3.7 Stability of salmonella bacteriophage in Ca-alginate beads under different temperatures and pH

The pH stability of the salmonella bacteriophage-entrapped and free phages was evaluated. The 100 mg of each sample and free phage (10^7 PFU/ml) were placed into SM buffer was adjusted to pH 2.5, 5, 5.5, 6.5, 7.5 and 8 (2.5 is the pH of the gizzard, 5 is the pH of the duodenum, 5.5 is the pH of the crop, 6.5 is the pH of the jejunum and 8 is the pH of the cecum) by using 1 M HCl or 0.5 M NaOH. All samples were incubated at 37 °C for 2 hours. Subsequently, the survival of free phage or entrapped phage in the Ca-alginate bead under each pH was measured by double agar overlay assay according to the method described above (3.3.4) The structure of the alginate polymer network is destroyed at pH 2.5 and 5, but the structure of calcium alginate is stable between pH 5.5 to 10 (Malektaj, Drozdov, Fini and Christiansen, 2024).

For thermal stability, the 100 mg of alginate beads were placed into 1 ml SM buffer pH 7.4 and subsequently at different temperatures including 4°C, 25°C, 37°C, 42°C and 50°C (At 4°C is the storage temperature, 25°C is the room temperature, 37°C is the human body temperature, 42°C is the chicken body temperature and 50°C is the representative of high temperature) for 24 hours (Dougan and Baker, 2014). The survival phages in each bead treatment were determined by the same method described above (No. 3.3.4).

3.3.8 Effect of bile salt on the stability of salmonella bacteriophage

The survival of free phages and entrapped phages in each Ca-alginate bead was determined. For free phage, 100 µl of phage suspension (10^7 PFU/ml) was placed into 4.9 ml of 0.01 M bile salt, 0.85% (w/v) NaCl pH to 6.8 as well as that the 100 mg of each Ca-alginate bead was placed into 4.9 ml bile salt solution. Afterward, all treatments were incubated at 41 °C with agitation at 120 rpm for 1 hour (Scanlan, Hall and Scanlan, 2019: Ma, Pacan, Wang, Xu, Huang and Korenevsky, 2008). The titer of survival phage under the bile salt solution was determined immediately by agar overlay assay according to the method described above (No. 3.3.4).

3.3.9 Stability of the phage in Ca-alginate beads under simulated gastric fluid (SGF)

The stability of the entrapped phages under simulated gastric fluid (SGF; pH 2.5) that composed of 3 mg/ml pepsin (pepsin enzyme, HIMEDIA®, Nashik, India) and 0.85% NaCl; pH to 2.5 that adjusted by using 1 M HCl (Colom et al., 2017) was determined. One hundred milligrams of Ca-alginate beads were placed into SGF and incubated in orbital shaker (S1500, Stuart, Stone, UK) at 120 rpm at 41 °C for 30, 60, 90 and 120 min and then removed SGF from the test tube and replace 2 ml of SM buffer pH 7.4 and left at room temperature for 5 min. The phage titers in each bead treatment were determined as described above (No. 3.3.4). The treatment was performed in triplicate and the results were presented in percentage of survival phages (Ma et al., 2008).

3.3.10 Phage releasing under simulated intestinal fluid (SIF)

The entrapped phage in Ca-alginate beads treatment were evaluated for the phage releasing under the simulated intestinal fluid (SIF) that consisted of 1 mg/ml of pancreatin enzyme (HIMEDIA®, Nashik, India) and 0.85% NaCl, pH to 6.8 that adjusted with 1 M HCl. The 100 mg of each Ca-alginate bead was incubated at 41 °C for 120 min on orbital shaker incubator at 120 rpm. The 100 ul of sample solutions were sampled at 30, 60, 90 and 120 min. The release of phage titers under SIF was examined by utilizing the double agar overlay assay as described above (No. 3.3.4).

3.3.11 The survival of entrapped phages in Ca-alginate beads during storage

The survival of the entrapped salmonella phage in Ca-alginate beads during storage at 4 °C in SM buffer was investigated for one month interval until 6 months. The phage titers were counted as described as above (3.3.4).

3.3.12 Statistical analysis.

The experiments were conducted in triplicate, and the results are presented as means ± SD. The resultes was evaluated by using one way analysis of variance (ANOVA) followed by Tukeys multiple comparison statiscal tests by using SPSS 26.0 for Windows (SPSS Inc., Chicago, USA). Significant differences were assessed if $p < 0.05$ (95% confidence interval).

3.4 Results and Discussion

3.4.1 Entrapment efficiency (EE)

Salmonella phages were entrapped in Ca-alginate beads without or with soybean isolate protein (SPI). The entrapment efficiency (EE) of each encapsulation bead was evaluated **(Table 3.1)**. The EE of each treatment was not significantly different, around 97-98%. This result showed that the addition of SPI to Ca-alginate beads did not affect the EE of Ca-alginate beads to entrapped salmonella phages. Alginate polymer bind to calcium ions (Ca^{2+}) know as an egg-box mechanism, forming a three-dimensional (3D) network capable of entrapping phage particles (20–200 nm) (Guliy and Evstigneeva, 2025; Selimoglu and Elibol, 2010). Therefore, the addition of proteins, such as soy protein isolate (SPI), may not significantly alter the gel structure in terms of pore size or cross-linking density, which are factors affecting entrapment efficiency (EE). Moreover, SPI tends to function as a stabilizer, enhancing the long-term stability of the encapsulated material rather than increasing the initial EE (Szekalska, Puciłowska, Szymańska, Ciosek and Winnicka, 2016; Babot et al., 2023).

