EFFECTS OF SOY PROTEIN ISOLATE AND CASSAVA STARCH ADDED IN Ca-ALGINATE BEADS ON STABILITY OF SALMONELLA PHAGE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Food Technology
Suranaree University of Technology
Academic Year 2024

ผลของการเติมโปรตีนถั่วเหลืองไอโซเลต และแป้งมันสำปะหลังในเม็ดเจลแคลเซียม อัลจิเนตต่อความคงตัวของฟาจแซลโมเนลลา

นางสาวภาณุการ์ มลาวัลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ภาณุการ์ มลาวัลย์: ผลของการเติมโปรตีนถั่วเหลืองไอโซเลต และแป้งมันสำปะหลังในเม็ดเจลแคลเซียมอัลจิเนตต่อความคงตัวของฟาจแซลโมเนลลา (EFFECTS OF SOY PROTEIN ISOLATE AND CASSAVA STARCH ADDED IN Ca-ALGINATE BEADS ON STABILITY OF SALMONELLA PHAGE) อาจารย์ที่ปรึกษา: ดร.พัชรินทร์ ศิริงาน , 96 หน้า.

คำสำคัญ : การกักเก็บ, โปรตีนถั่วเหลืองไอโซเลต, แป้งมันสำปะหลัง, เม็ดเจลแคลเซียมอัลจิเนต, ความคงตัวของฟาจ

ฟาจแซลโมเนลลา (*vB_salP-pYM*) ที่มีศักยภาพในการควบคุมเชื้อ *Salmonella* enterica serovar Typhimurium ซึ่งก่อโรคในคนและทั่วไปพบแซลโมเนลลาอาศัยอยู่ในทางเดินอาหารโดยเฉพาะในสัตว์ปีก การป้องกันเชื้อแซลโมเนลลาโดยใช้ฟาจ นิยมให้ทางปากผ่านทางเดินอาหาร (Oral route administration) เข้าไปยังลำไส้เล็กซึ่งเป็นจุดที่มีมักพบเชื้อแซลโมเนลลา ซึ่งฟาจนี้ไม่สามารถอยู่รอดในสภาวะความเป็นกรดในกระเพาะอาหารได้ ดังนั้นวัตถุประสงค์ของงานวิจัยนี้คือเพื่อประเมินการกักเก็บฟาจด้วยเม็ดเจลแคลเซียมอัลจิเนต (Ca-alginate beads) โดยศึกษาผลการเติมโปรตีนถั่วเหลืองไอโซเลต (SPI) หรือแป้งมันสำปะหลัง (CS) ต่อความคงตัวของฟาจแซลโมเนลลาในเม็ดเจลแคลเซียมอัลจิเนตมีร้อยละประสิทธิภาพการการกักเก็บฟาจในเม็ดเจลแคลเซียมอัลจิเนตที่มีการเติมโปรตีนถั่วเหลืองไอโซเลตหรือแป้งมันสำปะหลัง มีค่า >97% และไม่แตกต่างกันกับการกักเก็บในเม็ดเจลที่ไม่เติม ($p > 0.05$) แสดงว่าการเติมโปรตีนถั่วเหลืองไอโซเลตหรือแป้งมันสำปะหลังไม่มีผลต่อประสิทธิภาฟการกักเก็บฟาจ นอกจากนี้พบว่าการเติม SPI และ CS ในเม็ดเจลแคลเซียมอัลจิเนตที่ความเข้มข้น 0.15% และ 0.30%(w/v) สามารถเพิ่มความคงตัวของฟาจในสภาวะกรด (pH 2.5) และที่อุณหภูมิ 50 องศาเซลเซียส ได้ดีกว่าฟาจอิสระและฟาจที่กักเก็บในเม็ดเจลแคลเซียมอัลจิเนตที่ไม่เติมเพิ่มอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ความคงตัวของฟาจภายใต้สภาวะจำลองของกระเพาะ พบว่าการเติมแป้งมันสำปะหลังที่ความเข้มข้น 0.30%(w/v) ฟาจที่ถูกกักเก็บสามารถอยู่รอดได้มากกว่า 60% ที่ 120 นาที ได้นานกว่าการเติมโปรตีนถั่วเหลืองไอโซเลต 0.30%(w/v) ซึ่งทำให้ฟาจอยู่รอดได้แค่ 60 นาที แต่ยังนานกว่าฟาจที่ถูกกักเก็บในเม็ดเจลที่ไม่เติมโปรตีนและแป้ง ซึ่งอยู่รอดประมาณ 40% หลังบ่ม 30 นาที และผลการประเมินความสามารถในการปลดปล่อยฟาจในระบบจำลองของลำไส้เล็ก พบว่าการเติมโปรตีนถั่วเหลืองไอโซเลตและแป้งมันสำปะหลังในเม็ดเจลสามารถลดระยะเวลาการปลดปล่อยฟาจในลำไส้เล็กได้เร็วกว่าการกักเก็บในเม็ดเจลแคลเซียมอัลจิเนตที่ไม่เติมภายในเวลา 30 นาที และ 120 นาที ตามลำดับ และยังเพิ่มความอยู่รอดในเกลือน้ำดีของฟาจที่ถูกกักเก็บได้มากกว่า 90% ในเม็ดเจลแคลเซียมอัลจิเนตที่มีการเติมที่ความเข้มข้น 0.3% (w/v) ของ SPI หรือ CS นอกจากนี้การเติม 0.3%(w/v) SPI หรือ CS ในเม็ดเจลแคลเซียมอัลจิเนต ทำให้ฟาจที่ถูกกักเก็บสามารถอยู่รอดโดยมีการลดลงเพียงเล็กน้อยในระยะเวลา

การเก็บรักษาหกเดือนที่อุณหภูมิ 4 องศาเซลเซียส ($p<0.05$) แสดงให้เห็นว่าการเติมโปรตีนถั่วเหลืองไอโซเลตหรือแป้งมันสำปะหลังสามารถกักเก็บฟาจแซลโมเนลลาได้อย่างมีประสิทธิภาพ สามารถเพิ่มการอยู่รอดในสภาวะการจำลองในกระเพาะอาหาร โดยเฉพาะการเติมแป้งมันปะหลัง และสามารถลดระยะเวลาการปลดปล่อยฟาจที่ถูกกักเก็บในลำไส้เล็กที่เป็นเป้าหมายได้เร็วขึ้น โดยฟาจสามารถอยู่รอดได้สามารถเข้าไปควบคุมการติดเชื้อแซลโมเนลลาในระบบทางเดินอาหาร แต่อย่างไรก็ตามควรมีการศึกษาเพิ่มการจำลองระบบทางเดินอาหารทั้งระบบ เพื่อสำรวจการอยู่รอดของฟาจที่ถูกกักเก็บในเม็ดเจลแคลเซียมอัลจิเนตที่มีการเติมโปรตีนถั่วเหลืองไอโซเลตและแป้งมันสำปะหลังได้ เพื่อนำไปประยุกต์ใช้ในการควบคุมโรคติดเชื้อ (phage therapy)

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา .....

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา .....

PANUKAR MALAWAN: EFFECTS OF SOY PROTEIN ISOLATE AND CASSAVA STARCH ADDED IN Ca-ALGINATE BEADS ON STABILITY OF SALMONELLA PHAGE. THESIS ADVISOR: PATCHARIN SIRINGAN, Ph.D., Number of 96 page PP.

Keyword: Entrapment, Soy protein isolate, Cassava starch, Ca-alginate beads, Phage stability

Salmonella phage (*vB_salP-p*YM) with the potential to control *Salmonella enterica* serovar Typhimurium — a pathogen in humans commonly found in the gastrointestinal tract, especially in poultry — is typically administered orally to reach the small intestine, where *Salmonella* is often found. However, this phage cannot survive the acidic conditions of the stomach. Therefore, the objective of this study was to evaluate the entrapment of the phage using calcium alginate beads and to investigate the effects of adding soy protein isolate (SPI) or cassava starch (CS) on the stability of the phage in the calcium alginate beads. The entrapment efficiency of the phage in calcium alginate beads with added SPI or CS was greater than 97%, with no significant difference compared to beads without additives ($p > 0.05$), indicating that SPI or CS does not affect phage entrapment efficiency. Additionally, the incorporation of 0.15% and 0.30% (w/v) SPI or CS significantly improved phage stability under acidic conditions (pH 2.5) and at 50°C compared to free phages and those entrapped without additives ($p < 0.05$). Under simulated gastric conditions, cassava starch at 0.30% (w/v) allowed over 60% of entrapped phages to survive after 120 minutes, longer than SPI at the same concentration, which maintained survival for only 60 minutes. Both were more effective than phages entrapped without additives, which showed approximately 40% survival after 30 minutes. In simulated small intestine conditions, the both addition of SPI or CS to the beads significantly accelerated the release of phages within 30 minutes, while phages that entrapped in beads without additives were released at 120 minutes. Furthermore, these additions improved phage survival in bile salt solution to over 90% at a concentration of 0.30% (w/v) SPI or CS. Moreover, adding 0.30% (w/v) SPI or CS to the calcium alginate beads resulted in only a slight reduction in phage viability during six months of storage at 4°C ($p < 0.05$). This demonstrates that SPI or CS can effectively entrap Salmonella phages, enhance their survival under simulated gastric conditions — particularly with cassava starch — and accelerate their release in the targeted site (small intestine), allowing the phages to control *Salmonella* infections in the gastrointestinal tract.

However, further studies simulating the entire gastrointestinal system are recommended to explore the survival of phages entrapped in calcium alginate beads with SPI and CS for future applications in phage therapy for infectious disease control.

School of Food Technology
Academic Year 2024

Student's Signature .....Pancukar.....
Advisor's Signature .....Patcharin Srirugsa.....

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my advisor, Dr. Patcharin Siringan for excellent supervision, patience and dedication in helping me accomplish my research and thesis. She provided advice on academics throughout the course of my study. In addition, the valuable lesson she has taught me through research is greatly appreciated. I would also like to thank all members of the proposed committee for their excellent advice and suggestions.

I would also like to thank faculty members at the School of Food Technology, Suranaree University of Technology, for their scientific discussion, instrumental support and other help with kindness. Financial support from the One Research One Grant (OROG) scholarship.

Many thanks go to all my friends at Suranaree University of Technology for their scientific discussion and friendships. Finally, I wish to thank my beloved parents, sisters and relatives for their moral support, understanding, inspiration and encouragement.

Panukar Malawan

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CHAPTER 1

INTRODUCTION

1.1 Background and significance of the study

Bacteriophages, or phages, are the most abundant biological entities on earth and represent a substantial reservoir of genetic and ecological diversity (Hassan, ElNaby, Elela, Abouelwafa, Sersy, and Fisheries, 2020). Their history spans over a century, starting with their discovery in 1915 by Frederick Twort and independently by Félix d'Hérelle in 1917. Initially studied for their potential as antibacterial agents, phages were used in phage therapy, particularly in the former Soviet Union, before the widespread use of antibiotics (Ofir and Sorek, 2018; Letarov, 2020). Phages act as natural antagonists of bacteria, demonstrating high host specificity. Upon recognizing a suitable host, their receptor-binding proteins mediate the injection of phage genetic material into the bacterial cell. Lytic, or virulent, phages then replicate their genome, assemble new phage particles, and ultimately lyse the host cell to release progeny virions (Gutiérrez and Calap, 2020; Kasman and Porter, 2022). In the early 20th century, initial attempts to employ bacteriophages for therapeutic purposes were constrained by limited understanding of phage–host interaction mechanisms. As antibiotic therapies become increasingly effective and widely adopted, scientific interest in the bactericidal potential of phages gradually declines (Połaska and Sokołowska, 2019). The growing concern over antibiotic resistance has sparked renewed interest in phage therapy as a potential solution. However, phage therapy had limitations due to the sensitivity of phages to stressful environmental factors. Phages were destroyed at high temperatures and low pH. 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).

Salmonella enterica subsp. enterica remains a major public health concern, particularly in developing countries, where it continues to be a leading cause of foodborne illness. (Eng, Pusparajah, Mutalib, Ser, Chan and Lee, 2015). This bacterial

subspecies is classified into numerous serovars based on variations in surface antigens, which contribute to its widespread prevalence and epidemiological diversity (Relaño et al., 2021). As a foodborne pathogen, *S. enterica* causes infectious gastroenteritis worldwide and is primarily transmitted through the consumption of contaminated animal-derived products, especially poultry products such as raw chicken and eggs. Upon ingestion, the bacteria survive the acidic gastric environment and colonize the small intestine, leading to gastrointestinal symptoms that range from mild discomfort to severe illness (Vargas, Sánchez, Hernández and Barragán, 2020; Dougan and Baker, 2014). Patients commonly present with clinical symptoms such as nausea, vomiting, diarrhea occasionally hemorrhagic and fever (Fàbrega and Vila, 2013). The World Health Organization (WHO) reports that *Salmonella* infections affect an estimated 550 million individuals annually, including 220 million children under five years of age (Makalatia et al., 2021). The rising incidence of *S. enterica* infections constitutes a critical public health concern globally, with substantial implications for food safety, especially in poultry products, which represent a key source of dietary protein worldwide (House, Bishop, Parry, Dougan and Wain, 2001). The primary treatment for *Salmonella* infections relies on antimicrobial therapy (Lamichhane et al., 2024). However, inappropriate and excessive use of antibiotics in human and veterinary medicine contributes significantly to the escalating problem of antimicrobial resistance (Ahmed, Hussein, Qurbani, Ibrahim, Fareeq and Mahmood, 2024). Forecasts suggest that by 2050, antibiotic-resistant pathogens may account for approximately 1.91 million deaths annually, with an additional 8.22 million fatalities attributed to complications associated with antimicrobial resistance (Naddaf, 2024).

Consequently, the World Health Organization (WHO) underscores the urgent need to develop innovative strategies to mitigate this escalating antimicrobial resistance crisis (Vargas et al., 2020). Biocontrol serves as an alternative strategy to reduce reliance on antibiotics, with bacteriophages (phages) emerging as a promising therapeutic modality (Kimminau et al., 2020). On September 21, 2016, the United Nations General Assembly convenes to address the pressing issue of antibiotic resistance, acknowledging it as one of the most significant global challenges and threats. Among the key recommendations is the reintroduction of phage therapy, which presents several advantages over antibiotics, including host specificity, self-

amplification, biofilm degradation, and low toxicity to humans (Lin, Koskella and Lin, 2017).

Numerous studies explore the potential of phage therapy in mitigating *Salmonella* contamination. For instance, Kimminau et al. (2020) investigated the application of bacteriophages at a concentration of 10^8 PFU/g in chicken feed (1 kg/ton and 1.5 kg/ton of phages) contaminated with *Salmonella enteritidis*. The feed was administered to chicks, and after one-week, fecal samples and organ tissues (liver and spleen) were collected to assess bacterial reduction. The results demonstrated a significant reduction in *Salmonella enteritidis* prevalence in feces ($P<0.001$), underscoring the efficacy of phages in mitigating pathogen contamination in chicken feed during passage through the digestive system, even in the absence of antibiotics. Similarly, Colom et al., (2017) encapsulated bacteriophages using alginate/$CaCO_3$ and evaluated their stability in comparison to free phages in simulated gastric fluid at pH 2.8. The results revealed that free phages exhibited a substantial reduction of 5–8 log PFU/mL after 60 minutes, whereas encapsulated phages showed a comparatively smaller reduction of 3–3.5 log PFU/g. Although encapsulation enhanced phage survival, a notable loss still occurred within the first hour of incubation. These findings underscore that, although oral administration of phage therapy is feasible, the low pH of the stomach, along with the enzymatic activity of bile and the small intestine, remains a critical barrier to its effectiveness (Colom et al., 2017). Consequently, ongoing research into protective strategies and materials is essential to enhance phage stability in harsh gastrointestinal environments, thereby enabling the optimal utilization of phage therapy.

Over the past decade, extensive research was conducted on cell encapsulation within porous materials, demonstrating its effectiveness in minimizing cell loss and enhancing the stability of biotherapeutic agents. Various biopolymers including polysaccharides, synthetic polymers, and proteins were widely employed as encapsulating materials due to their capacity to provide protective barriers against environmental stressors. Among these, the most commonly utilized encapsulation systems involved the formation of small polymeric beads ranging from 1 to 5 mm in diameter. Notably, encapsulation using natural polymers received considerable attention, as it offered a gentle, biocompatible approach that preserved the viability of living cells (Willaert and Baron, 1996). One of the most promising applications of

encapsulation technology is phage immobilization, which prevents the leakage of encapsulated bacteriophages while enabling their controlled release at the target site. Alginate, a natural polysaccharide, is widely recognized as a preferred polymer for phage encapsulation due to its acid resistance and its ability to support the sustained release of viable microorganisms such as probiotics, bacteria, and phages into the gastrointestinal tract (Zhou, Xu, Yu, Han, Liu and Qu, 2022). Several studies investigate the potential of encapsulation to enhance the survival of beneficial microorganisms under harsh digestive conditions. Zhang, Zheng, Gu, Ni, Wang and Tang, (2015) investigated the encapsulation of the probiotic strain *Enterococcus faecalis* HZNU P2 utilizing a composite matrix of alginate and soy protein isolate. Their study demonstrated that the encapsulated probiotics retained high viability following two hours of incubation in simulated gastric fluid (pH 2), whereas the unencapsulated counterparts exhibited a viability reduction of approximately 2 log CFU/mL. Furthermore, upon exposured to a bile salt solution (pH 2.5) for the same duration, encapsulated probiotics exhibited only a minimal decline in viability, while unencapsulated cells failed to survive. These findings suggested that the incorporation of soy protein isolate into the alginate matrix significantly enhanced the resilience of probiotics under harsh gastrointestinal conditions. Despite these promising outcomes, the use of plant-based proteins as encapsulation materials remains relatively limited. In a related study, Vazquez, Calleros, Buendia, Chavez, Ramirez and Carter, (2015) investigated the potential application of cassava starch–modified sodium alginate for the encapsulation of chlorogenic acid (CGA) and assessed its release behavior under simulated gastrointestinal conditions (pH 2.3 and bile salt solution) over a two-hour period. Their findings indicated that the optimal formulation, incorporating 0.75% cassava starch, significantly minimized CGA leakage compared to sodium alginate alone. These results highlighted the promising role of cassava starch and other plant-derived biopolymers in enhancing the protective efficacy of alginate-based encapsulation systems. Overall, these studies highlight the feasibility of utilizing alginate-based encapsulation, supplemented with soy protein isolate or cassava starch, as an effective strategy to improve the stability and controlled release of therapeutic agents, including bacteriophages and probiotics, within the gastrointestinal tract. Ongoing research is needed to optimize

encapsulation formulations and evaluate their practical applications in food safety and biomedical interventions.

The aim of this study is to evaluate the efficacy of calcium alginate beads, modified with varying concentrations of soy protein isolate and cassava starch, in protecting salmonella bacteriophage under different pH conditions, temperatures, and simulated chicken digestive environments, as well as their ability to release viable phages in the target small intestine.

1.2 Research objectives

1.2.1 To study the entrapment efficacy of phages in Ca-alginate beads and Ca-alginate beads with soy protein isolate (SPI) or cassava starch (CS).

1.2.2 To study the stability of salmonella bacteriophage in modified beads under different temperatures and pH levels.

1.2.3 To study the stability of salmonella bacteriophage in modified beads under simulated gastric fluid and bile salt solution.

1.2.4 To study the release of salmonella bacteriophage from modified beads under simulated intestinal fluid.

1.2.5 To study the stability of salmonella bacteriophage in modified beads for six months at 4°C.

1.3 Research hypothesis

The Ca-alginate beads modified with soy protein isolate (SPI) or cassava starch (CS) have a higher entrapment efficiency of *SalP-p*YM phage compared to pure Ca-alginate beads. The stability of *SalP-p*YM phage entrapped in modified Ca-alginate beads under various temperatures and pHs is higher than that of pure Ca-alginate beads. The stability of *SalP-p*YM phage entrapped in modified calcium alginate beads is higher than pure Ca-alginate beads when evaluated under simulated gastrointestinal conditions. Modified calcium alginate beads are more effective in preserving the long-term stability of *SalP-p*YM phage during storage at 4°C for six months compared to pure Ca-alginate beads. The Ca-alginate beads modified with SPI or CS could be applied by mixing with chicken feed to reduce *S.* Typhimurium contamination.

1.4 Expected results

The study's expected result is that Ca-alginate beads modified with soy protein isolate (SPI) or cassava starch (CS) can enhance the entrapment efficiency and increase the survival number of salmonella phages under high temperature and low pH. The survival of phages in the gastrointestinal tract was increased and their release in the intestine was accelerated.

1.5 References

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CHAPTER 2
LITERATURE REVIEWS

2.1 *Salmonella* spp.

Salmonella, a genus within the *Enterobacteriaceae* family, was first identified by American veterinary pathologist Daniel Elmer Salmon in 1885 during his investigations into the etiology of swine cholera (Bintsis, 2017). The genus is classified into two species: *Salmonella enterica* and *Salmonella bongori*, the latter being primarily associated with cold-blooded animals (Grey, Tee, Phillips, Micalizzi and Armstrong, 2024). *Salmonella enterica* is further categorized into six subspecies: *S. enterica subsp. enterica, salamae, arizonae, diarizonae, houtenae*, and *indica*, as shown in **Figure 2.1**. Notably, nearly all *Salmonella* pathogens affecting humans and domestic animals are classified under *S. enterica* subsp. *enterica* (Fàbrega and Vila, 2013; Bintsis, 2017). The antigenic composition of *Salmonella* plays a crucial role in its classification and pathogenicity. The three primary antigens include: H (flagellar) antigen: determined by flagellar proteins and capable of phase variation, classified into phase 1 and phase 2, allowing *Salmonella* to alternate between these phases. O (somatic) antigen: located on the outer membrane and defined by the structure of lipopolysaccharides (LPS), essential for serogroup classification. Vi (capsular) antigen: a superficial antigen that overlays the O antigen and plays a role in virulence. The O antigen is primarily used for determining serogroups, while the H antigen complex is critical for defining serovars (serotypes) of *Salmonell*a strains (Giannella, 1996).

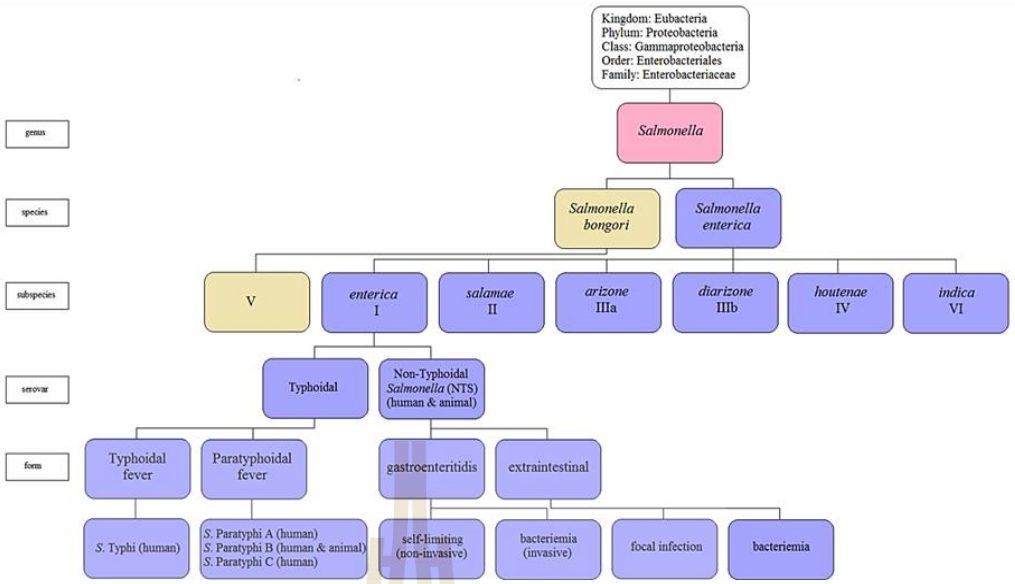

Figure 2.1 The Classification of *Salmonella* according to the most recent classification by the Centers for Disease Control and Prevention (CDC). The genus *Salmonella* is composed of two primary species: *Salmonella bongori* and *Salmonella enterica*.
Source: Wójcicki et al. (2021)

Salmonella is a rod-shaped, non-sporulating, facultatively anaerobic, gram-negative bacterium that exhibits motility through peritrichous flagella. The bacterial cells range in size from 0.7 to 1.5 μm in width and 2.2 to 5.0 μm in length, while colonies typically measure 2–4 mm in diameter (Arachchi and Wanigatunge, 2020; Grey et al., 2024). Metabolically, *Salmonella* ferments glucose and produces gas, utilizing citrate as its sole carbon source. However, it does not ferment salicin, sucrose, or lactose (Crump and Wain, 2016). The cell wall structure of *Salmonella* consists of lipids, lipopolysaccharides (LPS), proteins, and lipoproteins, with endotoxins *present* in the LPS and lipid components (Shaji, Selvaraj and Shanmugasundaram, 2023).

Salmonella grows on blood agar and MacConkey agar and can be selectively isolated using Xylose Lysine Deoxycholate (XLD) agar, Salmonella Shigella Agar (SSA), and Bismuth Sulfite Agar (BSA). On these selective media, *Salmonella* ferments glucose and mannose but does not ferment lactose or sucrose. The bacterium thrives at an optimal temperature of 35°C to 40°C but can survive within a broader range of 2°C to 54°C. While extremely low temperatures inhibit its growth, freezing does not necessarily eliminate the organism. *Salmonella* also exhibits acid resistance, withstanding pH levels as low as 1.0 to 4.6, and demonstrates high resistance to desiccation. Additionally, the bacterium is resistant to heat and alcohol, further

contributing to its environmental persistence and pathogenic potential (Cosby, Cox, Harrison, Wilson, Buhr and Cray, 2015; Oludairo et al., 2022).

Typhoid fever, also known as enteric fever, is a systemic infection caused by *Salmonella enterica* serotype *Typhi* (*S. Typhi*). According to the World Health Organization (WHO, 2023), typhoid fever affects over 9 million people worldwide and results in approximately 110,000 deaths annually. The disease remains a major cause of community-acquired bloodstream infections in south and southeast Asia and can lead to severe, potentially life-threatening complications (Ching, Bansal and Bhandari, 2017). Clinically, patients with typhoid fever typically present with prolonged fever, headache, abdominal discomfort, and general lethargy. Unlike other *Salmonella* serotypes, *S. Typhi* is human-specific and restricted to this host (House et al., 2001).

Nontyphoidal *Salmonella* (NTS) infections are primarily caused by *S.* Typhimurium, *S. Newport*, *S. Heidelberg*, and *S. Javiana* (Bush and Pertejo, 2024). Unlike *S. Typhi*, NTS infections are zoonotic and transmitted through contaminated food and water, particularly poultry, eggs, meat, and vegetables. Transmission can also occur via direct person-to-person contact (Dudhane, Bankar, Shelke and Badge, 2023). NTS commonly causes gastroenteritis, which is typically self-limiting and does not require antimicrobial treatment. However, in some cases, NTS leads to invasive infections, including bacteremia, osteomyelitis, and meningitis, which necessitate antimicrobial therapy (Chen, Wang, Su and Chiu, 2013). The genetic and genomic evolution of *Salmonella* has contributed to increased virulence and multidrug resistance (MDR), making NTS infections a significant public health concern. Antimicrobial resistance (AMR) in NTS varies across serotypes and antibiotics, with *S. Enteritidis* generally exhibiting greater susceptibility to antimicrobial agents compared to other serotypes. In contrast, *S.* Typhimurium shows a high resistance rate and is widely distributed globally (Chen et al., 2013). The rising prevalence of antibiotic-resistant *Salmonella* strains highlights the need for alternative treatment strategies and enhanced surveillance programs to mitigate the impact of AMR.

2.2 Virulence factors of *Salmonella* spp.

The pathogenicity of *Salmonella* is highly complex, involving a network of virulence genes that enable the bacterium to invade host cells, evade immune

responses, and establish systemic infections. Studies on *Salmonella* pathogenicity mechanisms have identified specific genes associated with virulence, which contribute to the bacterium's ability to cause disease (Kombade and Kaur, 2021). The severity of infection varies depending on both the host's physiological state and the virulence of the bacterial strain, which is determined by genetic elements known as virulence factors (Asten and Dijk, 2005).

A key component of *Salmonella* virulence is the presence of *Salmonella* Pathogenicity Islands (SPIs) clusters of virulence-related genes located on the *Salmonella* chromosome. These genetic regions play a crucial role in bacterial invasion, intracellular survival, and systemic dissemination. To date, 17 SPIs (SPI-1 to SPI-17) have been identified. These islands are typically inserted into tRNA genes, exhibit G+C content ratios between 37% and 47%, and display distinct codon usage compared to the core genome. Although the precise origin of SPIs remains unclear, it has been hypothesized that they were acquired through horizontal gene transfer, potentially from bacteriophages or plasmids (Amavisit, 2005; Ramatla et al., 2024; Marcus et al., 2000; Kombade and Kaur, 2021). Several SPIs have been extensively studied due to their critical roles in *Salmonella* virulence. For example:

- SPI-1 encodes genes responsible for host cell invasion and macrophage destruction, facilitating bacterial entry into epithelial cells.
- SPI-2 contains genes that enable *Salmonella* to survive and replicate within macrophages, contributing to systemic infection.
- SPI-3 plays a role in *Salmonella* survival within macrophages and in low-magnesium environments, which are critical for intracellular persistence.
- SPI-4 contains genes suspected to enhance *Salmonella*'s ability to persist within host tissues.
- SPI-5 houses genes involved in the pathogenesis of gastrointestinal infections, promoting inflammation and diarrhea.

The presence and function of these SPIs highlight the intricate molecular mechanisms that enable *Salmonella* to colonize hosts, evade immune responses, and establish infection. Understanding these virulence determinants is essential for

developing targeted strategies to combat *Salmonella* infections and mitigate their public health impact.

The Type III Secretion System (T3SS) is a highly specialized protein complex that plays a critical role in *Salmonella* pathogenesis. This structure consists of multiple subunits, comprising approximately 20 distinct bacterial proteins that collectively form the T3SS apparatus. These structural proteins provide the framework for the secretion system. In addition to these components, *Salmonella* utilizes translocator proteins to facilitate the transfer of bacterial effector proteins into the host cell cytoplasm (**Figure 2.2)**. These effector proteins manipulate host cell processes to promote bacterial survival and infection (Coburn, Sekirov and Finlay, 2007). The T3SS functions as a syringe-like mechanism, enabling *Salmonella* to translocate effector proteins directly into the intestinal epithelial cells of the host. Once inside the host cytosol, these effector proteins modulate signaling pathways to create a more favorable environment for bacterial invasion and persistence. This process is essential for *Salmonella* pathogenicity, as it allows the bacterium to evade immune defenses, establish infection, and maintain intracellular survival (Bao, Wang, Zhao and Liu, 2020; Kaur and Jain, 2012). The structural organization and function of the T3SS underscore its importance as a virulence factor in *Salmonella* infections. Understanding the molecular mechanisms of this system provides valuable insights into pathogen host interactions and may contribute to the development of targeted therapeutic strategies. Bao et al. (2020) have reported that *Salmonella enterica* encodes two T3SS gene clusters, designated as T3SS-1 and T3SS-2, located on SPI-1 and SPI-2, respectively. The T3SS-1 gene cluster plays a critical role during the early phase of infection, facilitating the invasion of intestinal epithelial cells and M cells, as well as initiating proinflammatory responses. In contrast, T3SS-2 is involved in later stages of infection, promoting intracellular survival and replication within host phagocytic cells.

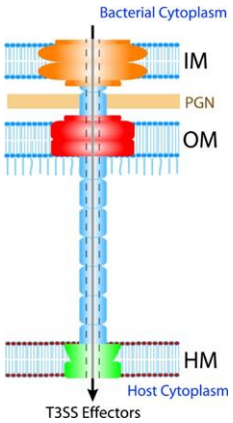

Figure 2.2 The Type III Secretion System (T3SS) consists of ring structures spanning both the inner membrane (IM) and outer membrane (OM) of the bacterium. A hollow cylindrical structure, known as the basal body, connects these membrane-spanning rings to the translocon complex (needle tip), which functions to form a pore in the membrane of the infected host cell (Bao et al., 2020).

Source: Alsubhi, (2021).

Fimbriae, also known as pili, are filamentous surface structures that play a crucial role in *Salmonella* adhesion and colonization. These structures range from 2–8 nm in width and 0.5–10 μm in length and are primarily composed of repetitive, helically arranged proteins known as fibrils. The genes responsible for fimbrial biosynthesis, structure, and assembly are typically organized within a 7–9 kb operon, comprising 8–11 genes (Asten and Dijk, 2005). In *Salmonella*, 13 predicted fimbrial loci have been identified, several of which are expressed in vivo and contribute to biofilm formation, host cell attachment, and intestinal colonization. However, these fimbriae do not appear to be directly involved in intracellular survival (Ibarra and Mortimer, 2009). Humphries, Townsend, Kingsley, Nicholson, Tsolis and Bäumler, (2001) demonstrated that fimbrial-mediated adhesion contributes to the infection process of *Salmonella* Typhimurium in intestinal epithelial cells. Their findings indicate that fimbriae are essential for bacterial invasion of tissue culture cells and may facilitate the targeting and selection of specific host cells during the early stages of infection.

Virulence plasmids are mobile genetic elements that enhance bacterial pathogenicity by encoding virulence factors such as toxins, adhesion molecules, and immune evasion mechanisms. These plasmids are commonly found in pathogenic bacterial strains, including *Escherichia coli*, *Salmonella*, and *Yersinia* (Clark, Pazdernik

and McGehee, 2018). Among *Salmonella* serovars, *S.* Typhimurium, *S. Enteritidis*, and *S. Choleraesuis* are recognized as significant human pathogens due to their possession of virulence plasmids. These plasmids harbor a distinct genetic locus known as the *Salmonella* plasmid virulence (SPV) locus, which comprises the *spvRABCD* gene cluster. The SPV genes play a critical role in promoting bacterial replication within host cells during extra-intestinal infections and contribute to systemic dissemination. In addition, virulence plasmids encode fimbrial genes (*pefBACDI*) and serum resistance factors (*traT*), further enhancing the pathogenic potential of *Salmonella* (Kaur and Jain, 2012). The role of the SPV locus in *Salmonella* virulence has been demonstrated by Silva, Puente and Calva, (2017), who investigated its function in zebrafish larvae infected with *S. Typhimurium*. Their findings revealed that the *spv* operon significantly increases bacterial virulence and survival by suppressing the host's innate immune response. Specifically, *S.* Typhimurium strains carrying an intact SPV locus were shown to inhibit neutrophil and macrophage activity, impairing the host's ability to clear the infection. Moreover, the *spv* genes interfered with early autophagosome formation, effectively neutralizing the host's immune defense mechanisms.

Fimbriae and virulence plasmids collectively play crucial roles in *Salmonella* pathogenicity by facilitating host cell adhesion, immune evasion, and intracellular survival. The SPV locus and its associated genes significantly enhance the bacterium's ability to persist and proliferate within host tissues, making them key factors in systemic *Salmonella* infections. A deeper understanding of these virulence determinants is essential for the development of targeted antimicrobial strategies to combat *Salmonella*-related diseases.

2.3 *Salmonella spp.* outbreaks

Salmonella infection, or salmonellosis, typically has an incubation period ranging from 12 to 72 hours, though in some cases it may extend to 60–120 hours. Outbreaks of salmonellosis are frequently associated with the consumption of contaminated food or water. Numerous animals including poultry, pigs, and cattle serve as reservoirs for *Salmonella* (Wei, Huang, Liao, Liu and Chiou, (2014), posing significant threats to both public health and economies worldwide (Popa and Papa, 2021).

Foodborne outbreaks commonly occur in enclosed communities where centrally prepared meals are served to large groups, such as student dormitories, elderly care homes, prisons, hospitals, and nursing facilities (Kunwar, Singh, Mangla and Hiremath, 2013). The Centers for Disease Control and Prevention (CDC) has identified nontyphoidal *Salmonella* as a major contributor to foodborne illnesses in the United States. Between 2012 and 2019, 27 *Salmonella* outbreaks were linked to beef, resulting in 254 hospitalizations and two fatalities. Ground beef was implicated in 73% of these cases, with non-intact raw ground beef responsible for 44% and intact raw beef for 22% (Canning et al., 2023). Previous studies have also associated *Salmonella* outbreaks with various food items, including eggs, cheese, dry cereals, packaged ice cream, fresh sprouts, fruit juices, cantaloupes, and other fresh produce (Acheson and Hohmann, 2001). Contaminated eggs, even those appearing normal, may carry *Salmonella* due to vertical transmission from infected hens via the ovaries (Vargas et al., 2020).