Table 3.1 Entrapment efficiency for bacteriophage *vB_salP-pYM* in Ca-alginate beads.

Sodium alginate (w/v) (%)	Soy protein isolate (w/v) (%)	Entrapment efficiency
3	0	97.67 ± 0.58^{ns}
3	0.15	98.34 ± 0.15^{ns}
3	0.3	98.12 ± 0.19^{ns}

SPI = soy protein isolate; ns means each treatment is not significantly different at $p < 0.05$. Data are means ± standard deviation of three replications.

3.4.2 Ca-alginate beads size and morphological characterization of Ca-alginate beads via scanning electron microscopy (SEM)

The average diameter of the fresh beads were measured using vernier caliper. Diameters of the extruded Ca-alginate beads containing 0% SPI, 0.15% w/v SPI and 0.3% w/v SPI (n=10) were 2.9 ± 0.25, 3.1 ± 0.20, and 3 ± 0.16 mm, respectively **(Figure 3.1)**. The average size of Ca-alginate beads was not significantly different (p> 0.5). The bead's shape was round, with smooth surfaces. The added SPI in Ca-alginate beads was less transparent than control beads. The results demonstrated that the addition of SPI did not influence the size or shape of the beads. Volić et al. (2018) revealed that a significant decrease in hydrogel size was observed when increasing SPI concentrations (1% to 1.5% w/v). However, the SPI 0.15% and 0.3% SPI added Ca-alginate beads were not affected in morphology or size of beads.

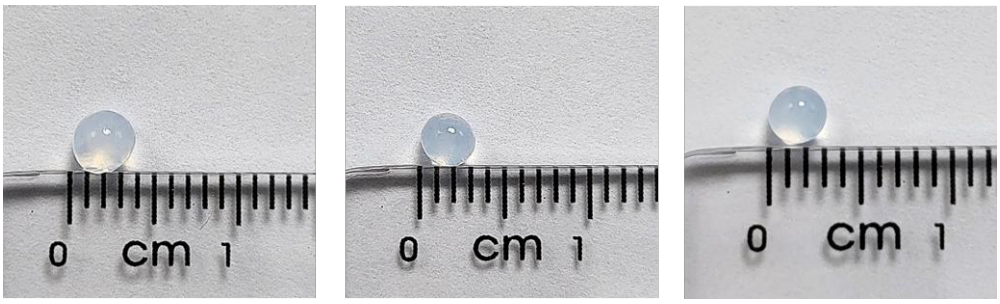

Figure 3.1 Illustration of Ca-alginate beads. A: 0% SPI, B: 0.15% w/v SPI and C: 0.3% w/v SPI.

Scanning electron microscopy (SEM) provided a detailed visualization of both surface and internal structures **(Figure 3.2)**. At 5000× magnification,

the surface of Ca-alginate beads exhibited relatively uniform folds, rough textures, and ridges across the entire surface area in all treatments. These features suggested that rapid drying or structural collapse may have occurred during sample preparation. The external surface of Ca-alginate beads containing SPI at both concentrations did not differ significantly from that of the control.

The internal structure of all treatments exhibited a network composed of many small fibers interconnected into a porous matrix, resulting in the arrangement of alginate polymers when crosslinked with calcium ions (Ca^{2+}). The alginate fibers were interconnected in a three-dimensional structure, indicating the mechanical strength of the matrix and its ability to retain embedded phages. Voids or pores were distributed throughout the network, which may influence the permeability properties of phage particles. Tang et al. (2013) reported that, based on SEM imaging, alginate-whey microspheres exhibited a denser but less homogeneous internal structure, whereas pure alginate microspheres showed a more porous and orderly internal configuration.

The internal pore size of the Ca-alginate beads was measured using the ImageJ software shown in **Table 3.2** The average pore sizes of beads were 232.41 μm for beads with 0.15% (w/v) SPI, 265.64 μm for beads with 0.3% (w/v) SPI and 258.74 μm for beads without SPI. The results show that the adding of SPI into Ca-alginate beads does not affect the internal structure's pore size.