Most pathogenic strains belong to *Salmonella enterica*, which exhibits host specificity: *S. Enteritidis* is prevalent in poultry, *S.* Typhimurium in horses, *S. Anatum* in cattle, and *S. Weltevreden* in seafood (Karodia, Shaik and Qekwana, 2024). Transmission primarily occurs through ingestion of food or water contaminated with feces. In natural settings, *Salmonella* infects a wide range of animals, including birds, mammals, and humans. Environmental contamination often originates from manure of infected birds, rodents, and other wildlife (Lamichhane et al., 2024). According to Oludairo et al., (2023), approximately 60% of human pathogens are zoonotic, with wildlife playing a critical role in transmission. Of the 1,415 known human infectious agents, 61% are zoonotic, and over 70% of emerging zoonotic diseases originate from wild animal reservoirs. Wildlife can act as asymptomatic carriers of enteric pathogens, increasing environmental contamination risks, especially in water sources. These pathogens may also be transmitted during the handling, processing, or consumption of undercooked wild game. To mitigate the risk of *Salmonella* transmission in animal farming, Relaño et al. (2023) proposed various health control strategies, including acidification of animal feed using probiotics, prebiotics, or phytobiotics. Additional interventions include the use of bacteriophages, vaccination,
and implementation of stringent biosecurity measures particularly in poultry and swine farming.

Food contamination by pathogenic bacteria remains a significant global public health concern. *Salmonella* is consistently identified among the most prevalent agents of foodborne illness (Milho, Silva, Melo, Santos, Azeredo and Sillankorva, 2018). Canning et al. (2023) reported 53 *Salmonella* outbreaks in the United States from 1990 to 2014, resulting in 2,630 illnesses, 387 hospitalizations, and five deaths. Among affected individuals, 85% had contact with chicks, while 38% reported contact with ducklings. High-risk behaviors included keeping poultry indoors (46%) and kissing birds (13%). Popa and Papa, (2021) noted a *Salmonella* outbreak in Chile in 2019, linked to contaminated sushi, which resulted in 80 reported cases. Similarly, in the United States, over 1,000 cases were recorded in August 2019 due to indoor poultry exposure, with an additional 473 cases reported in June 2020. Oh and Park, (2017) highlighted an increasing number of outbreaks linked to diverse food items, including alfalfa sprouts, cucumbers, papayas, mangoes, and even dried goods such as peanut butter, chia powder, and pistachios. From 2008 to 2017, the incidence of *Salmonella* outbreaks linked to fresh produce increased approximately 4.3-fold. These findings emphasize the need for novel strategies to prevent and control *Salmonella* contamination across the food supply chain.

2.4 *Salmonella* Typhimurium

Salmonella Typhimurium is a major etiological agent of foodborne illnesses worldwide, frequently resulting in hospitalizations and fatalities. It is considered a generalist pathogen capable of causing gastroenteritis in both humans and a wide range of mammalian hosts (Won and Lee, 2017). Infection typically occurs via the ingestion of contaminated food or water, enabling the bacteria to traverse the gastrointestinal tract and invade the intestinal mucosa, thereby triggering disease symptoms (Fàbrega et al., 2013). Although it primarily colonizes the intestines of humans and warm-blooded animals, *S.* Typhimurium has also been isolated from reptiles and insects. Major sources of contamination include eggs, meat, dairy products, vegetables, and water (Popa and Papa, 2021). In the United States, *S.* Typhimurium is one of the leading causes of hospitalizations and deaths resulting from foodborne infections. Despite its significant public health burden, no effective vaccine currently exists for the prevention of *S.* Typhimurium induced gastroenteritis. Moreover, the pathogen's increasing resistance to multiple antibiotics limits treatment

options and underscores the urgency of developing novel therapeutic strategies (Makalatia et al., 2021; Anderson and Kendall, 2017; McClelland et al., 2001).

The *S.* Typhimurium LT2 strain, originally isolated in the 1940s, has served as a key model organism in the study of *Salmonella* biology. It was instrumental in the early discovery of phage-mediated transduction. Genomic analysis of the LT2 strain reveals a chromosome consisting of 4,857,432 base pairs (bp) and a virulence plasmid (pSLT) containing 93,939 bp, with both genomic elements exhibiting a G+C content of approximately 53%. Notably, the chromosome includes ribosomal RNA clusters accounting for 7% of its content (McClelland et al., 2001). The pathogenicity of *S.* Typhimurium is largely attributed to virulence factors encoded within *Salmonella* pathogenicity islands (SPIs), which are horizontally acquired gene clusters integrated into specific loci of the bacterial chromosome. To date, 23 SPIs have been identified, with SPI-1 and SPI-2 being the most extensively studied. These two SPIs encode components of the Type III Secretion System (T3SS), a needle-like protein complex often described as a molecular syringe. Through this apparatus, *Salmonella* delivers effector proteins into host cells to manipulate cellular processes, promote intracellular survival, and enhance bacterial replication (Shaji et al., 2023).

2.5 Antibiotic resistance of *Salmonella* bacteria

Antibiotic resistance poses a significant threat to global public health. The molecular mechanisms underlying the emergence of antibiotic resistance are complex, leading to alterations in both the structural and functional characteristics of bacterial cells. In their 2019 AR Threats Report, the Centers for Disease Control and Prevention (CDC) classify antibiotic resistance in drug-resistant, non-typhoidal *Salmonella* and *Salmonella* serotype Typhi as serious threats (Chaudhari, Singh and Kodgire, 2023). The misuse of antibiotics in animal production and clinical practice contributes to this resistance. Experts predict that antimicrobial-resistant pathogens could cause up to 10 million deaths worldwide by 2050 (Vargas et al., 2020). In the 1980s, non-typhoidal *Salmonella* were generally susceptible to antibiotics. In contrast, during the 1990s, clinical investigations by the CDC revealed increasing antibiotic resistance in *Salmonella* (Acheson and Hohmann, 2001).

In 2023, Fatima et al. conducted a study on the prevalence and drug resistance of *Salmonella* in raw meat samples from Lahore, Pakistan. They found

Salmonella in 57 out of 111 samples, including 64.28% in beef, 60% in buffalo and goat meat, and 45.83% in poultry. The researchers identified multiple pathogens, with *Salmonella enterica* serovar Typhimurium being the most common (45.4%). Strain identification using VITEK and 16S rRNA gene sequencing revealed that these *Salmonella* strains were multidrug-resistant (MDR) and extensively drug-resistant (XDR). This underscores the significant presence of drug-resistant *Salmonella* in raw meat from Lahore. Consistent with the findings of Qin et al. (2022), who investigated the prevalence and antibiotic resistance of *Salmonella* Typhimurium in China a major cause of global food poisoning data from 2011 to 2021 were collected from humans, animals, food, and the environment, with isolates mainly from Guangdong, Guangxi, Jiangsu, and Shanghai. Using a random-effects model, they estimated the resistance rate of *S.* Typhimurium to be 75% or higher. Antibiotics showing resistance included tetracycline, ampicillin, sulfisoxazole, and streptomycin. The study highlights the increasing severity of drug resistance in *S.* Typhimurium and emphasizes the necessity for rational antibiotic use and the development of alternative treatments.

2.6 Bacteriophages

Bacteriophages, or phages, are the most abundant entities in the biosphere and represent a ubiquitous aspect of prokaryotic life. Phages are viruses that infect and replicate within bacterial hosts. They have garnered significant interest among scientists due to their role in fundamental molecular biology research, their involvement in horizontal gene transfer and bacterial evolution, and their potential as novel therapeutic agents (Clokie, Millard, Letarov and Heaphy, 2011). Phages were first described in 1915 by Frederick Twort, who observed a substance capable of converting *Micrococcus* colonies into a clear form an effect caused by the destruction of bacterial cells by viruses. In 1917, Félix d'Hérelle reported a bacterial virus, which he named "bacteriophage," that lysed cells of *Shigella* spp. (Cisek, Dąbrowska, Gregorczyk and Wyżewski, 2017). Bacteriophages are classified based on morphology (**Figure 2.3**) and genetics. The majority belong to tailed phages that contain double-stranded DNA (dsDNA) and fall under the order *Caudovirales*. This order includes three families: 1. *Myoviridae*, recognized for their contractile tails; 2. *Podoviridae*,

distinguished by short tails; 3. *Siphoviridae*, characterized by long, non-contractile tails (Giri, 2021).

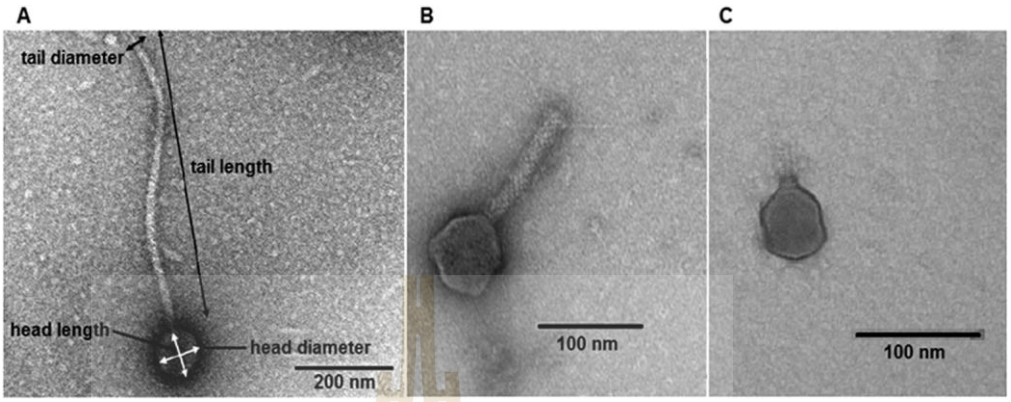

Figure 2.3 classification bacteriophages based on morphology;
A: *Siphoviride*, B: *Myoviridae* and C: *Podoviridae*.
Source: Kurek et al. (2016)

Bacteriophages exhibit two distinct life cycles: lytic (virulent) and lysogenic (temperate) (Britannica, 2023). The lytic cycle is characterized by viral specificity. Initially, lytic phages adsorb to host bacteria by binding their attachment sites to receptor sites on the bacterial surface. Certain bacteriophages employ enzymes to penetrate the bacterial cell wall, enabling them to inject their genome into the cytoplasm. The phage then replicates its genome and hijacks the host's metabolic machinery to synthesize viral enzymes and structural components. These components assemble during maturation, and eventually, a phage-encoded lysozyme degrades the bacterial cell wall, resulting in osmotic lysis and the release of new virions. In contrast, in the lysogenic cycle, bacteriophages integrate their DNA into the host bacterium's genome, becoming a noninfectious prophage. After genome injection, instead of immediately disrupting the host's cellular processes, the phage DNA incorporates into the bacterial chromosome. At this stage, a repressor protein inhibits the expression of genes required for replication. As a result, the prophage replicates passively along with the host genome, ensuring its transmission to all daughter cells (Gary Kaiser, 2024). A diagram illustrating both life cycles is presented in **Figure 2.4**.

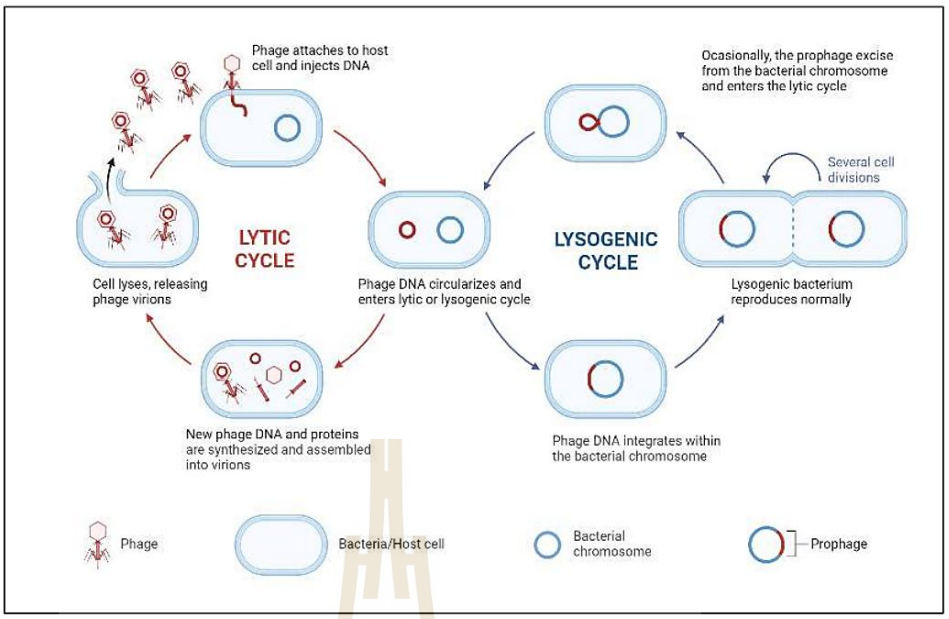

Figure 2.4 Diagram of the bacteriophage life cycle. Lytic cycle: The bacteriophage infects a bacterial cell, resulting in the synthesis of multiple new viral particles. The cycle concludes with the lysis of the host cell, releasing the newly formed virions. Lysogenic cycle: The bacteriophage genome integrates into the host bacterium's chromosome, forming a prophage. In this latent state, the viral DNA replicates passively alongside the host genome without causing immediate damage to the host. **Source:** Alsubhi, (2021).

2.7 Bacteriophages: a solution for bacterial treatment

Bacteriophages were first identified as antibacterial agents in 1915. However, following the discovery of penicillin in 1928 and its mass production in the 1940s, interest in bacteriophage therapy declined. Soon after the beginning of the antibiotic era, an increase in drug-resistant bacteria was observed. Unlike antibiotics, phages specifically target and infect particular bacterial species, making them a promising alternative for the treatment of bacterial infections (Alsubhi, 2021). In addition to their therapeutic potential, phages serve various functions. One such application is phage typing, a method that uses bacteriophages to identify bacterial species based on their specific interactions with bacterial receptors. This technique allows identification at both the genus and species levels (Merwe, Helden, Warren, Sampson and Pittius, 2014). Another application is phage biocontrol, which involves the use of phages to manage bacteria or fungi responsible for plant diseases. This strategy reduces the reliance on chemical treatments in the food industry and helps lower

microbial contamination in fruits, vegetables, and minimally processed foods without compromising sensory qualities such as taste and aroma (Adhya, Merril and Biswas, 2014). Phage therapy, which utilizes lytic phages to eliminate pathogenic bacteria, has proven effective in treating infectious diseases in both humans and animals (Lin et al., 2017).

Phage therapy refers to the use of bacteriophages, particularly lytic phages and their lytic proteins, to eliminate multidrug-resistant (MDR) bacteria. It can be applied either as a standalone treatment or in combination with antibiotics (Hibstu, Belew, Akelew and Mengist, 2022). According to Alsubhi (2021), phages may be employed alone or alongside antibiotic therapy. The first documented application of this treatment occurred in the Soviet Union, where phages were successfully used to treat patients with various bacterial infections. However, early publications mostly in Russian were not widely disseminated in Western scientific communities. Lin et al. (2017) highlight several advantages of phage therapy over conventional antibiotics, including host specificity, self-amplification, biofilm degradation, and low toxicity to humans.

At the Eliava Phage Therapy Center in Tbilisi, phages have been used to treat infections caused by *Enterococcus faecalis* (various serovars), *E. coli*, *Proteus vulgaris*, *P. mirabilis*, *Pseudomonas aeruginosa*, multiple *Salmonella* serovars, *Shigella flexneri*, and *Shigella sonnei*, with positive outcomes (Cisek et al., 2017). Several reviews, such as Liang et al. (2023), support the application of phage therapy in China, where significant therapeutic efficacy has been demonstrated in both veterinary and human clinical settings. Recent research by Young, Hall, Merabishvilli, Pirnay, Clark, and Jones, (2023) investigates phage therapy in diabetic patients at high risk of limb amputation. Patients consented to receive phage therapy alongside antibiotics, with phage solutions used to irrigate wounds instead of saline. The study reported positive outcomes in 9 out of 10 patients, showing improved infection control and wound healing without side effects. Nevertheless, limitations remain, as phages are sensitive to environmental stressors. According to Ranveer et al. (2024), phages can be inactivated at high temperatures and low pH, posing challenges for storage and application.

2.8 Salmonella bacteriophages

Salmonella is a foodborne pathogen responsible for significant disease outbreaks in both humans and animals. The pathogen has developed resistance to many commercially available antibiotics, presenting a substantial public health challenge. In contrast, salmonella-specific bacteriophages offer a promising alternative due to their high specificity, strong lytic activity, and safety for consumption (Baskaran and Karthik, 2022). More than 90% of salmonella phages isolated from the environment are tailed, lytic phages belonging to the order *Caudovirales* (Ackermann, 2007; Baskaran and Karthik, 2022). These phages are morphologically classified into three families: Myoviridae: These phages have icosahedral heads measuring approximately 60.7 nm and contractile tails. The tails measure 157 × 17 nm when uncontracted and include a sheath measuring 150 × 17 nm with 4 nm cross-striations when contracted. These phages lack collars and base plates and typically produce small plaques (<1 mm) without halos. Siphoviridae: These possess icosahedral heads (~62.5 nm) and long, rigid, non-contractile tails (120 × 7 nm) with 4 nm striations. Their tails feature a 20 nm wide baseplate with spikes and often form plaques with halos. Podoviridae: These also have icosahedral heads (~62.5 nm) and short tails (~13 nm) with a 20 nm wide base plate bearing three prongs. The plaques range from pinpoint size to 1 mm and are often accompanied by halos (Lappe, Doran, O'connor, O'hare and Cormican, 2009).

Non-typhoidal *Salmonella* is a leading cause of human diarrhea, with poultry being the primary reservoir. Therefore, eliminating *Salmonella* from poultry farms is vital for improving public health and food safety. Effective phage-based biocontrol depends on the isolation of lytic phages with high titers and broad host ranges (Gunathilake et al., 2024). To expand the phage library and ensure phage safety, continuous isolation of new phages is essential (Unverdi, Erol, Kaskatepe and Babacan, 2024). Mhone et al, (2022) examined the stability of ten *Salmonella enteritidis* phages isolated from Kenyan poultry farms under different pH levels, temperatures, and simulated gastric and intestinal fluid (SGF and SIF) conditions. Their results indicated a significant loss of phage activity at pH 2–3, while stability was

maintained at pH 5–9 and temperatures between 25–42°C for up to 12 hours. In SGF, infectivity declined after 20 minutes, whereas phages remained stable in SIF for up to two hours. Consistent with these findings, Kwaśnicka et al, (2020) reported two newly isolated bacteriophages vB_Sen-TO17 and vB_Sen-E22 from chicken feces. These phages infected multiple *Salmonella enterica* serovars and exhibited robust lytic activity. They efficiently adsorbed to host cells (>95%) within 10 minutes at 42°C and demonstrated stability across a wide range of environmental conditions, including pH levels 3–12, temperatures from −80°C to 60°C, and exposure to ethanol, chloroform, and DMSO. These features highlight their potential for phage therapy targeting *S. enterica* in poultry.