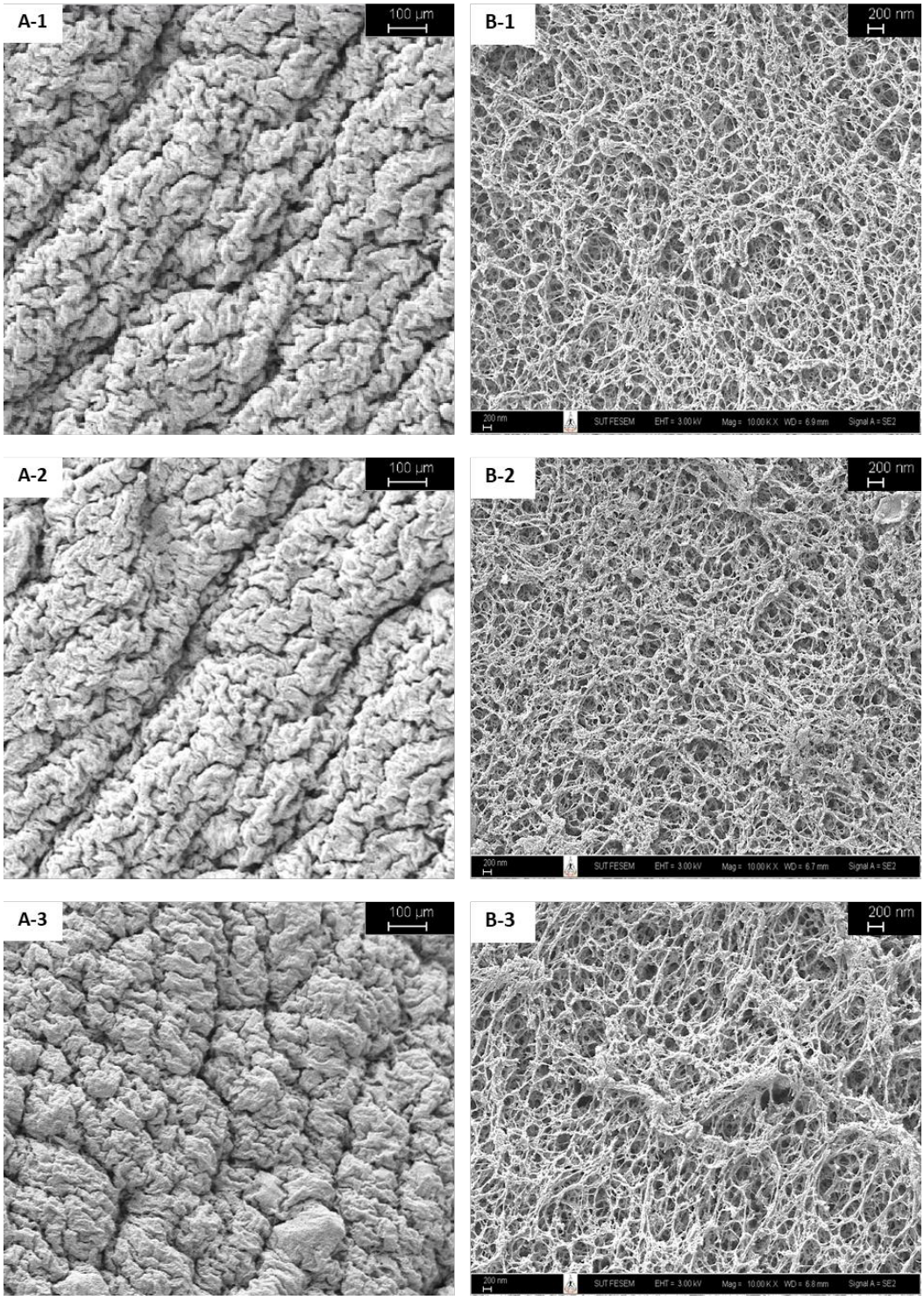

Figure 3.2 The SEM image surface and internal structure of Ca-alginate beads. Images A is surface structure at 5000x magnification and images B is internal structure at 10000x magnification. 1: 0% w/v SPI, 2: 0.15% w/v SPI and 3: 0.3% w/v SPI.

Table 3.2 Internal morphology and pore size analysis of Ca-alginate beads.

Treatment (T)	Min (μm)	Max (μm)	Mean diameter (μm)
0%SPI	174.00	376.67	258.74 ± 59.22 ^{ns}
0.15%SPI	183.62	304.25	232.41 ± 36.77 ^{ns}
0.3%SPI	136.02	386.26	265.64 ± 79.39 ^{ns}

SPI = soy protein isolate. Data are means diameter ± standard deviation of ten replications; ns means not significantly different ($p \leq 0.05$).

3.4.3 Lytic assay of entrapped salmonella phage.

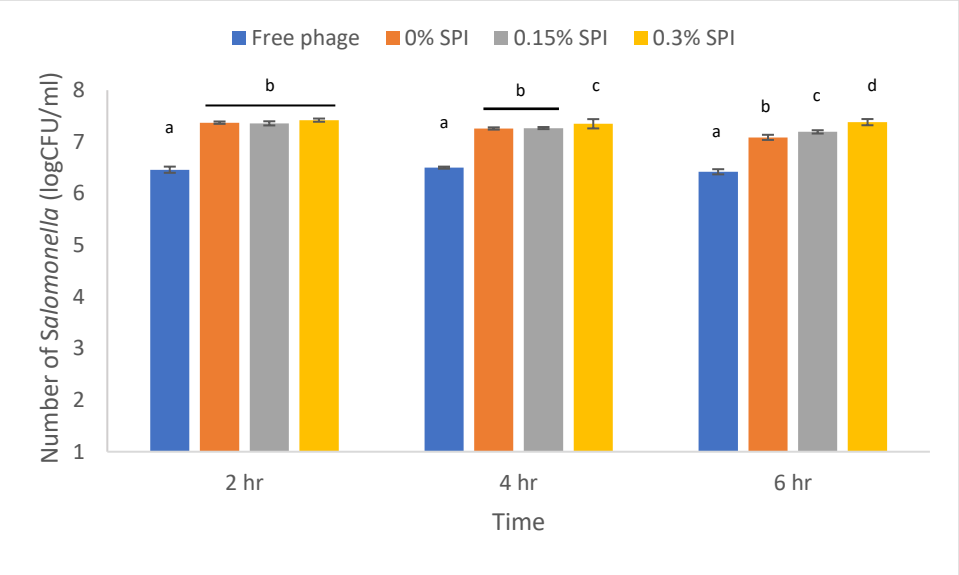

Figure 3.3 Lytic of entrapped salmonella phage on number of *Salmonella* Typhimurium; The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different at the same time ($p \leq 0.05$).

All treatments were placed in SM buffer (pH 7.4), followed by the addition of *Salmonella* Typhimurium cells at a concentration of 10^7 PFU/mL. The samples were then incubated at 41°C for 6 hours to evaluate the lytic activity of both free and entrapped salmonella phages **(Figure 3.3)**. After 2 hours of incubation, a significant reduction in Salmonella cell numbers was observed in the treatment with free phages, whereas in other treatments, the bacterial count was high due to phages not being able to release from the Ca-alginate beads. After 4 hours, the *Salmonella* count incubated with the free phage was significantly lower compared to Ca-alginate beads and after 6 hours, the *Salmonella* count in all treatments was significantly

different. Notably, the Ca-alginate beads with SPI could be effective in entrapping phages. The addition of SPI with oppositely charged alginate can form a complex due to electrostatic attraction, resulting in improved overall structure and strength (Albano, Cavallieri and Nicoletti, 2019). This suggests that Ca-alginate, with or without SPI, can entrap salmonella phages, which is consistent with our previous evaluations of entrapment efficiency. Similarly, Abdelsattar, Abdelrahman, Dawoud, Connerton and Shibiny, (2019) evaluated the lytic activities of free and encapsulated phages against Escherichia coli at 3, 6, and 10 hours under simulated intestinal conditions. Their results showed that free phages could reduce *E. coli* after 3 hours. The phages encapsulated in chitosan-alginate beads could be capable of releasing phages gradually against *E. coli*. This chitosan-alginate could not prevent the release of phage particles.

3.4.4 Stability of salmonella bacteriophage under different temperatures

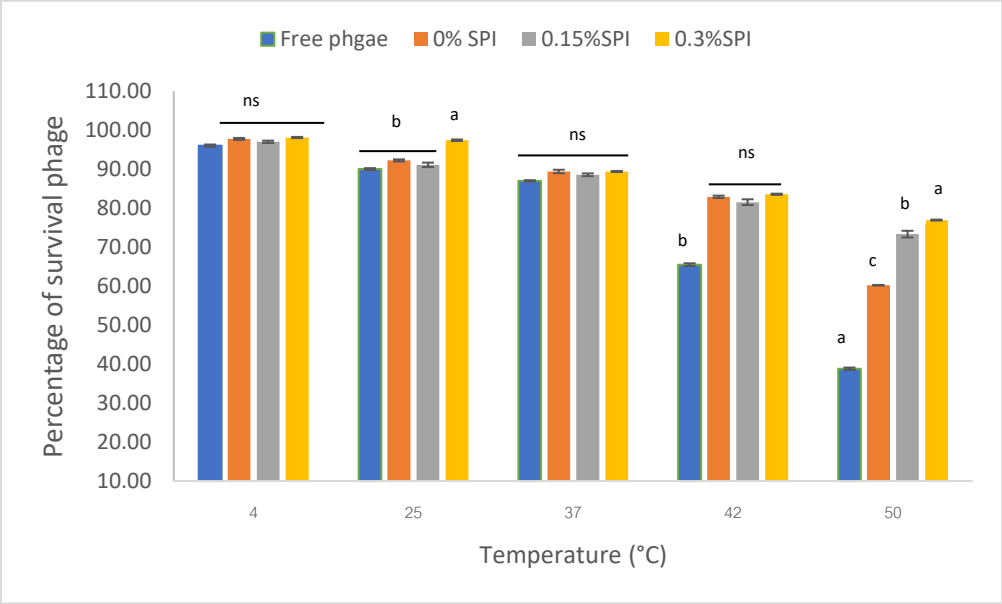

Figure 3.4 The survival of salmonella bacteriophage in the modified bead at various temperatures. The results corresponded to the mean ± standard deviation (n=3). ns means not significantly different, different letter means significantly different among the same temperature ($p \leq 0.05$).

The Ca-alginate beads modified with or without SPI were evaluated at various temperatures for 24 hours. (**Figure 3.4**). The number of salmonella phages in entrapped Ca-alginate beads was not significantly different at 4 and 37 °C. The titers

of salmonella phage in each treatment at 25, 42 and 50 °C were significantly different. The addition of SPI to the Ca-alginate beads could protect salmonella phages at high temperatures better than only Ca-alginate encapsulation. The free phages were incubated at various temperatures. At 42 and 50 °C, phage survivability was decreased significantly by approximately 65.50% and 38.81%, respectively. These results suggested that the addition of SPI in Ca-alginate could increase the efficiency of entrapment of salmonella phage, leading to protection of phages at high temperatures. The denatured proteins acted as fillers within the cross-linked network of alginate polymers and calcium ions. The negative charges of the proteins enhanced bond formation with the positive charges of calcium chloride, thereby strengthening the overall structure (Lin, Kelly, Maidannyk and Miao, 2021). As a result, incorporating SPI into Ca-alginate beads could improve the stability and survival of salmonella phages. Zhou et al. (2022) reported that encapsulated salmonella phage SL01 in alginate (ALG) mixed with carrageenan (CG) and formed by the extrusion method showed an increase in the survival of these phages under high temperatures, better than when using alginate alone for phages. Thus, SPI and sodium alginate were mixed and then autoclaved at 121°C for 15 minutes, leading to protein denaturing and incorporating with alginate polymer (Zhang et al., 2023).