Several studies have explored the application of salmonella phages for pathogen control in poultry. Pelyuntha, Ngasaman, Yingkajorn, Chukiatsiri, Benjakul and Vongkamjan, (2021) isolated phages from various environmental sources at a broiler farm in Songkhla Province, including cloacal swabs, rice husk bedding, and wastewater treatment ponds. Phage cocktails formulated from these isolates effectively targeted five *Salmonella* serovars *Kentucky, Saintpaul*, *Schwarzengrund, Corvallis*, and Typhimurium reducing bacterial counts by 2.2–2.8 log units within six hours. Importantly, no resistance to the phage cocktail was observed. Similarly, Kumar et al. (2020) investigated phage NINP13076, isolated from sewage water, for its ability to reduce *Salmonella* contamination. Treatment with this phage significantly decreased *Salmonella* levels on raw chicken skin (from 6.7 to 5.4 log CFU/g in 3 hours) and in carrot salad (by 1 log CFU/g in 4 hours). These results support its potential use as a biocontrol agent in food safety applications. However, several challenges limit the commercial development of phage therapy. For instance, producing phages free from integrase genes, antibiotic resistance genes, or phage-encoded toxins remains difficult (Principi et al., 2019).

2.9 Extrusion technique

Microencapsulation techniques are commonly divided into three main categories. The first includes physical approaches like spray drying, extrusion, freeze-drying (lyophilization), supercritical fluid precipitation, and solvent evaporation. The second group comprises physico-chemical methods, such as coacervation, liposome formation, and ionic gelation. Lastly, chemical techniques involve processes like

interfacial polymerization and the formation of molecular inclusion complexes (Łętocha, Miastkowska and Sikora, 2022).

Extrusion techniques (external gelation), which include air jet, nozzle extrusion, centrifugation, electrostatic, and vibrational extrusion, have been widely employed in the formation of hydrogels. Among these methods, the extrusion or dripping technique is the most commonly utilized approach for producing alginate-based encapsulated hydrogels (**Figure 2.5**). In this technique, a suspension of hydrogel precursor is extruded through a nozzle, generating droplets that subsequently fall into a gelling solution typically containing divalent cations such as calcium chloride ($CaCl_2$) either by gravitational force or with external assistance. The size of the resulting microspheres is influenced by several parameters, including the concentration of the solution, the nozzle diameter, and the surface tension of the droplets (Paiboon, Surassmo, Ruktanonchai, Kappl and Soottitantawat, 2023; Lee, Ma, Khoo, Abdullah, Kahar and Hamid, 2021; Lee, Ravindra and Chan, 2013).

Bhushan, Parshad, Qazi and Gupta, (2008) demonstrate that immobilizing Arthrobacter sp. (ABL) cells, which produce lipase, within calcium alginate microspheres using the extrusion method enhances the enzyme's stability across a range of temperatures, pH levels, and storage durations when compared to the free enzyme. Similarly, Machado, Silva, Vicente, Soares, Pinheiro and Cerqueira, (2022) report that an extract of Spirulina sp. LEB-18 encapsulated using calcium alginate beads via the same technique shows higher bioaccessibility of phenolic compounds than in their free form. These studies indicate that the extrusion method is effective in protecting and maintaining the functional properties of bioactive substances.

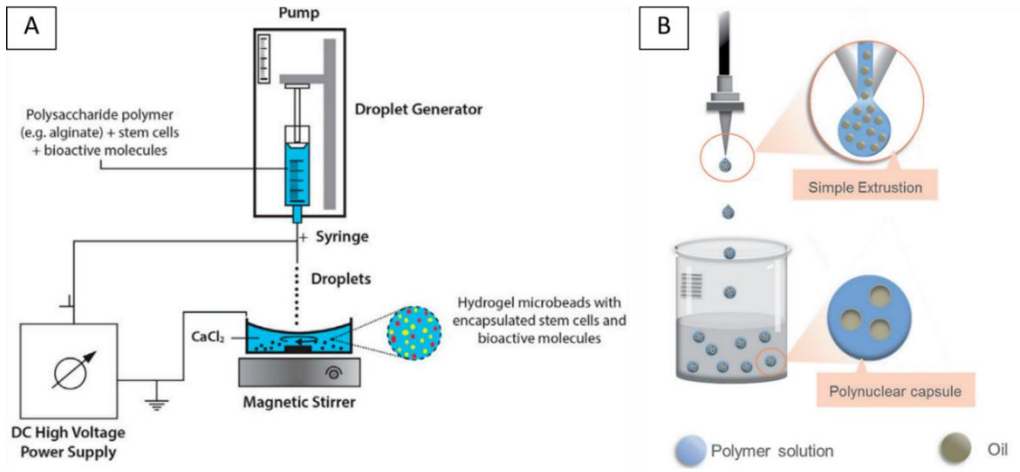

Figure 2.5 illustrates the encapsulation of an active compound within a hydrogel matrix, demonstrating two extrusion methods: (A) the electrostatic or electrospray droplet extrusion approach, and (B) the nozzle-based extrusion technique.
Source: Martins, Poncelet, Rodrigues and Renard, (2017) and Lee et al. (2021)

2.10 Production of Ca-alginate beads by the extrusion technique.

Alginate is a linear, anionic, water-soluble, and hydrophilic polysaccharide composed of 1,4-linked β-D-mannuronic acid (M-block) and 1,4-linked α-L-guluronic acid (G-block). These monomeric units can appear in homopolymer regions (M-blocks or G-blocks) or as alternating M-G sequences (**Figure 2.6**). One of alginate's most notable properties is its ability to form hydrogels in the presence of divalent cations, most commonly calcium ions (Ca^{2+}). The G-blocks in alginate chains preferentially bind with Ca^{2+} ions to form a cross-linked structure known as the "egg-box" model, which contributes significantly to gel strength (**Figure 2.7**) (Gajić, Savić and Svirčev, 2023; Tomić, Radić, Vuković, Filipović, Runic and Vukomanović, 2023). In contrast, M-blocks exhibit weaker interactions with divalent cations (Zhou et al., 2022). Alginate has been widely used due to its favorable characteristics, including biocompatibility, biodegradability, and non-toxicity. It is generally recognized as safe for biomedical and pharmaceutical applications. Among natural polymers employed for the preparation of microparticles, alginates have gained considerable attention owing to their excellent gelling behavior, viscosity, water retention capacity and stabilizing properties, which support their use in various industrial processes. Alginate microparticles are particularly suitable for encapsulation purposes, as they are environmentally benign and compatible with living systems (Łętocha et al., 2022; Frent et al., 2022).

The formation of alginate beads occurs through gelation, which may involve either covalent or ionic crosslinking. However, ionic crosslinking is more widely utilized because it offers a simpler process under mild conditions, typically performed at room temperature or up to 100°C. Notably, only the G-block segments, composed of consecutive guluronic acid residues, participate in the crosslinking process owing to their favorable three-dimensional configuration. This mechanism is often described by the "egg-box" model, which illustrates the coordination between divalent cations and G-blocks of neighboring polymer chains (Zhang, Grossier, Candoni and Veesler,

2020). Divalent cations such as calcium, barium and magnesium are commonly used as ionic crosslinkers to form ionic bridges between guluronic acid (G) units in the alginate polymer chain (Lee et al., 2021).

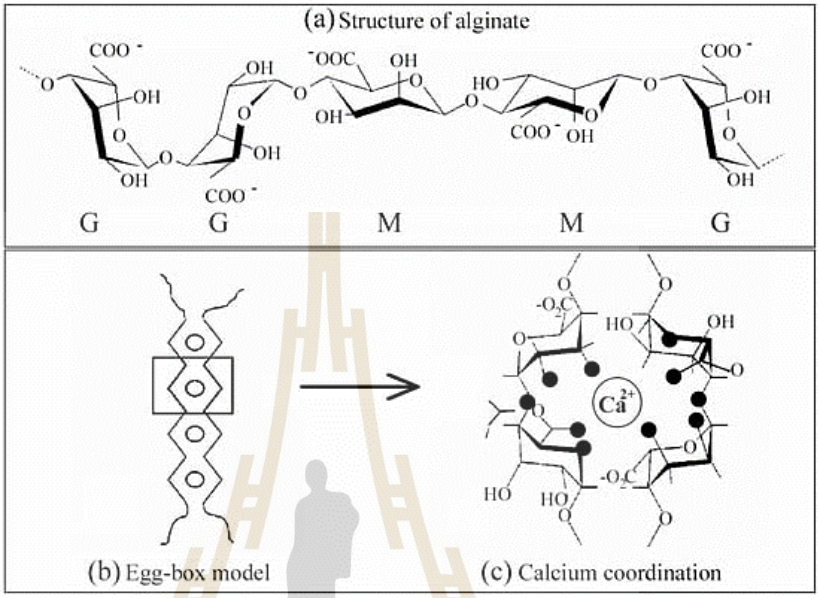

Figure 2.6 The structure of alginate and its gelation mechanism. (a) Schematic representation of M-blocks (β-D-mannuronic acid) and G-blocks (α-L-guluronic acid) in alginate chains; (b) The "egg-box" model illustrating cross-linking between G-blocks and Ca^{2+} ions; (c) Calcium coordination sites involved in the formation of alginate hydrogels.

Source: Hurtado, Aljabali, Mishra, Tambuwala and Aroca, (2022)

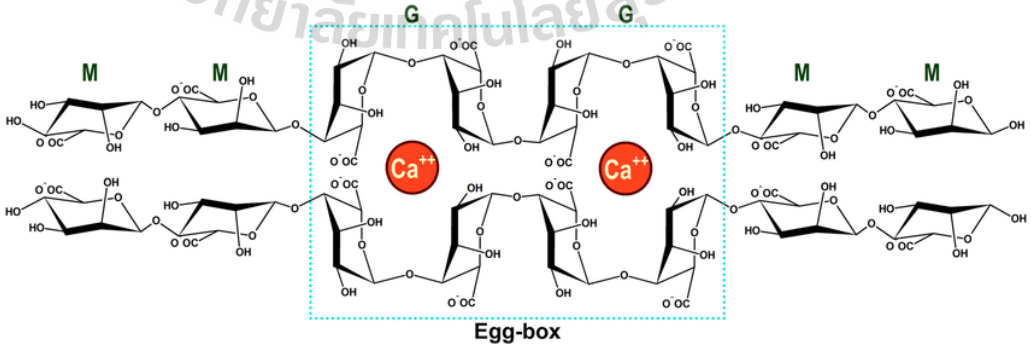

Figure 2.7 The gelation process of alginate is explained through the "egg-box" model, which illustrates the interaction between calcium ions (Ca^{2+}) and the alginate polymer chains. Calcium ions substitute sodium ions (Na^{+}) in the alginate matrix and subsequently form crosslinks by binding with guluronic acid blocks on adjacent alginate chains.

Source: Lee et al. (2019)

The mechanism underlying alginate gelation in the presence of divalent cations, such as calcium ions (Ca^{2+}), is well described by the "egg-box" model. In this model, guluronic acid (G) residues along alginate chains align in a way that allows them to chelate divalent ions within a structured cavity. These cavities are specifically formed by sequences rich in GG-blocks, which enable a highly specific and stable binding site for the cations. The cross-linking occurs as each Ca^{2+} ion simultaneously coordinates with carboxyl groups from adjacent G residues on two opposing polymer chains, resulting in a three-dimensional network that forms the basis of the hydrogel structure. It has been observed that at least 8 to 20 contiguous G residues are typically required to generate a mechanically stable and cohesive hydrogel network (Colin, Akpo, Perrin, Cornu and Cambedouzou, 2024).

2.11 Entrapment

Entrapment is a widely employed technique for the immobilization of materials and is considered one of the most popular methods for the preparation of immobilized systems. This approach involves incorporating target materials, such as biomass, into carrier matrices to form discrete particles or defined structures. The entrapment process serves to prevent material leakage and protect the entrapped substances from unfavorable environmental conditions. Based on the nature of the carrier system, entrapment methods can be classified into two main categories: gel entrapment and semipermeable membrane or capsule entrapment. This approach helps prevent leakage and protects the encapsulated material from adverse environmental effects. Common carriers and matrices used in entrapment include organic polymers, silica, membrane devices, and microcapsules (Schmidt and Mosbach, 1977; Liu, Yang, Zhang, Tang, He and Liang, 2022).

Entrapment is characterized by the immobilization of a material commonly termed the payload, active internal phase, or core within the structural framework of another substance, forming particles of nanoscale (nanoencapsulation), microscale (microencapsulation), or even millimeter dimensions. The enclosing material is referred to by various terms, including coating, membrane, shell, capsule, exterior phase, or matrix (Al, Saeinasab and Sefat, 2022). Entrapment has gained widespread

application across numerous fields. Rivera, Talavera, Jiménez, Cruz, Bernardino and Pacheco, (2021) described microbial entrapment, also known as bioencapsulation, as embedding microorganisms within a semi-permeable matrix that facilitates the transfer of nutrients and bioactive agents between the internal and external environments. While such structures are predominantly spherical, they can also appear in tubular or oval forms of varying sizes.

The main advantages identified in numerous studies include the protection of active ingredients from degradation (e.g., heat, acid, enzymes), controlled release, enhanced stability and shelf life, reduced side effects (e.g., some drugs do not irritate the stomach), and targeted delivery. Moreover, these materials have been shown to remain stable and can be utilized in various forms, including pure compounds, crude extracts, enzymes, whole cells, and microorganisms (Kailasapathy, 2009; Siddiqui, Singh, Bahmid, Mehany, Shyu and Assadpour, 2023; Pegg and Shahidi, 2007). Despite its benefits, entrapment has limitations. For instance, polymers may undergo denaturation, leading to leakage of the encapsulated substances. Furthermore, microencapsulation may hinder diffusion processes (**Figure 2.8**) (Schmidt and Mosbach, 1977; Homaei, 2015). Examples of situations where probiotic microorganisms confer health benefits when present in appropriate amounts (Nedović, Kalušević, Manojlović, Lević and Bugarski, 2010). However, their viability is often compromised by environmental stresses, including fluctuations in pH, mechanical force, transport conditions, and digestive enzymes. Entrapment provides a protective mechanism that mitigates these challenges without adversely affecting food texture or taste. It also enhances the survival of sensitive microorganisms, such as *Lactobacilli* and *Bifidobacteria*.

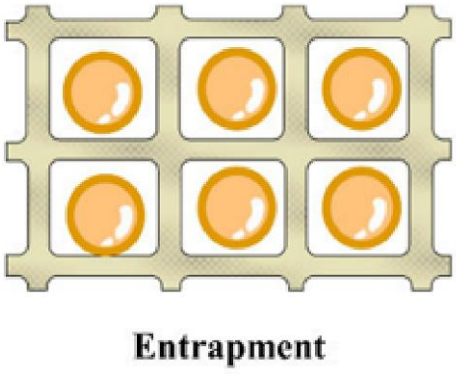

Figure 2.8 Illustration showing entrapment methods.

Source: Liu et al. (2022).

Gel entrapment is a form of irreversible immobilization in which materials are entrapped within polymer matrices or fibers. These matrices typically feature a lattice structure that allows the diffusion of substrates and products while preventing the escape of immobilized materials. Natural polymers such as alginate, chitosan, and agar are frequently employed in this context due to their capacity to form gels at relatively low temperatures (Chaudhary, Rana, Vaidya, Ghabru, Rana and Dipta, 2019). An effective entrapment method should ensure the retention of the encapsulated substance and maintain mechanical, chemical, and biological stability (Freeman, 1986). Recent studies have demonstrated the application of entrapment in food packaging. Alves, Cerqueira, Pastrana and Sillankorva, (2020) investigated the incorporation of bacteriophages and cinnamaldehyde (CNMA) into sodium alginate-based films to inhibit *Salmonella enteritidis* and *Escherichia coli*. The results revealed that CNMA did not impair phage activity, and the combined treatment effectively reduced bacterial populations. These findings underscore the potential of entrapment technologies in enhancing food safety.

The global rise in antibiotic resistance has revived interest in the therapeutic application of bacteriophages. However, phages are inherently unstable in solution and are vulnerable to degradation during processing and storage (Malik, Sokolov, Vinner, Mancuso, Cinquerrui and Vladisavljevic, 2017). Entrapment addresses these issues by protecting phages from the harsh gastric environment, including low pH and enzymatic activity. Entrapment is typically achieved using alginate-based microspheres or liposomes. Several studies have confirmed the viability of phage entrapment in food systems. For example, the integration of *E. coli* specific phages into whey protein isolate (WPI) films and chitosan matrices has been shown to preserve phage activity throughout storage (Alves et al., 2020). Encapsulated phages also exhibited enhanced antimicrobial efficacy when applied to vegetable surfaces, meat, and fish feed. Encapsulation of phage K in alginate has demonstrated significant antibacterial activity, particularly under acidic gastric conditions, where it outperformed non-encapsulated phages (Loh, Gondil, Manohar, Khan, Yang and Leptihn, 2021). Moreover, a phage cocktail encapsulated in alginate/$CaCO_3$ microcapsules was effectively used in broiler chickens infected with *Salmonella*. Pulit, Mituła, Śliwka, Łaba and Skaradzińska, (2015) further reported that phages

encapsulated in biopolymer matrices such as alginate and pectin, with optional oleic acid emulsification and high-methoxyl pectin coating exhibited high encapsulation efficiency (1.2×10^7 PFU/bead). These encapsulated phages retained infectivity after 30 minutes at pH 1.6, demonstrating superior resistance to pepsin digestion compared to free phages.

2.12 Entrapment with alginate beads

A major challenge in phage delivery systems is maintaining phage viability during processing, storage, and gastrointestinal transit. This limitation has prompted the development of various encapsulation strategies, including nanosomes, liposomes, and hydrogel-based systems. Among these, alginate (ALG) has emerged as a preferred biopolymer due to its biocompatibility, affordability, and low toxicity (Garcia et al., 2021). Alginate is a linear, anionic, water-soluble, and hydrophilic polysaccharide. Alginate is predominantly extracted from brown seaweeds belonging to the Phaeophyceae family, although certain bacterial genera, such as *Pseudomonas* and *Azotobacter*, also produce it. One of alginate's most notable properties is its ability to form hydrogels in the presence of divalent cations, most commonly calcium ions (Ca^{2+}) (Gajić et al., 2023; Tomić et al., 2023). Alginate is considered a highly effective material for entrapment applications. It exhibits resistance to acidic gastric conditions and allows for controlled release of live phages into the small intestine (Zhou et al., 2022). However, a key limitation of alginate gels is their relatively low mechanical strength, which can compromise their protective capability in harsh environments. To improve gel robustness, the typical method involves dropping alginate solution into a calcium chloride ($CaCl_2$) bath, which induces ionic cross-linking and significantly enhances the mechanical stability of the resulting beads (Ching et al., 2017). Nevertheless, this approach alone may not sufficiently shield phages from extreme gastric acidity. For this reason, the incorporation of additional polymers in combination with alginate has been recommended to enhance protective effects and overall encapsulation efficiency (Samtlebe, Ergin, Wagner, Neve, Küçükçetin and Franz, 2016).