3.4.5 Stability of salmonella bacteriophage under various pHs.

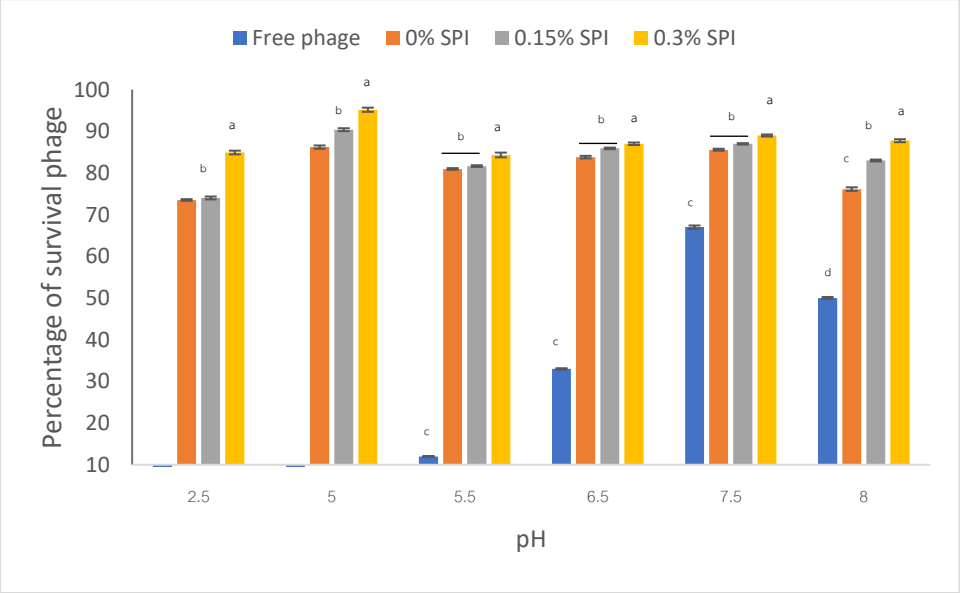

Figure 3.5 The stability of salmonella bacteriophage in Ca-alginate bead under various pHs. The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different at same pHs ($p \leq 0.05$).

The stability of Ca-alginate beads entrapping phages and incubated under various pH levels at 37 °C for 2 hours **(Figure 3.5)** was evaluated. The viability of salmonella phages entrapped in Ca-alginate beads with or without SPI was investigated under pH conditions of 2.5 (gizzard), 5 (duodenum), 5.5 (crop), 6.5 (jejunum), and 8 (cecum). Ca-alginate beads containing 0.3% w/v SPI showed the highest phage survivability compared to other treatments at all tested pH levels. At pH 2.5 and 5, free phages did not survive after 2 hours. These results indicated that incorporating SPI into Ca-alginate beads enhanced phage viability across a range of pH conditions, which simulate the pH values of each organ in the chicken's digestive system. At low pH, Ca-alginate beads were shrunk due to protonation of free carboxylate groups on alginate and a reduction in repulsive forces between alginate monomers and Ca^{2+} (Lin et al., 2020). The addition of SPI molecules increased the number of available bonding sites within the egg-box structure, thereby enhancing the crosslinking density. This stronger and more stable network reduced bead porosity, minimizing the leakage of salmonella phages (Babot et al., 2023; Shahbazizadeh, Tabasi, and Noghabi, 2022; Li, Chen, Su, Wang, He, Liu, 2021).

3.4.6 Effect of bile salt on the stability of entrapped salmonella phage

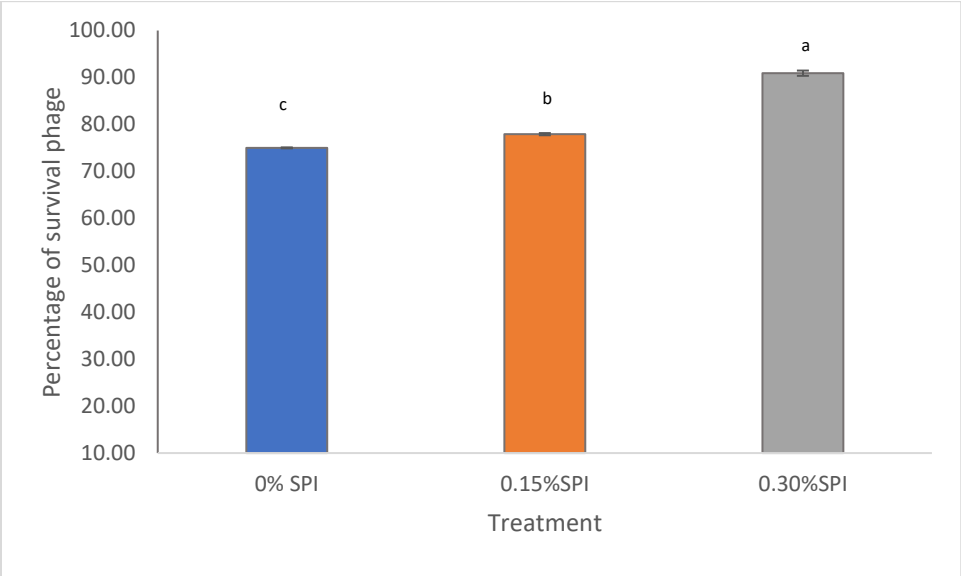

Figure 3.6 The number of phages remaining in Ca-alginate beads were incubated in bile salt. The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different among the same condition ($p \leq 0.05$).

The stability of phages in Ca-alginate beads were evaluated under a 0.01 M bile salt solution (pH 6.8) at 41 °C for 1 hour **(Figure 3.6)**. The percentage of phages remaining in Ca-alginate beads without SPI was 75%, while beads containing 0.15% w/v and 0.3% w/v SPI retained 77.92% and 90.91% of phages, respectively. These results indicated that entrapment in Ca-alginate beads enhanced phage stability, particularly when supplemented with SPI. When Ca-alginate beads are exposed to bile solution, their structure changes, primarily involving swelling and partial dissolution. The hydrogel swells as bile components, bile salts and enzymes, penetrate the polymer matrix, which can disrupt the hydrogen bonds that contribute to the hydrogel's structure (Shivakumara and Demappa, 2019). Mixing sodium alginate with soy protein in hydrogel molecules of soy protein isolate enhances the mechanical strength and stability of the Ca-alginate beads, preventing them from degrading in the bile salt environment (Lin et al., 2021). Similarly, Tang et al. (2013) reported that alginate/whey protein microspheres were evaluated under 1% and 2% bile salt solutions for 1 and 3 hours, resulting in a partial reduction in the viability of phage Felix O1.

3.4.7 Stability of the phage in Ca-alginate beads under simulated gastric fluid (SGF).

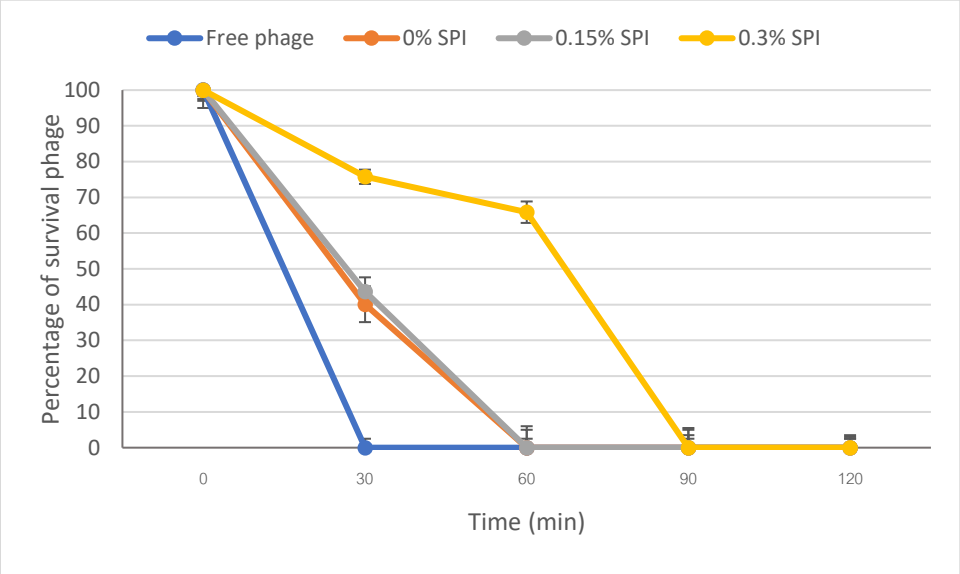

Figure 3.7 The survival of salmonella phage in the modified beads under SGF. The results corresponded to the mean ± standard deviation (n=3).

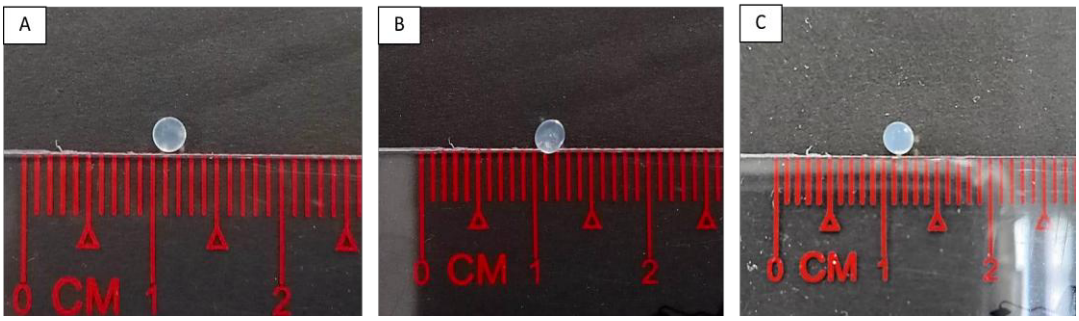

Figure 3.8 Morphology of Ca-alginate beads after in vitro incubation in simulated gastric fluid (SGF) for 120 min. A is Ca-alginate beads without SPI, B is Ca-alginate beads wit 0.15% w/v SPI and C is Ca-alginate beads wit 0.3% w/v SPI.