2.12.1 Alginate beads modified by soy protein isolate and cassava starch

In 2022, Zhou et al. reported an early study on the encapsulation of bacteriophages using an ionic gelation technique, where calcium ions served as cross-linking agents. In this work, *Salmonella* spp. Felix O1 phages were successfully encapsulated within a matrix composed of chitosan, alginate, and calcium chloride. The encapsulated phages were shown to be safely delivered to the small intestine, demonstrating the potential of this method for targeted delivery applications (Garcia et al., 2021).

Similarly, Zhang et al. (2015) investigated the encapsulation of *Enterococcus faecalis* HZNU P2 using a composite of alginate and soy protein isolate (SPI). The study evaluated the survival of the encapsulated bacteria in simulated gastric fluid (SGF) and their release behavior in simulated intestinal fluid (SIF). At low pH values (2.5 and 2.0), the viability of encapsulated *E. faecalis* HZNU P2 remained stable after two hours of incubation, while the viability of free cells declined from 11 to 9.85 log CFU/mL. In SIF, the encapsulated bacteria were completely released within one hour, indicating effective protection and targeted release. Volić et al. (2018) explored the encapsulation of thyme essential oil using alginate–SPI beads formed by electrostatic extrusion followed by gelation with calcium ions. Alginate and SPI concentrations ranged from 1–2.5 wt.% and 0–1.5 wt.%, respectively. The beads were air-dried at room temperature until they reached a constant mass. The encapsulation efficiency, based on total polyphenol content, ranged from 72% to 80%. In SIF, 42% to 55% of the thyme essential oil was released over 2.5 hours, accompanied by bead swelling and partial matrix degradation. Consistent with these findings, Jin et al. (2023) reported that calcium alginate–SPI microgels exhibited higher encapsulation efficiency and greater storage stability of β-carotene compared to calcium alginate microgels alone. The addition of SPI resulted in enhanced resistance to SGF, evidenced by reduced gel size shrinkage in gastric conditions and minimized swelling in intestinal conditions. These results suggest that incorporating SPI into alginate-based systems improves the performance of controlled-release formulations and enhances protection in gastrointestinal environments.

Several studies have investigated the combination of cassava starch (CS) and alginate for use in encapsulation systems. Riyajan, (2017) highlighted the potential of this biopolymer mixture in various delivery applications. Vazquez et al. (2015)

explored the modification of alginate beads by incorporating different weight ratios of cassava starch (alginate: cassava starch; 1:0, 0.75:0.25, 0.5:0.5, and 0.25:0.75) for the encapsulation of chlorogenic acid (CGA). Among the tested formulations, the beads containing the highest cassava starch content (0.25:0.75) demonstrated the most effective control of CGA release under simulated gastrointestinal conditions. This outcome was attributed to increased viscosity resulting from the higher starch content, which reduced CGA diffusion and minimized leakage from the matrix. More recently, Fang et al. (2022) developed a novel oral drug delivery system for cancer therapy using carboxymethyl cassava starch–alginate beads enriched with magnesium ferrite ($MgFe_2O_4$) nanoparticles for the encapsulation of doxorubicin (Dox). In vitro experiments revealed that the beads remained stable in simulated gastric fluid (SGF) while enabling sustained drug release in simulated intestinal fluid (SIF). Additionally, cytotoxicity assays and confocal laser scanning microscopy showed that the delivery system did not adversely affect normal cells, but exhibited selective cytotoxic effects against colon cancer cells (HCT116). These findings support the application of cassava starch alginate-based systems in targeted and controlled drug delivery.

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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.

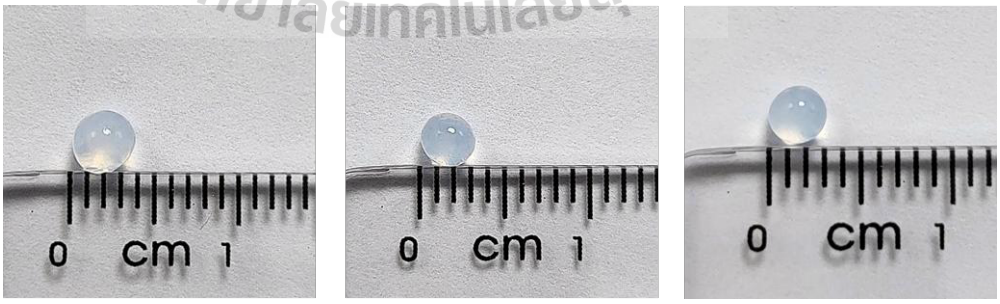

Figure 3.1 Illustration of Ca-alginate beads. A: 0% SPI, B: 0.15% w/v SPI and C: 0.3% w/v SPI.

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.

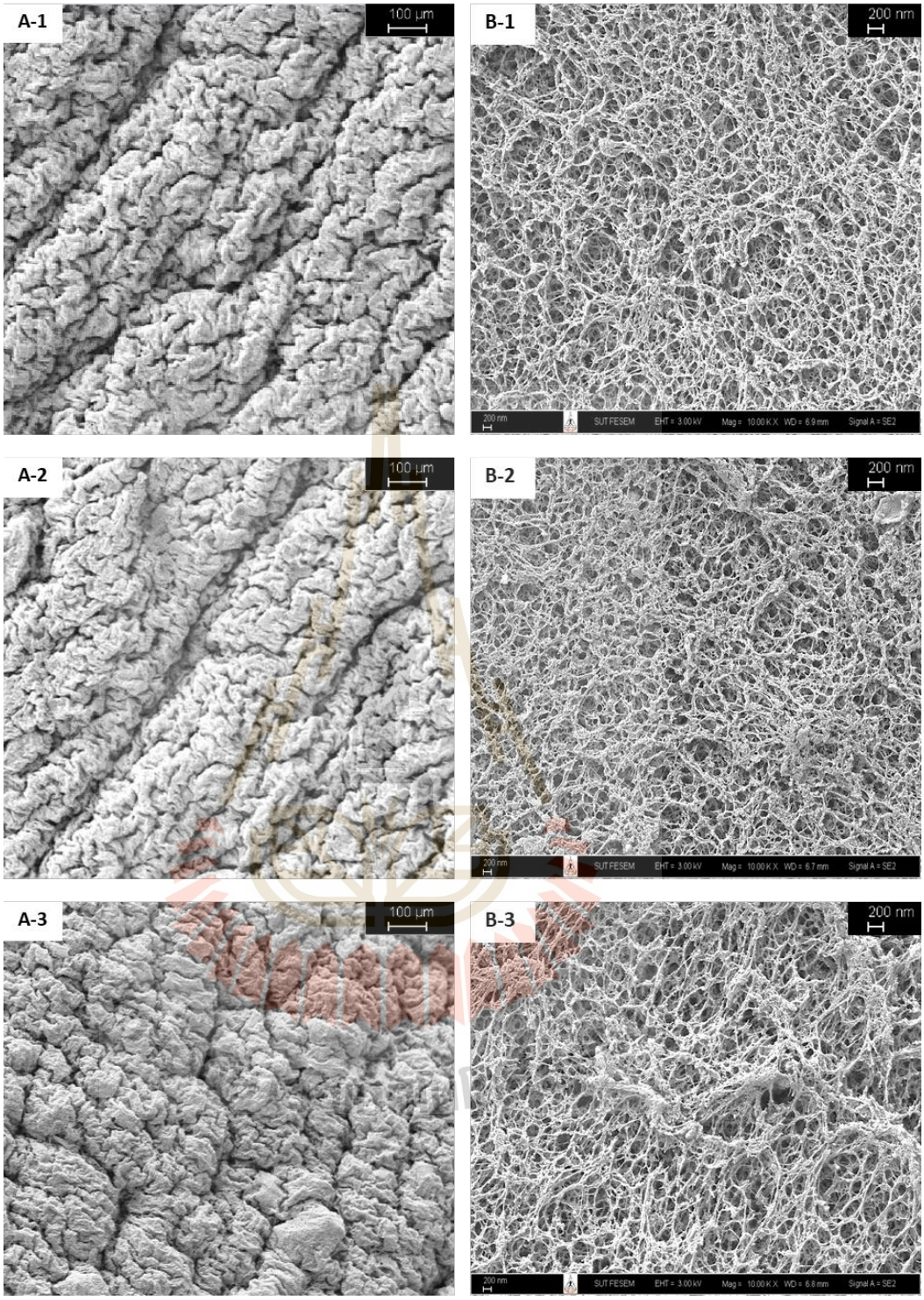

Figure 3.2 The SEM image surface and internal structure of Ca-alginate beads. Images A is surface structure at 5000x magnification and images B is internal structure at 10000x magnification. 1: 0% w/v SPI, 2: 0.15% w/v SPI and 3: 0.3% w/v SPI.

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.

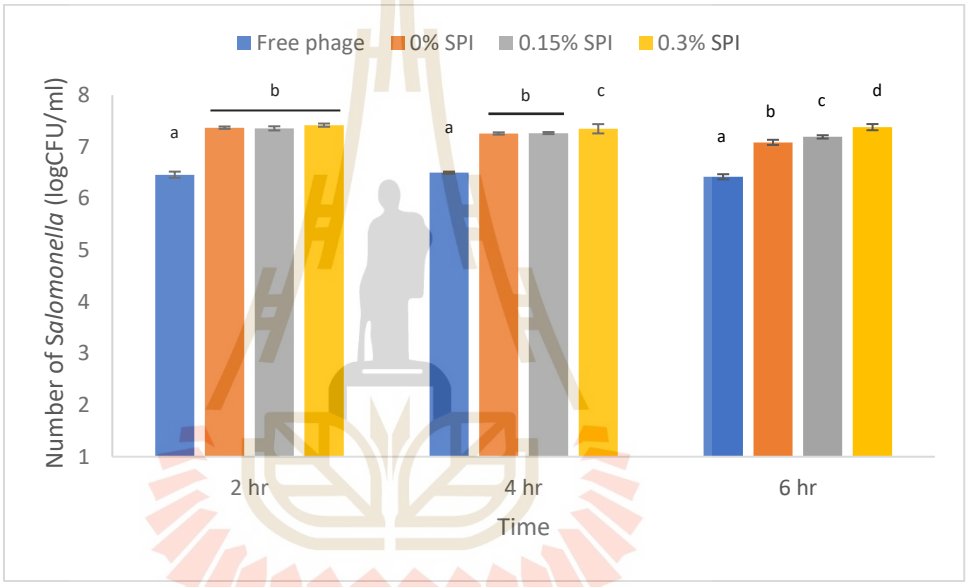

Figure 3.3 Lytic of entrapped salmonella phage on number of *Salmonella* Typhimurium; The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different at the same time ($p \leq 0.05$).

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

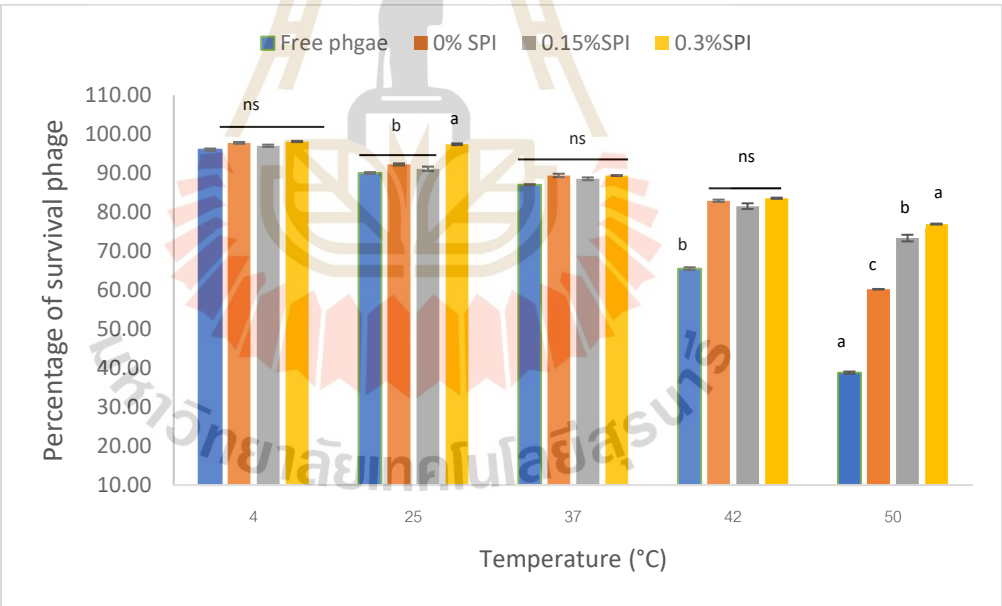

Figure 3.4 The survival of salmonella bacteriophage in the modified bead at various temperatures. The results corresponded to the mean ± standard deviation (n=3). ns means not significantly different, different letter means significantly different among the same temperature ($p \leq 0.05$).

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.

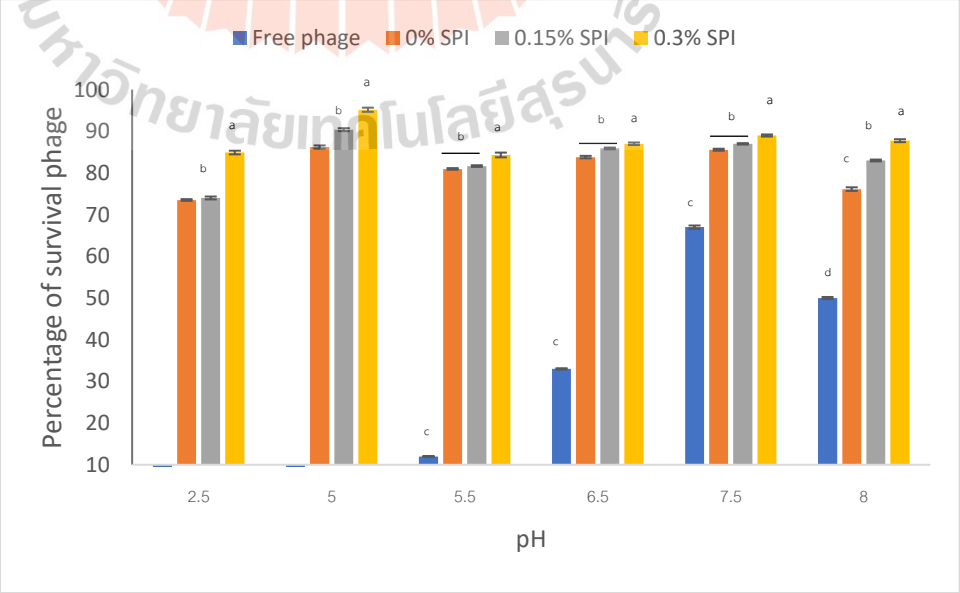

Figure 3.5 The stability of salmonella bacteriophage in Ca-alginate bead under various pHs. The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different at same pHs ($p \leq 0.05$).

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

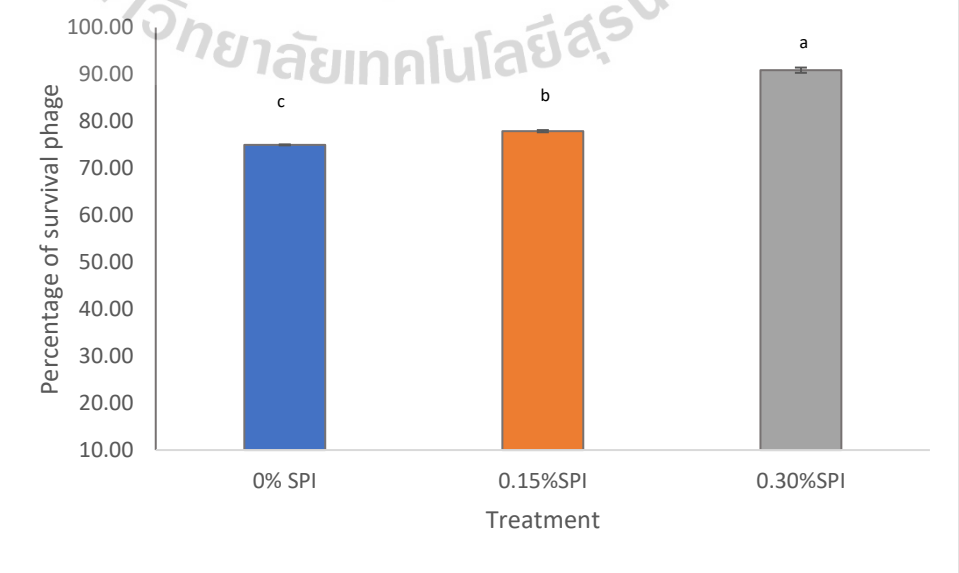

Figure 3.6 The number of phages remaining in Ca-alginate beads were incubated in bile salt. The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different among the same condition ($p \leq 0.05$).

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).

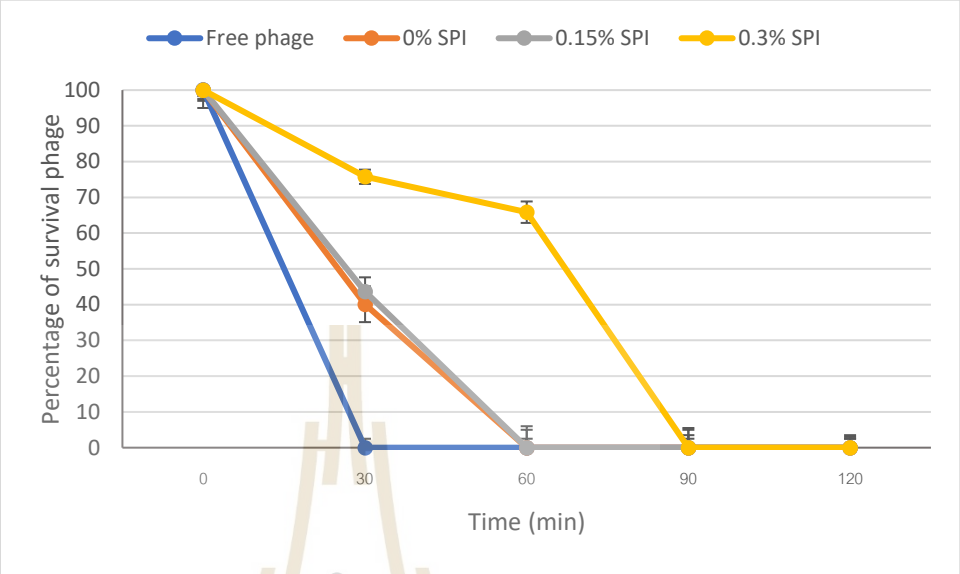

Figure 3.7 The survival of salmonella phage in the modified beads under SGF. The results corresponded to the mean ± standard deviation (n=3).

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).

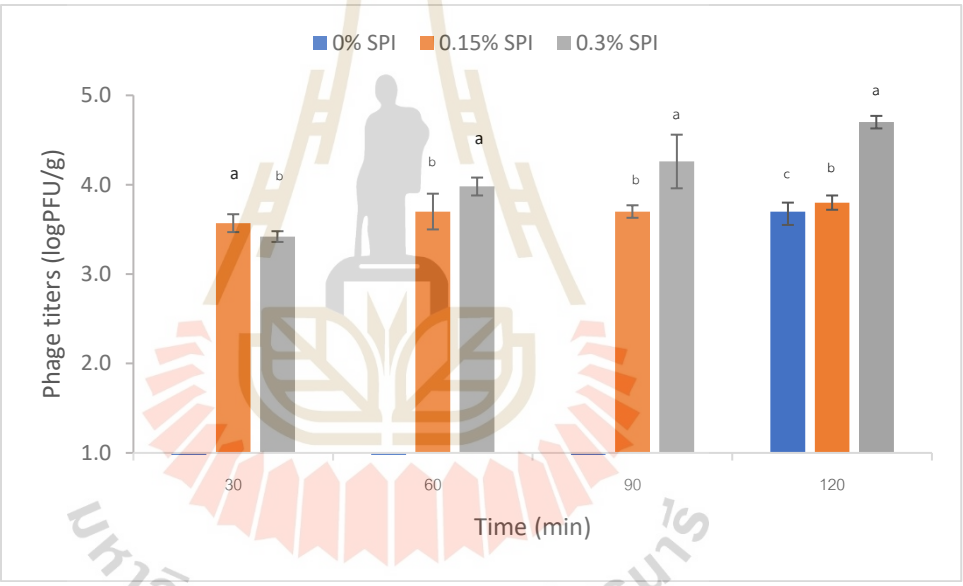

Figure 3.9 The release of phages was entrapped with Ca-alginate beads in simulated intestinal fluid at pH 6.8. The results corresponded to the mean ± standard deviation (n=3), different letter means significantly different under the same time ($p \leq 0.05$).

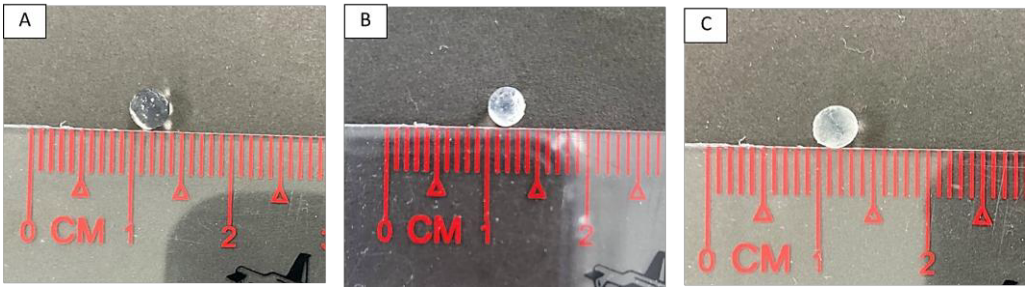

Figure 3.10 Morphology of Ca-alginate beads after in vitro incubation in simulated intestinal fluid (SIF) for 120 min. A is Ca-alginate beads without SPI, B is Ca-alginate beads wit 0.15% w/v SPI and C is Ca-alginate beads wit 0.3% w/v SPI.

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.

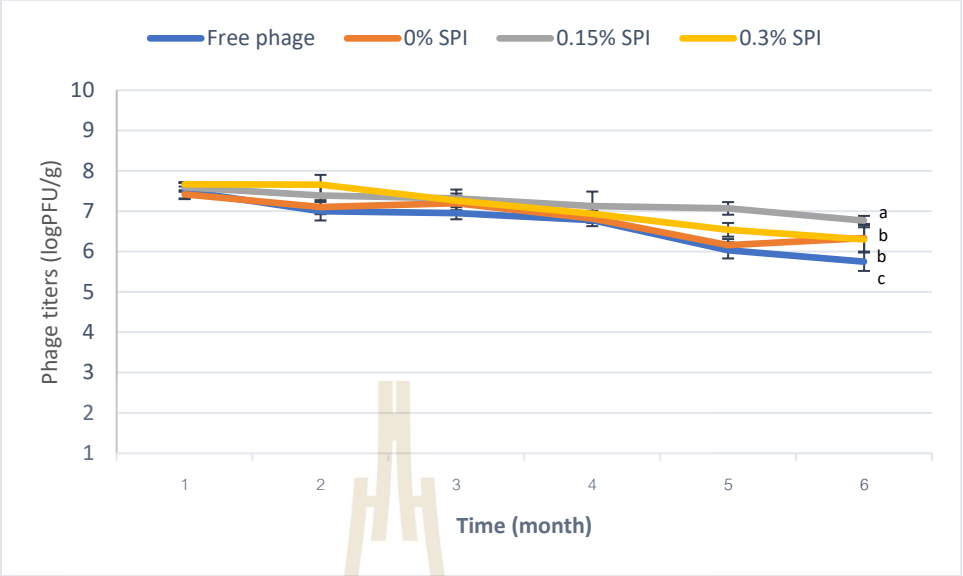

Figure 3.11 Phage survival in the Ca-alginate beads during storage at 4 °C for 6 months.

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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CHAPTER 4

EFFECT OF CASSAVA STARCH ADDED IN CA-ALGINATE BEADS ON STABILITY ENTRAPPED *vB_salP-p*YM BACTERIOPHAGE.

4.1 Abstract

This study investigated the effect of cassava starch (CS) incorporation on the stability and entrapment efficiency of salmonella bacteriophage *vB_salP-p*YM in Caalginate beads under various conditions. All treatments showed high entrapment efficiency (>97%), indicating that the addition of CS did not significantly affect the entrapment efficiency. The morphological shape of Ca-alginate beads with/without CS was illustrated by SEM. These results showed that the addition of CS in Ca-alginate beads was able to increase the porosity and pore size of the internal structure. The addition of CS in Ca-alginate beads could improve the stability of phages at high temperature, acidic and alkaline pH better than only Ca-alginate beads. In addition, the CS incorporated with Ca-alginate could support the phage stability exposure to SGF and also accelerate phage release in SIF that are targeted for colonization of salmonella phages. The entrapped phages in Ca-alginate with CS prolonged the shelf life of phages during six months of storage. These results suggested that cassava starch could be a promising biopolymer addition for improving the stability and application of alginate entrapped bacteriophages, with potential applications in therapy and food safety.

Keywords: Cassava starch, Ca-alginate beads, Salmonella bacteriophage, Entrapment.

4.2 Introduction

Bacteriophages, or phages, are viruses that infect and lyse bacteria through rapid replication within their hosts. Consequently, phage therapy has been proposed as a method to control *Salmonella* infections in poultry. Numerous studies have demonstrated the efficacy of phages in reducing colonization of *Salmonella enterica* in chickens (Colom, Sarabia, Otero, Cortés, Maspoch and Llagostera, 2015). Bacteriophage have been increased research as an alternative method for pathogen

control for phage therapy in animals and processing aids in the food industry for phage therapy for enteric bacterial infections in animals was interesting. The challenge of phage therapy was oral administration because of survivability when exposed to the gastrointestinal tract, including gastric juice, bile salt and intestinal conditions (Colom et al., 2017). Encapsulation of phages was applied for the prevention of loss of phage viability (Loh et al., 2021). Ca-alginate beads were not stabilized under acidic conditions. Alginate stability can be increased by (1) combining it with other natural polymers like proteins, glycerol, or starch, and (2) coating an extra layer of beads (Moghtader, Eğri and Piskin, 2017). Biswas and Sahoo, (2016) reported that cassava starch–alginate beads were used to deliver metoprolol tartrate (MT), exposure to simulated gastric fluid (pH 1.2), resulting in protection. The entrapment efficiency was increased from 58% to 88% and the release duration was from 45 minutes to 3–4 hours. These findings suggested that blending alginate beads with cassava starch can enhance stability and prolong the release of MT in gastric juice. Additionally, Rohman, Kaewtatip, Kantachote and Tantirungkij, (2021) reported that *Rhodopseudomonas palustris* KTSSR54 bacteria, used as a biofertilizer, were successfully entrapped within alginate/cassava starch hydrogels, leading to reduced cell loss. The entrapment efficiency of cassava starch in these hydrogels was 70.83%, compared to approximately 50.56% without starch. Therefore, the objective of this study to evaluate the effectiveness of cassava starch incorporation with Ca-alginate for protecting phages under various temperatures, pH, bile salt, gastrointestinal environment and storage at 4°C.

4.3 Material and Methods

4.3.1 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.

4.3.2 *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 et al., 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)

4.3.3 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 et al., 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

4.3.4 Entrapments efficiency of bacteriophage

The phage suspension was entrapped into sodium alginate (HIMEDIA®, Nashik, India) with or cassava starch (CS; Kriangkrai company limited, Nakornprathom, Thailand) by modified from method of Ma et al., (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

4.3.5 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.

4.3.6 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.

4.3.7 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 (4.3.4) 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 et al., 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. 4.3.4).

4.3.8 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 et al., 2019: Ma et al., 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. 4.3.4).

4.3.9 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 consposed 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. 4.3.4). The treatment was

performed in triplicate and the results were presented in percentage of survival phages (Ma et al., 2008).

4.3.10 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. 4.3.4).

4.3.11 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 (4.3.4).

4.3.12 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 $p \leq 0.05$ (95% confidence interval).

4.4 Result and Discussion

4.4.1 Entrapment efficiency (EE)

Salmonella phages were entrapped in Ca-alginate beads containing 0%, 0.15%, and 0.3% (w/v) cassava starch (CS). The entrapment efficiency (EE) of each

treatment was evaluated **(Table 4.1)**. There were no significant differences in EE among treatments, approximately 97.67 to 98.00%, respectively. These results indicated that the addition of CS to Ca-alginate beads did not significantly affect their ability to entrap salmonella phages. This EE was higher than the alginate/carrageenan microcapsules for phage encapsulation, with an efficiency of about 95% (Zhou et al., 2022).

Table 4.1 Entrapment efficiency for bacteriophage *vB_salP-p*YM in Ca-alginate beads.

Sodium alginate (w/v) (%)	Cassava starch (w/v) (%)	Entrapment efficiency
3	0	97.67 ± 0.58^{ns}
3	0.15	98.12 ± 0.19 ^{ns}
3	0.3	98.00 ± 1.00 ^{ns}

Data are means ± standard deviation of three replications. ns means each treatment is not significantly different at $p< 0.05$.

4.4.2 Ca-alginate beads size and morphological structure of Ca-alginate beads via scanning electron microscopy (SEM)

The diameters of the extruded beads containing 0% CS, 0.15% (w/v) CS, and 0.3% (w/v) CS were 2.9 ± 0.25 mm, 3.0 ± 0.55 mm, and 2.8 ± 0.8 mm, respectively (**Figure. 4.1**). All beads exhibited nearly perfect roundness, smooth surfaces, and a clear appearance. These results demonstrated that the addition of CS did not influence the size or shape of the beads.

On the other hand, Vazquez et al. (2015) reported that the mixture of alginate and cassava starch significantly increases in viscosity, resulting in a wide diameter and abnormal shape, compared to only alginate. When increasing the biopolymer in gel beads of alginate leads to obtaining the extended size of beads (Biswas and Sahoo, 2016)

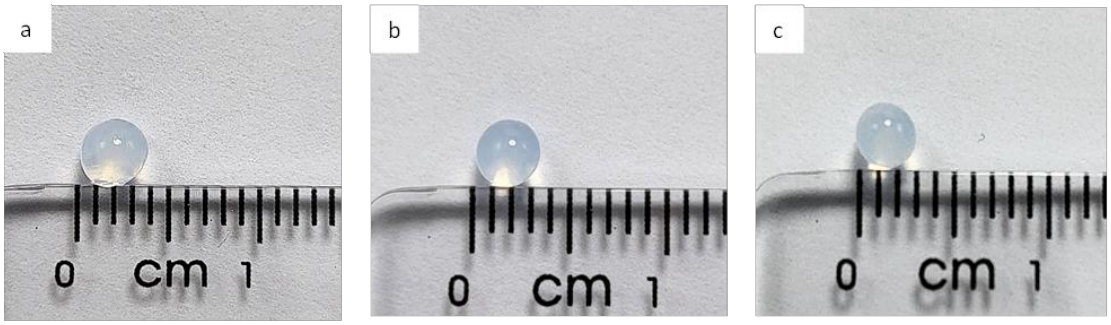

Figure 4.1 Illustration of Ca-alginate beads. A: 0% CS, B: 0.15% w/v CS and C: 0.3% w/v CS.

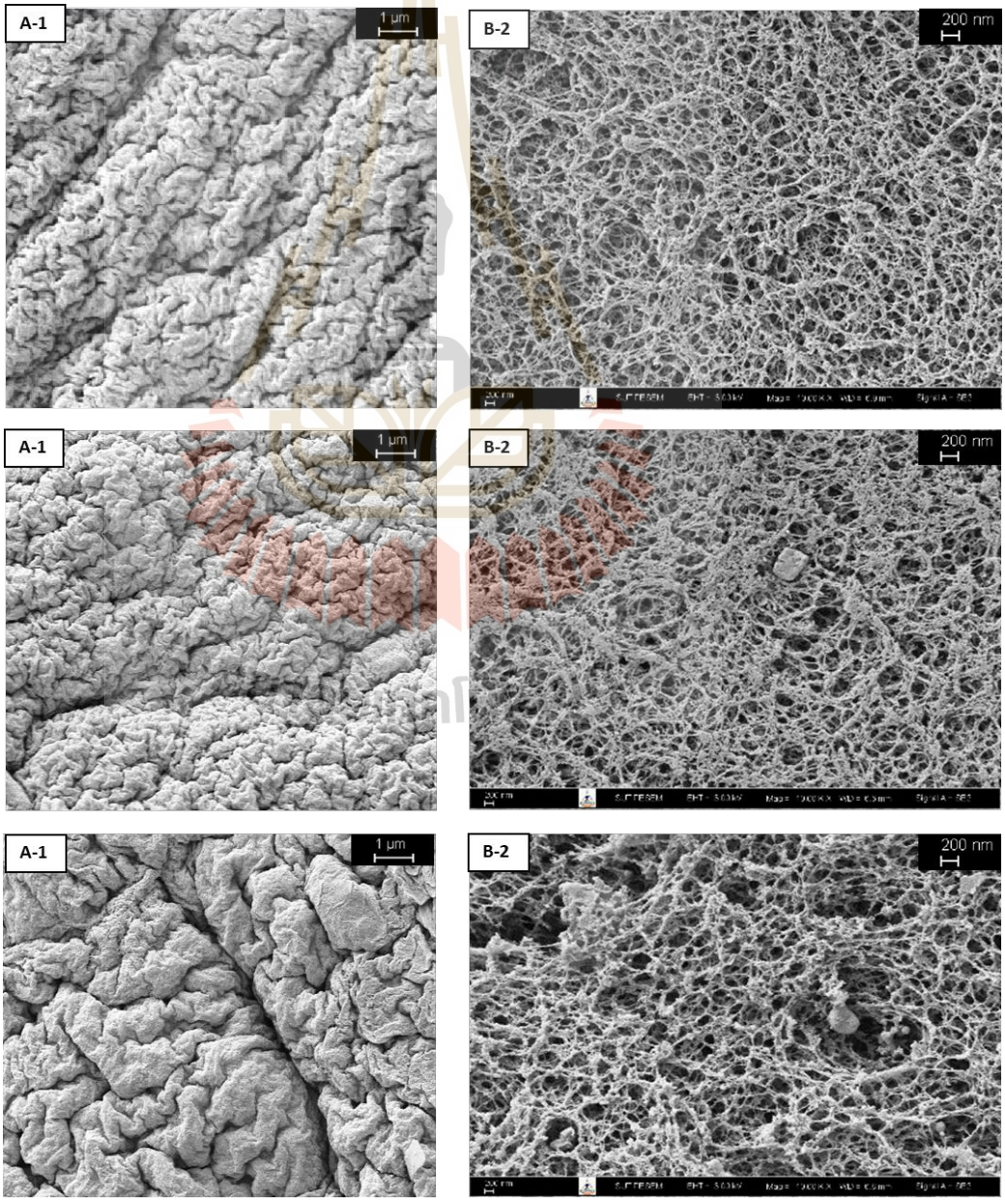

Figure 4.2 The SEM image surface and internal structure of Ca-alginate beads. Images A is surface structure at 5000x magnification and images B is internal structure at 10000x magnification. 1: 0% w/v CS, 2: 0.15% w/v CS and 3: 0.3% w/v CS.

Scanning electron microscopy (SEM) revealed detailed visualizations of both the surface and internal structures of the beads (**Figure 4.2**). The surface of all treatments exhibited a wrinkled, rough, and cracked morphology. The pore size of the internal structure of the Ca-alginate beads was measured by using ImageJ software, with 10 replicates for each treatment (**Table 4.2**). The average pore sizes of Ca-alginate beads added with CS 0.15% and 0.3% were 225.50 and 330.10 μm, respectively, compared to only Ca-alginate beads, about 235.90 μm, which means that the incorporation of a higher concentration of CS led to an increase in pore size. The presence of amylose and amylopectin in cassava starch may contribute to increased viscosity in the alginate solution. When cassava starch is incorporated into alginate, it can fill the 'egg-box' structure and stretch the alginate polymer chains (Belia, Fransiska and Suprianto, 2024). These results reveal that the wide-pore internal structure of CS added Ca-alginate beads was observed (**Figure 21; B2**).

Similarly, Tiamwong, Yukhajon, Noisong, Subsadsana and Sansuk, (2023) found that incorporating cassava starch into Ca-alginate led to increased pore size within the internal structure of the beads. The cassava starch insertion into the molecular arrangement of alginate during cross-linking with calcium ions resulted in the formation of wide spaces, which were observed under SEM.