The stability of entrapped phages with or without SPI in Ca-alginate beads was evaluated under SGF (3 mg/ml pepsin in 0.85% NaCl), pH 2.5, at 41°C for 2 hours (**Figure 3.7**) and the morphology of Ca-alginate beads was illustrated (**Figure 3.8**). No survival was detected for free phages after incubation for 30 minutes. For Ca-alginate beads without SPI and with 0.15% and 0.3% w/v SPI, the percentage survival of phages was 40 ± 0.52%, 44 ± 0.4%, and 76 ± 0.22%, respectively. After 60 minutes, the survival of phage in entrapped Ca-alginate beads with 0.3% SPI remained approximately 66 ± 0.3%.

Afterward, the phage was not detected in any treatment. These results indicated that incorporating soy protein isolate (SPI) into the beads enhanced the survival of phages when exposed to simulated gastric fluid (SGF) for 60 minutes. Although alginate beads could reduce the direct contact of phages with gastric acid, diffusion of hydrogen ions via porous surfaces is too small, leading to a decrease in internal pH and subsequent loss of phage viability (Ma et al., 2008). Enzymatic hydrolysis by pepsin, exposed to the gastric juice, resulted in protein degradation, an increase in porosity and the release of phage (Nissen and J., 1986).

3.4.8 Evaluation releasing of phages from Ca-alginate beads in simulated intestinal fluid (SIF).

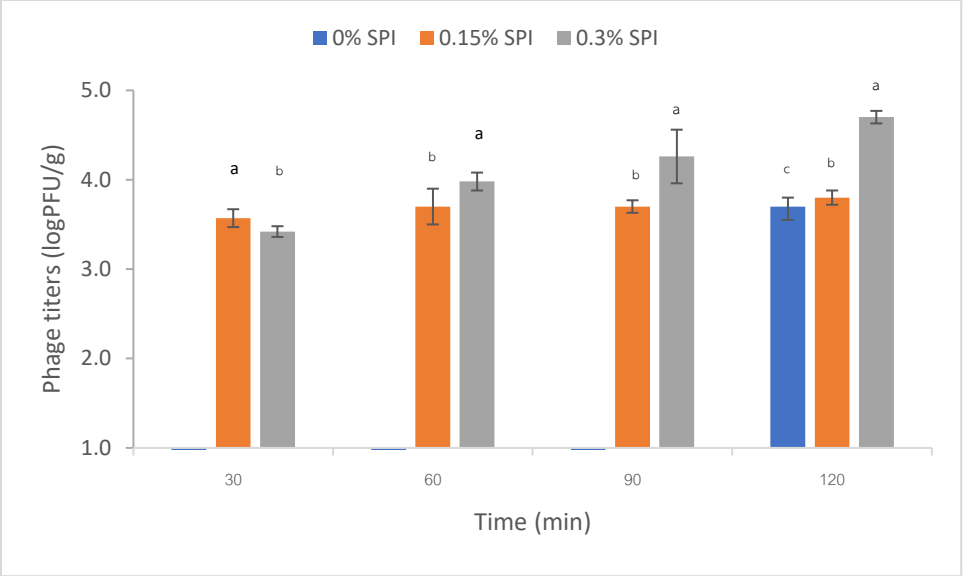

Figure 3.9 The release of phages was entrapped with Ca-alginate beads in simulated intestinal fluid at pH 6.8. The results corresponded to the mean ± standard deviation (n=3), different letter means significantly different under the same time ($p \leq 0.05$).

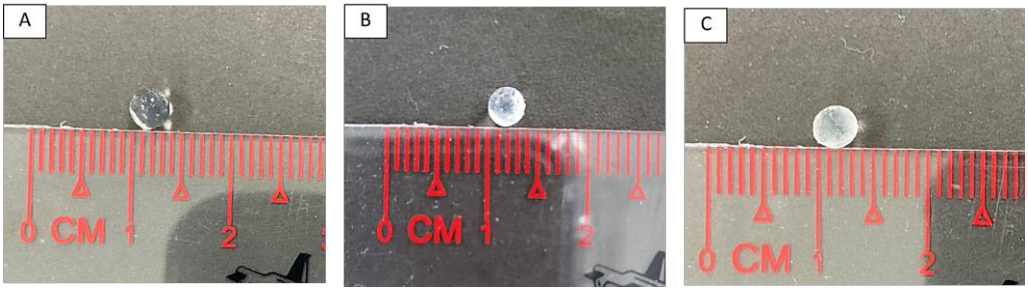

Figure 3.10 Morphology of Ca-alginate beads after in vitro incubation in simulated intestinal fluid (SIF) for 120 min. A is Ca-alginate beads without SPI, B is Ca-alginate beads wit 0.15% w/v SPI and C is Ca-alginate beads wit 0.3% w/v SPI.

Ca-alginate beads, with and without soy protein isolate (SPI), were incubated for 120 minutes in simulated intestinal fluid (SIF; 1 mg/mL pancreatin in 0.85% NaCl) at 41°C under pH 6.8 **(Figure 3.9)** and the morphology of Ca-alginate beads were illustrated **(Figure 3.10)**. After 90 minutes of incubation, Ca-alginate beads without SPI did not release any detectable phages. In contrast, phages entrapped in Ca-alginate beads containing 0.15% and 0.3% (w/v) SPI were released as early as 30 minutes, with release levels increasing further at 60 and 90 minutes. After 120 minutes, phage titers released from beads without SPI reached 3.7 ± 0.1 log PFU/g, whereas those from beads containing 0.15% and 0.3% SPI reached 3.8 ± 0.08 and 4.7 ± 0.7 log PFU/g, respectively. These results indicated that the inclusion of SPI in Ca-alginate beads could contribute to phage release better than only alginate.