Table 4.2 Surface morphology and pore size analysis of Ca-alginate beads.

Treatment (T)	Min	Max	Mean diameter(μm)
0% CS	135.24	375.47	235.90 ± 0.85 [b]
0.15% CS	141.84	357.14	225.50 ± 0.65 [a]
0.3% CS	131.68	726.34	330.10 ± 0.54 [c]

CS = cassava starch. Data are means diameter ± standard deviation of ten replications; different letter mean significantly different ($p \leq 0.05$).

4.4.3 Lytic assay of entrapped salmonella phage.

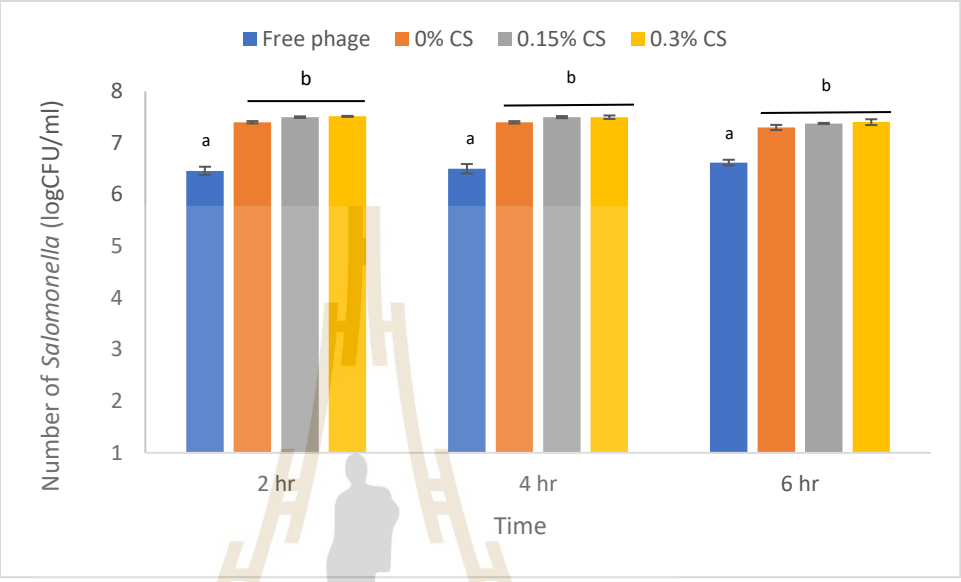

Figure 4.3 Lytic of entrapped salmonella phage on number of *Salmonella* Typhimurium; The results corresponded to the mean ± standard deviation (n=3). Different letter means significantly different at the same time ($p \leq 0.05$).

All treatments were placed in SM buffer (pH 7.4) and *Salmonella* Typhimurium cells were added 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 the free phage and entrapped salmonella phages (**Figure 4.3**). After 2 hours, the number of *Salmonella* incubated with free phages decreased rapidly compared to those incubated with Ca-alginate beads. *Salmonella* incubated with Ca-alginate beads remained close to the initial cell count after 6 hours of incubation, as the phages were not released from the Ca-alginate beads. The results showed that the number of *Salmonella* was significantly different from that of *Salmonella* incubated with free phages at 2, 4, and 6 hours. The number of *Salmonella* did not differ significantly between treatments with Ca-alginate beads for 6 hours. These results indicated that Ca-alginate, with or without CS, can entrap salmonella phages, which is consistent

with our previous evaluations of entrapment efficiency. Because the ionic gelation of sodium alginate with $CaCl_2$ forms a cross-linked network through ionic bonds effective in encapsulating salmonella phages. When CS is added to Ca-alginate beads. The hydroxyl groups (-OH) on starch molecules can form hydrogen bonds with the carboxyl groups (-COOH) on alginate chains. These interactions create a network of cross-linked polymer chains, increasing the overall strength and stability of the material (Merakchi, Bettayeb, Drouiche, Adour and Lounici, 2019). The homogeneous distribution of starch molecules within the gaps of the alginate matrix results in improved encapsulation efficiency (Vazquez et al., 2015). In contrast, Zhou et al. (2022) encapsulated phages in alginate (ALG)/**K**-carrageenan (CG) beads and evaluated their lytic activity under simulated intestinal conditions. In that study, both free and encapsulated phages demonstrated efficacy in reducing *S.* Typhimurium LT2 populations.

4.4.4 Stability of salmonella bacteriophage under different temperatures.

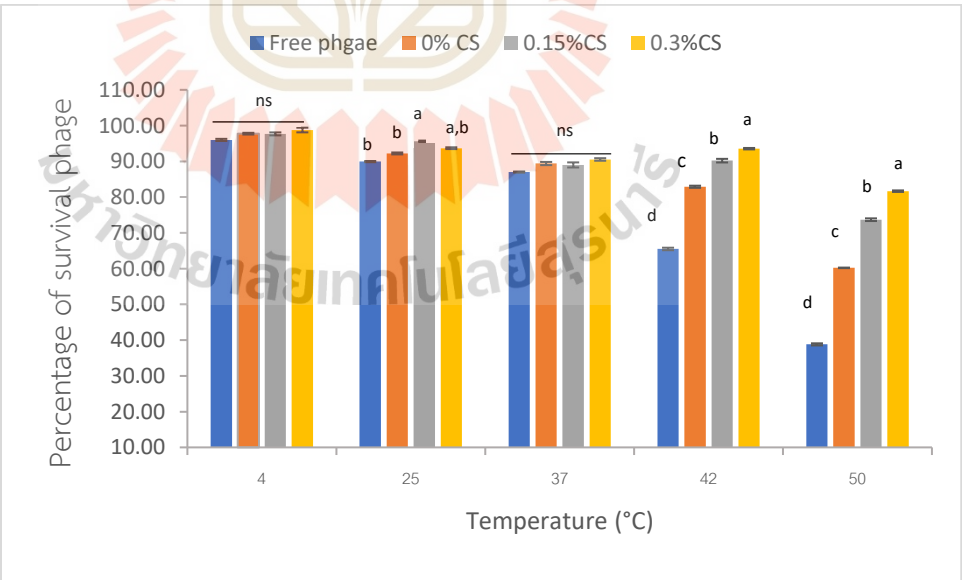

Figure 4.4 The survival of salmonella bacteriophage in the modified bead at various temperatures. The results corresponded to the mean ± standard deviation (n=3). ns means not significantly different, different letter mean significantly different among the same temperature ($p \leq 0.05$).

The Ca-alginate beads, modified with or without chitosan (CS), were evaluated under various temperatures for 24 hours (**Figure 4.4**). The salmonella phages entrapped in Ca-alginate beads were not significantly different at 4 and 37 °C. At 25 °C, the survival numbers of phages in Ca-alginate beads containing 0.15% and 0.3% CS were higher than those without CS and free phages. At 42 and 50 °C, the titers of surviving salmonella phages were significantly different among treatments, particularly the addition of CS in Ca-alginate beads at both concentrations. The number of free phages decreased when the temperature increased due to thermal damage by approximately 65.50% and 38.81%, respectively. These results suggest that incorporating CS into Ca-alginate beads improves the thermal stability of salmonella phages. Because when the starch was autoclaved, the amylopectin from the starch interacted with the carboxyl groups of the alginates and acted as a filler in the egg box. This interaction enhances the crosslinking between starch and alginate, thereby increasing gel stability and reducing syneresis (Belia et al., 2024). Consistent with Abdelsattar et al. (2019), the *E. coli* ZSEC5 bacteriophage encapsulated in chitosan/alginate beads exhibited a 0.8 log reduction in PFU/mL after exposure to 80 °C. These findings suggest that incorporating starch into the matrix enhances phage stability.

4.4.5 Stability of salmonella bacteriophage under various pHs.

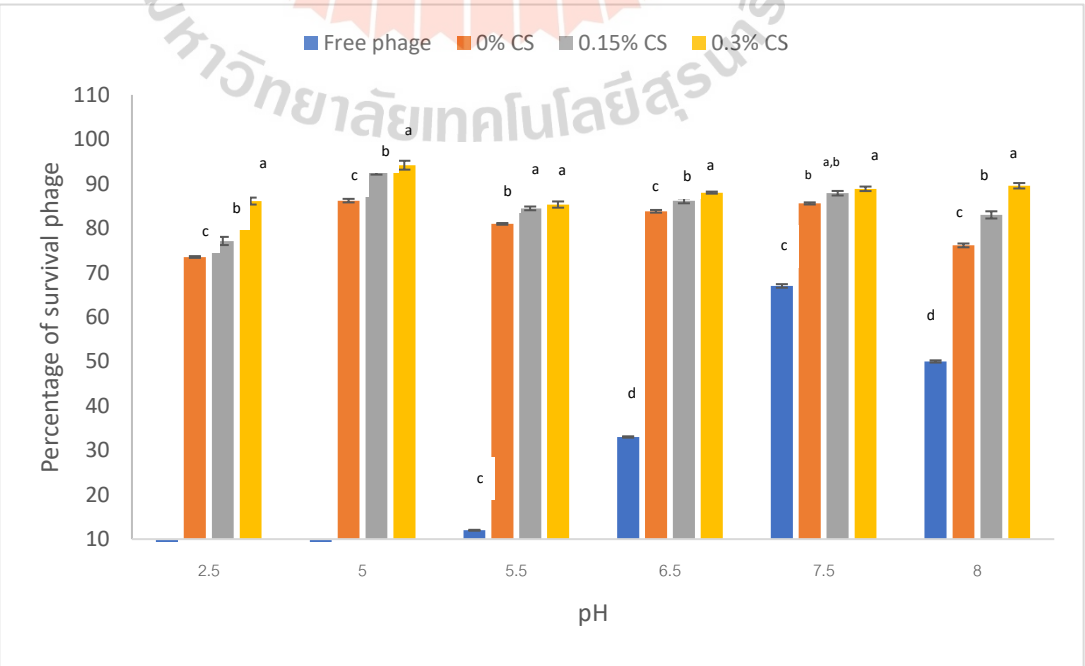

Figure 4.5 The stability of salmonella bacteriophage in a Ca-alginate bead under various pHs. The results corresponded to the mean ± standard deviation (n=3). Different letters mean significantly different at the same pHs ($p \leq 0.05$).

The stability of salmonella bacteriophage entrapped in Ca-alginate beads was evaluated at 37°C for 2 hours under various pH conditions 2.5 (gizzard), 5 (duodenum), 5.5 (crop), 6.5 (jejunum), and 8 (cecum). (**Figure 4.5**). At pH 2.5 and 5.0, free phages were not detected; the entrapped phages in Ca-alginate were examined, resulting in a higher percentage of surviving phages than free phages at all determined pH. It was notable that the addition of CS in Ca-alginate beads could increase the percentage of survival better than only Ca-alginate entrapment. These findings indicate that entrapment in Ca-alginate, particularly with 0.3% CS, enhances phage stability under a wide range of pH conditions. Mixing sodium alginate with cassava starch in hydrogels can improve their performance, especially at low pH, due to reduced shrinkage of the hydrogel matrix. The hydroxyl groups in starch interact with the carboxyl groups in SA through hydrogen bonding, creating a more complex and robust biopolymeric network and cassava starch acts as a structural component, preventing the hydrogel from shrinking excessively. Especially the acidic condition of the chicken's digestive system, where SA can be more susceptible to degradation (Khlibsuwan, Tansena and Pongjanyakul, 2018; Fang et al., 2022). Ma et al. (2008) reported that the survival of bacteriophage in chitosan/alginate microcapsules was enhanced for phage stability under acidic conditions. As cassava starch was integrated into the egg-box structure. Hydrogen bonding and other interactions between hydroxyl groups in starch and carboxyl groups in alginate resulted in a denser and more stable matrix (Rohman et al., 2021; Abdelsattar et al., 2019). This modified matrix improved the protective capability of Ca-alginate beads for salmonella phages.

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

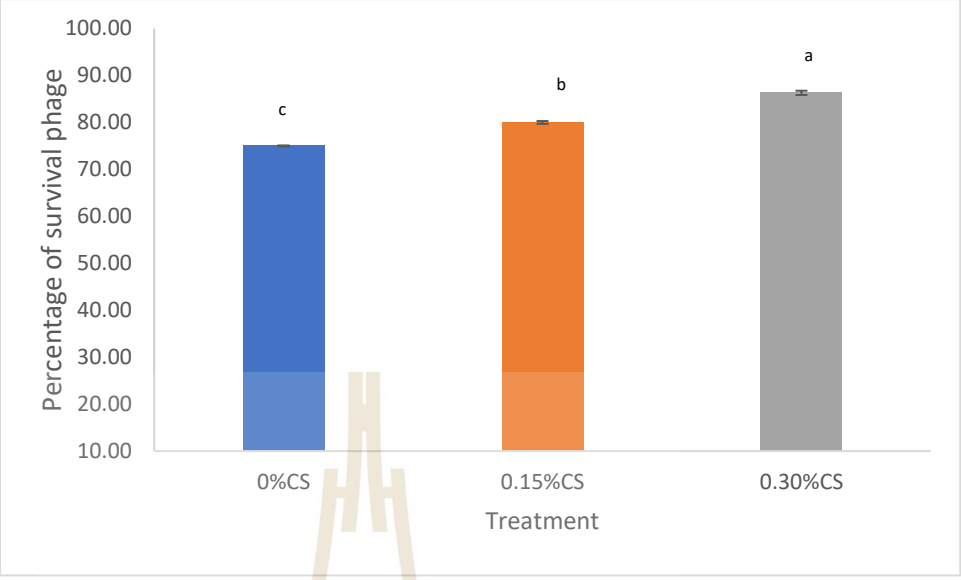

Figure 4.6 The number of phages remaining in Ca-alginate beads were incubated in bile salt solution (pH 6.8) at 41°C for 1 hour. The results corresponded to the mean ± standard deviation (n=3), different letter means significantly different at the same condition.

The stability of salmonella phages entrapped in Ca-alginate beads exposed to a 0.01 M bile salt was evaluated (**Figure 4.6**). The results indicated that the survival number of phages in Ca-alginate beads without CS was 75%, while the beads containing 0.15% and 0.3% (w/v) CS were 77.92% and 90.91%, respectively. The percentage of survival phages was increased because of the addition of CS in Ca-alginate beads. The entrapment of materials within a starch–alginate matrix improves mechanical strength and enhances crosslinking between sodium alginate and calcium ions (Ca^{2+}), forming complexes with hydroxyl groups (-OH) and oxygen atoms in the glycosidic bonds of amylose and amylopectin. These interactions contribute to increased gel strength and the formation of a more stable polymer network (Onyido, Ato and Nnamonu, 2012; Bagnolo, Almeida, Silva and Sato, 2023). Batalha et al. (2020) reported that when free phage UFV-AREG1 was exposed to 2% bile salt, survival decreased by 0.4 log, while the phage was encapsulated within alginate/carrageenan beads, the number of survival phages was equal to the initial number (10^9 PFU/g).

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

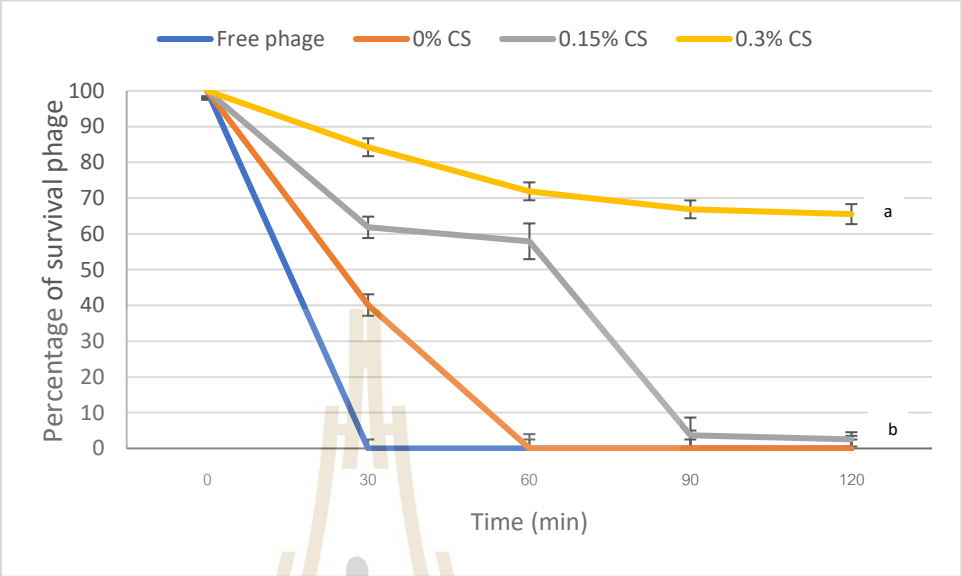

Figure 4.7 The survival of salmonella phage in the modified beads under SGF. The results corresponded to the mean ± standard deviation (n=3), different letter means significantly different at the same condition.

Figure 4.8 Morphology of Ca-alginate beads after in vitro incubation in simulated gastric fluid (SGF) for 120 min. A is Ca-alginate beads without CS, B is Ca-alginate beads wit 0.15% w/v CS and C is Ca-alginate beads wit 0.3% w/v CS.

The stability of entrapped phages was evaluated after incubation in SGF (3 mg/mL pepsin in 0.85% NaCl, pH 2.5) at 41°C for 2 hours **(Figure 4.7)**. The morphology of the Ca-alginate beads after incubation was shown in **Figure 4.8**. The free phages and phages entrapped in Ca-alginate beads with or without cassava starch (CS) were determined. The percentage of survival phages declined rapidly, becoming

undetectable within 30 minutes. Similarly, Ca-alginate beads without CS entrapped phages, which quickly lost viability and were inactivated within 60 minutes. In contrast, phage stability was better maintained in beads containing 0.15% CS than in free phages or 0% CS beads. However, the phages in these beads showed a considerable loss in viability over time, with their numbers decreasing to practically undetectable levels by 90 minutes. The beads with 0.3% CS provided the best level of protection, retaining a significant proportion of live phages even after 120 minutes. These findings indicated that 0.3% w/v of CS addition could enhanced phage stability under acidic conditions. Mixing sodium alginate with cassava starch in beads improves their stability and reduces degradation in simulated gastric fluid because the starch's hydroxyl groups form hydrogen bonds with the carboxyl groups of alginates, creating a denser, more complex network that resists degradation. Additionally, the starch acts as a structural component, preventing over-shrinkage of the alginate, which can improve the bead's ability to maintain its structure and function in a harsh environment (Fransiska, Belia, Nofika, Yohana and Ramayanti, 2025; Fang et al., 2022).

Bacteriophage Felix O1 encapsulated within chitosan-coated alginate microspheres exhibited significant protection when incubated in simulated gastric fluid (SGF, pH 2.4) containing pepsin for 90 minutes (Ma et al., 2008). The encapsulated phages showed only a 2.58 PFU/ml reduction, while no viable free phages were found after 5 minutes of exposure. Ca-alginate beads shrank towards the end of the evaluation period. Under acidic SGF conditions, the carboxylate groups ($-COO^-$) in the alginate structure were protonated to carboxylic groups (-COOH), reducing the adhesion between polymer chains linked by calcium ions (Ca^{2+}) and resulting in bead shrinkage (Lee and Mooney, 2012). Cassava starch's hydroxyl groups can create hydrogen bonds with alginate chains, which serve to bridge the gaps in the egg-box structure. As a result, the density and stability of the cross-links are increased (Lin et al., 2021), increasing phage protection under SGF conditions.

4.4.8 Evaluation of phage release from Ca-alginate beads in simulated intestinal fluid (SIF).

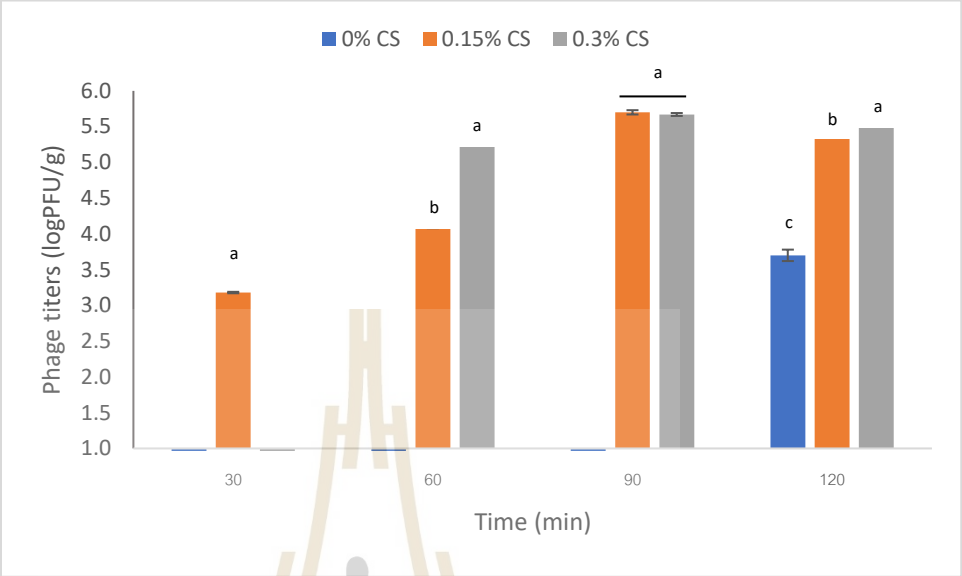

Figure 4.9 Phage stability released in simulated intestinal fluid at pH 6.8. The results corresponded to the mean ± standard deviation (n=3), different letters indicated significant differences at the same time ($p \leq 0.05$).

Figure 4.10 Morphology of Ca-alginate beads after in vitro incubation in simulated intestinal fluid (SIF) for 120 min. A is Ca-alginate beads without CS, B is Ca-alginate beads wit 0.15% w/v CS and C is Ca-alginate beads wit 0.3% w/v CS.

Ca-alginate beads, with and without cassava starch (CS), were incubated for 120 minutes in simulated intestinal fluid (SIF) (1 mg/mL pancreatin in 0.85% NaCl, pH 6.8) at 41°C (**Figure 4.9**) and the morphology of Ca-alginate beads was illustrated (**Figure 4.10**). After 30 minutes, only Ca-alginate beads containing 0.15% (w/v) CS released phages, with a concentration of 3.2 log PFU/g. At 60 minutes,

no phage release was observed from Ca-alginate beads without CS, whereas beads containing 0.15% and 0.3% (w/v) CS released 4.1 and 5.7 log PFU/g, respectively. The phage release was not detected in beads without CS, while beads with 0.15% and 0.3% CS could be determined to have approximately 5.7 log PFU/g after 90 minutes. After 120 minutes, phage release from beads without CS reached 3.7 log PFU/g for beads containing 0.15% and 0.3% CS, 5.7 and 6.1 log PFU/g were released, respectively. These results indicated that incorporating cassava starch into Ca-alginate beads could accelerate within 30 minutes in SIF compared to beads without starch. Furthermore, a CS concentration of 0.3% (w/v) led to greater phage release than 0.15% (w/v), suggesting a concentration-dependent effect. Cassava starch contains amylose and amylopectin (Wang et al., 2022), which fill the porous structure of the alginate gel when incorporated. Its highly hydrophilic nature enables cassava starch to promote swelling of the alginate-starch composite in aqueous environments such as SIF. This swelling enlarges the pore size of the hydrogel matrix and facilitates the diffusion of entrapped substances (Das et al., 2024; Poojari, Kulkarni and Wairkar, 2024). In SIF, amylase degrades the starch component enzymatically, disrupting the gel's structural integrity and accelerating the release of entrapped phages (Chen, Song, Huang and Guan, 2021). These combined mechanisms enhance the phage release profile observed in cassava starch–alginate composites under simulated intestinal conditions. Kim et al., (2015) reported that *Escherichia coli* O157:H7 bacteriophages were encapsulated in chitosan/alginate microspheres and evaluated their release in SIF. The results showed that chitosan/alginate microspheres accelerated phage release faster than pure alginate.

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

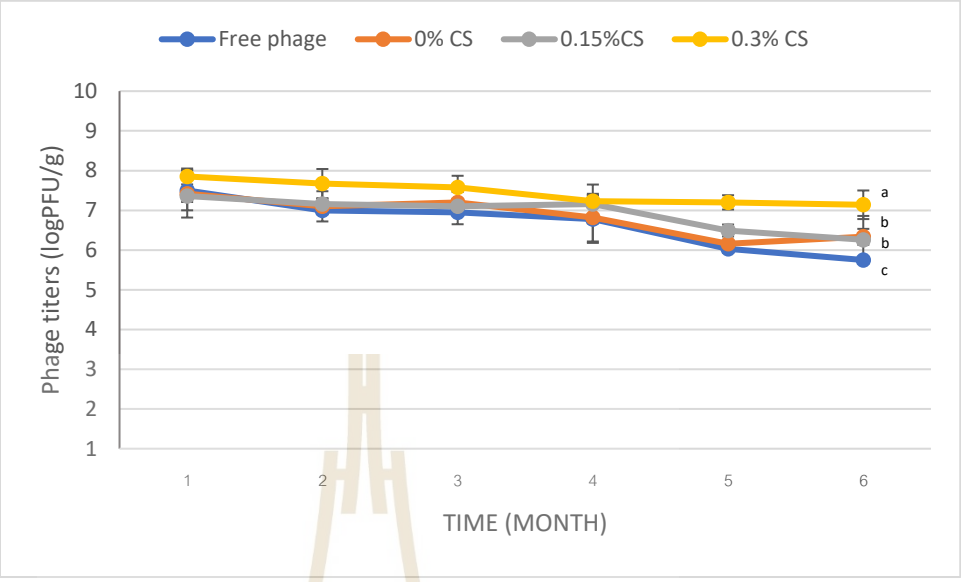

Figure 4.11 The survival of phages *vB_salP-p*YM in the Ca-alginate beads during storage at 4 °C for 6 months. The results corresponded to the mean ± standard deviation (n=3), different letters indicated significant differences at the same time ($p \leq 0.05$).

This study evaluated the survival of phages entrapped in Ca-alginate beads, with and without cassava starch (CS), during six months of storage at 4°C (**Figure 4.11**). After six months, the titer of free phages decreased by 2.25 log PFU/mL from an initial concentration of 8 log PFU/mL. In contrast, phages entrapped in Ca-alginate beads without CS exhibited a smaller reduction of 1.67 log PFU/g from the same initial concentration. The reduction in phage titer for beads supplemented with 0.15% and 0.3% (w/v) CS was 1.74 and 0.86 log PFU/g, respectively. Among all treatments, Ca-alginate beads with 0.3% CS demonstrated the highest phage stability over the six-month period. These results suggest that the incorporation of cassava starch improves phage stability during long-term cold storage.

Cassava starch functions as a filler in the alginate matrix, lowering permeability to tiny molecules while increasing network density. Furthermore, cassava starch has cryoprotective qualities, which serve to reduce phage damage caused by ice crystal formation during low-temperature storage (Anal and Singh, 2007; Ma et al.,

2008; Gogineni, Jain and Arora, 2020). Our findings are similar to those of Colom et al. (2015), who encapsulated bacteriophages UAB_Phi20, UAB_Phi78, and UAB_Phi87 in alginate/liposome films and preserved them at 4°C for three months. The encapsulated phages were highly stable, with no appreciable drop in titer from the initial level (10^{11} PFU/mL).

4.5 Conclusions

This study demonstrated that phage *vB_salP-p*YM entrapped in calcium-alginate beads, with and without CS, possessed high entrapment efficiency. CS addition to calcium-alginate beads enhanced survival of the bacteriophages against simulated gastric fluid (SGF), heat, low pH and bile salt. Moreover, CS-loaded beads enhanced phage release efficiency in simulated intestinal fluid (SIF) and enhanced phage viability after prolonged storage at low temperature. These findings showed that CS-loaded calcium-alginate beads represent a good way of protecting phages from degradation when administered orally, thereby moving forward their potential for application in phage therapy.

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CHAPTER 5
CONCLUSION AND RECOMMENDATION

5.1 Result of the entrapment efficacy of Ca-alginate beads with SPI or CS.

Salmonella phages were entrapped in Ca-alginate beads. The entrapment efficiency of each treatment was not significantly different, ranging from 97.67% to 98.34%. This result showed that the addition of SPI or CS to Ca-alginate beads did not affect the entrapment efficiency (EE) of Ca-alginate beads for salmonella phages.

Ca-alginate beads were placed in SM buffer (pH 7.4), followed by the addition of *Salmonella* Typhimurium at a concentration of 10^7 PFU/mL. The samples were then incubated at 41°C for 6 hours to evaluate phage efficacy. After 2 hours of incubation, a significant reduction in *Salmonella* counts was observed in the free phage treatment group, whereas the groups entrapped with Ca-alginate beads, either with or without additional biopolymers, exhibited higher bacterial counts, likely due to delayed phage release from the entrapment beads. At 4 hours, the free phage group continued to show markedly lower *Salmonella* levels compared to all bead treatments. After 6 hours, calcium-alginate beads containing 0.3% w/v CS were found to have the least release amount compared to other treatments.

5.2 Result of the stability of salmonella bacteriophage in Ca-alginate beads under different temperatures, pH levels, under simulated gastric fluid and bile salt solution.

The Ca-alginate beads, modified with or without soybean protein isolate (SPI) or chitosan (CS), were evaluated for their ability to protect entrapped salmonella phages under various temperatures for 24 hours. At 4°C and 37°C, the phage titers in all entrapped treatments showed no significant differences. However, at 25°C, the survival of phages in Ca-alginate beads containing 0.15% or 0.3% CS was higher than in beads without CS and in free phages. Similarly, at elevated temperatures (42 °C and 50 °C), the phage titers varied significantly among treatments.

Both SPI and CS enhanced the thermal protection of salmonella phages when incorporated into Ca-alginate beads, with improved survivability compared to Ca-alginate alone. In contrast, free phages exhibited a marked decline in survivability at high temperatures, with reductions of approximately 65.50% at 42 ℃ and 38.81% at 50 ℃, indicating substantial thermal damage. These findings suggest that the addition of either SPI or CS to Ca-alginate beads enhances the thermal stability of entrapped salmonella phages, making them more resilient to heat-induced inactivation.

The stability of salmonella bacteriophages entrapped in Ca-alginate beads, with or without modifications using SPI or CS, was evaluated under various pH conditions (2.5, 5.0, 5.5, 6.5, and 8.0) at 37 ℃ for 2 hours. At highly acidic conditions (pH 2.5 and 5.0), free phages were undetectable after incubation, indicating complete inactivation. In contrast, phages entrapped in Ca-alginate beads showed significantly higher survival across all tested pH levels. Notably, the incorporation of 0.3% (w/v) SPI or CS into the Ca-alginate beads improved phage viability compared to unmodified beads, with SPI and CS enhanced demonstrating the highest phage survivability. These findings suggest that modifying Ca-alginate beads with either SPI or CS enhances the pH tolerance of entrapped salmonella phages, providing better protection in both acidic and neutral environments.

The stability of salmonella phages entrapped in Ca-alginate beads, with or without supplementation of SPI or CS, was evaluated in a 0.01 M bile salt solution (pH 6.8) at 41 ℃ for 1 hour. The results showed that Ca-alginate beads without any additive retained approximately 75% of phages, while beads containing 0.15% and 0.3% (w/v) of either SPI or CS improved phage survivability to 77.92% and 90.91%, respectively. These findings suggest that the incorporation of SPI or CS into Ca-alginate beads significantly enhances the resistance of entrapped phages against bile salt exposure, thereby improving their overall stability under gastrointestinal-like conditions.

The stability of salmonella phages entrapped in Ca-alginate beads, with or without supplementation of SPI or CS, was evaluated under simulated gastric fluid (SGF; 3 mg/mL pepsin in 0.85% NaCl, pH 2.5) at 41 ℃ for 2 hours. Free phages were rapidly inactivated and became undetectable after 30 minutes of exposure. Similarly, phages entrapped in unmodified Ca-alginate beads lost viability over time, with no

detectable phages after 60 minutes. The incorporation of 0.15% (w/v) SPI or CS provided a modest improvement in survivability, with phage survival at 30 minutes ranging from 44 ± 0.4% (SPI) to higher levels observed with CS. Notably, beads containing 0.3% (w/v) SPI or CS offered significantly better protection. Phages entrapped with 0.3% SPI exhibited 76 ± 0.22% survival at 30 minutes and maintained approximately 66 ± 0.3% at 60 minutes before becoming undetectable. In comparison, 0.3% CS-entrapped phages showed the highest overall resistance, retaining viable phage populations up to 120 minutes. These results indicate that modifying Ca-alginate beads with 0.3% SPI or CS substantially improves phage stability under acidic gastric-like conditions, offering potential for oral delivery applications.

5.3 Result of the release of salmonella bacteriophage from modified beads under simulated intestinal fluid.

Ca-alginate beads, with and without SPI or CS, were incubated for 120 minutes in simulated intestinal fluid (SIF; 1 mg/mL pancreatin in 0.85% NaCl, pH 6.8) at 41°C. For beads containing SPI, phage release was not detected in samples without SPI until after 90 minutes. In contrast, beads containing 0.15% and 0.3% (w/v) SPI released phages as early as 30 minutes, with increasing release levels at 60 and 90 minutes. After 120 minutes, phage titers from beads without SPI reached 3.7 ± 0.1 log PFU/g, while those from beads with 0.15% and 0.3% SPI reached 3.8 ± 0.08 and 4.7 ± 0.7 log PFU/g, respectively, indicating that SPI enhanced phage release compared to alginate alone. Similarly, Ca-alginate beads with CS showed accelerated phage release compared to those without. At 30 minutes, only beads containing 0.15% CS exhibited phage release (3.2 log PFU/g), while no release was observed from CS-free beads. At 60 minutes, beads with 0.15% and 0.3% CS released 4.1 and 5.7 log PFU/g, respectively. This trend continued, with phage titers reaching approximately 5.7 log PFU/g at 90 minutes and at 120 minutes, 3.7, 5.7, and 6.1 log PFU/g for beads without CS and those with 0.15% and 0.3% CS, respectively. These results suggest that both SPI and CS can enhance the release of entrapped phages in SIF, with CS demonstrating a faster onset of release. Additionally, higher concentrations (0.3% w/v) of either additive led to greater phage release than lower concentrations (0.15% w/v).

5.4 Result of the stability of salmonella bacteriophage in modified beads for six months at 4°C.

The long-term survival of phages entrapped in Ca-alginate beads supplemented with SPI or CS was evaluated over a six-month storage period at 4°C. After six months, the concentration of free phages declined by 2.25 log PFU/mL from an initial level of 8 log PFU/mL. Phages entrapped in Ca-alginate beads without any additive showed a reduction of 1.67 log PFU/g. In the SPI-containing beads, phage titers decreased by 1.23 and 1.7 log PFU/g for 0.15% and 0.3% (w/v) SPI, respectively, with no significant differences observed among treatments during the first month of storage. These results suggest that the inclusion of SPI can enhance phage viability over long-term refrigeration, with 0.15% SPI showing the highest stability at the end of six months. Similarly, phages entrapped in Ca-alginate beads with CS also exhibited improved stability compared to beads without additives. The reductions in phage titers were 1.74 and 0.86 log PFU/g for beads containing 0.15% and 0.3% (w/v) CS, respectively. Overall, these findings demonstrate that both SPI and CS contribute to improved long-term stability of entrapped phages during storage at 4°C, with optimal performance observed at specific additive concentrations of 0.15% for SPI and 0.3% for CS.

5.5 Summary of findings

This study demonstrated that Ca-alginate beads effectively entrapped the bacteriophage vB_salP-pYM with consistently high entrapment efficiency across all treatments. The incorporation of soy protein isolate or cassava starch into the Ca-alginate matrix significantly enhanced phage viability under various stress conditions, including elevated temperature, acidic pH, and exposure to 0.01 M bile salts, compared to Ca-alginate beads alone. Furthermore, these modified beads promoted greater phage survival during simulated gastrointestinal transit and facilitated faster phage release in intestinal conditions. The addition of soy protein isolate or cassava starch also contributed to improved phage stability during prolonged cold storage. Overall, Ca-alginate beads supplemented with these biopolymers show promise as a delivery system for enhancing the oral viability of bacteriophages, supporting their potential use in phage therapy.

BIOGRAPHY

Miss Panukar Malawan was born on February 29, 1992, in Buriram Province, Thailand. She obtained her bachelor's degree in Science in Microbiology, Mahasarakham University, Mahasarakham, Thailand (2011-2015). During bachelor's degree, her research interest was entitled "Inhibition of the growth of pathogenic microorganisms using traditional herbal medicines.

In 2019, she received a One Research One Grant (OROG) scholarship to financially support her study at Suranaree University of Technology, Thailand and pursued the degree of Master of Food Technology under the supervision of Dr. Patcharin Siringan. During her Master's study, some parts of the results from this study were presented as a poster presentation and published in the Food Innovation Asia Conference 2024, Thailand.