During incubation in SIF, the beads could be absorbed water, swelled, and fully dissolved within 1 hour (Zhang et al., 2015). Alginate, a natural polymer composed of mannuronic acid (M) and guluronic acid (G) units, contains carboxyl groups (-COOH) that deprotonate at neutral to alkaline pH, forming negatively charged carboxylate ions ($-COO^-$). The resulting electrostatic repulsion along the polymer chains causes the network to expand and swell as it absorbs water (Mirdarikvande, Sadeghi, Godarzi, Alahyari, Shasavari and Khani, 2014; Shivakumara and Demappa, 2019). In addition, the beads released SPI molecules, which enzymes subsequently hydrolyzed, improving the hydrogel's properties due to a synergistic effect between the two polymers. (Volić et al., 2018). According to Kim, Jo and Ahn, (2015), the chitosan/alginate microspheres encapsulating *Escherichia coli* O157:H7 bacteriophages began releasing the phages in SIF after 30 minutes.

3.4.9 Stability of phages in the Ca-alginate bead during storage at 4 °C.

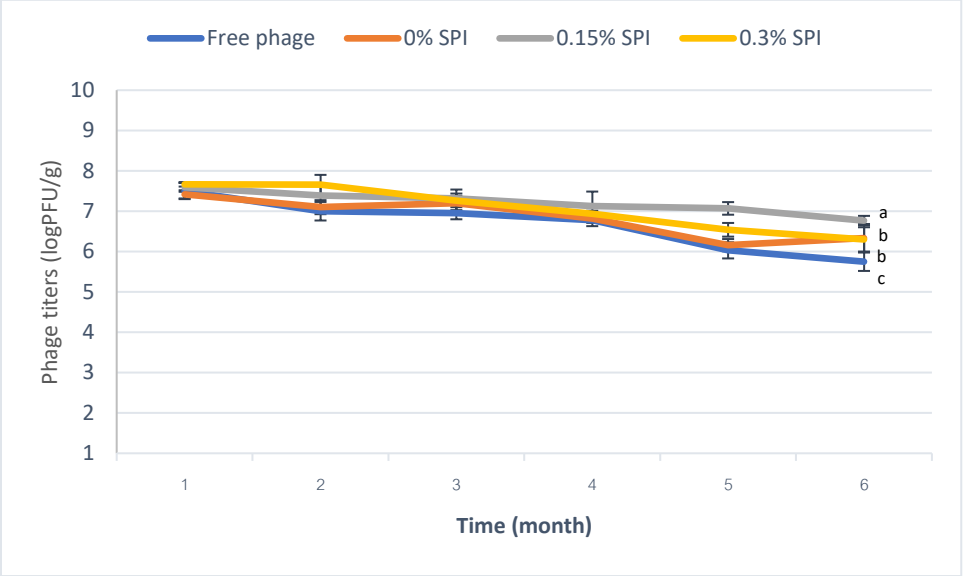

Figure 3.11 Phage survival in the Ca-alginate beads during storage at 4 °C for 6 months.

The survival of entrapped phages in Ca-alginate beads with and without soy protein isolate (SPI) was evaluated over 6 months storage period at 4°C **(Figure 3.11)**. After 6 months, the concentration of free phages had decreased by 2.25 log PFU/ml from an initial level of 8 log PFU/ml. Phages entrapped in Ca-alginate beads without SPI showed a reduction of 1.67 log PFU/g, while beads containing 0.15% and 0.3% (w/v) SPI exhibited decreases of 1.23 and 1.7 log PFU/g, respectively. During the first month of storage, phages titers were not observed significant different in all treatments. These results suggested that incorporating soy protein isolate (SPI) into Ca-alginate beads improved the long-term survival of entrapped salmonella phages at 4°C. Phages with 0.15% SPI showed the highest viability after six months, suggesting SPI enhances phage stability during extended storage. Combining sodium alginate with soy protein isolate (SPI) improves bead stability by forming a denser, more interconnected network. The alginate–SPI interaction creates a stronger, less temperature-sensitive hydrogel, making it more resistant to degradation at low temperatures (Jin et al., 2023).

Similarly, Zhang et al. (2023) investigated the stability of phages encapsulated in xanthan gum/sodium alginate/chito-oligosaccharide microspheres during storage at 4°C for 1.5 months. Their results demonstrated a reduction in the number of free phages by 2.88 log PFU/mL, whereas the number of encapsulated

phages decreased by only 1.02 log PFU/g. These findings indicated that entrapment improves the stability of phages compared to free phages. Due to covalent interactions between the carboxyl groups of alginates and the amide groups of soy protein, the soy protein fills gaps within the beads, resulting in a more stable and stronger structure (Volić et al., 2018).

3.5 Conclusions

This study has demonstrated that the phage *vB_salP-p*YM entrapped in calcium-alginate beads, both with and without SPI, exhibited high entrapment efficiency. The incorporation of SPI into the calcium-alginate beads enhanced the survival of the bacteriophages under simulated gastric fluid (SGF), high temperatures, low pH and bile salt. Furthermore, SPI loaded beads increased phage release efficiency in simulated intestinal fluid (SIF) and improved phage viability during long term storage at low temperatures. These findings suggested that SPI loaded calcium-alginate beads offer a promising strategy for protecting phages during oral administration, thereby supporting their potential application in phage therapy.

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