Development of Encapsulated *Salmonella* Bacteriophages to Reduce *Salmonella* spp. in Food Matrices

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ชื่อวิทยานิพนธ์ การพัฒนา *Salmonella* bacteriophages ในรูปแบบเอนแคปซูเลท เพื่อลดปริมาณ เชื้อ *Salmonella* spp. ในอาหาร
ผู้เขียน นางสาวกันติยา เพชรสง
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บทคัดย่อ

Salmonella Enteritidis และ *Salmonella* Typhimurium เป็นซีโรวาร์ที่สำคัญของเชื้อ *Salmonella* ซึ่งมักมีรายงานการปนเปื้อนและเป็นสาเหตุของการระบาดโรคติดต่อทางอาหาร แบคเทอริ-โอเฟจ (เฟจ) เป็นไวรัสที่มีความสามารถในการทำลายอย่างจำเพาะเจาะจงต่อแบคทีเรียเจ้าบ้าน ซึ่งเป็นคุณสมบัติพิเศษนอกเหนือจากยาปฏิชีวนะและสารเคมีอื่นๆ ในปัจจุบันการประยุกต์ใช้เฟจเป็นสารควบคุมทางชีวภาพได้รับความนิยมเป็นอย่างมากในอุตสาหกรรมอาหารงานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาแบคเทอริโอเฟจในรูปแบบเอนแคปซูเลทที่มีลักษณะเป็นผงแห้ง เพื่อใช้ในการควบคุมเชื้อ *Salmonella* สายพันธุ์ต่างๆ โดยได้ทำการแยกเฟจที่มีความจำเพาะต่อเชื้อ *Salmonella* จากสิ่งแวดล้อมภายในฟาร์มสัตว์ประเภทต่างๆ (สัตว์ปีก สุกร แพะ และ โค-กระบือ) ภายในจังหวัดสงขลา เพื่อศึกษาความชุกของเฟจในแหล่งดังกล่าว งานวิจัยนี้สามารถแยกได้ทั้งหมด 36 เฟจ ผลการทดลองชี้ให้เห็นว่าสิ่งแวดล้อมภายในฟาร์มสัตว์ดังกล่าวเป็นแหล่งที่อยู่สำคัญของเฟจที่มีความจำเพาะต่อเชื้อ *Salmonella* ในการศึกษาลักษณะทางฟีโนไทป์ เฟจที่แยกได้ทั้งหมดถูกนำมาทดสอบความสามารถในการทำลายเชื้อ *Salmonella* จำนวน 47 สายพันธุ์ (28 ซีโรวาร์) ที่แยกได้จากแหล่งต่างๆ (ฟาร์มสัตว์ โรงงานอุตสาหกรรมอาหารทะเล และคน) จากประเทศไทยและสหรัฐอเมริกา

ผลการทดลองพบว่าเฟจจำนวนหนึ่งมีความสามารถในการทำลายเชื้อ *Salmonella* จากแหล่งต่างๆได้หลากหลายสายพันธุ์ ทั้งนี้เฟจส่วนใหญ่มีความสามารถสูงในการทำลายเชื้อ *Salmonella* จากฟาร์มสัตว์ในประเทศไทย ข้อมูลนี้ชี้ให้เห็นความสัมพันธ์ระหว่างแหล่งที่อยู่ของแบคทีเรียเจ้าบ้านกับเฟจ ขนาดโดยประมาณของจีโนมของเฟจโดยเทคนิค pulsed-field gel electrophoresis (PFGE) อยู่ในช่วง 50 ± 2 kb ถึง 200 ± 2 kb ผลการทดลองชี้ให้เห็นว่าเฟจที่แยกได้มีความหลากหลายทางพันธุกรรมและมีความสามารถในการทำลายเชื้อ *Salmonella* ที่แยกได้จากแหล่งต่างๆได้หลากหลายสายพันธุ์ รวมถึงสายพันธุ์สำคัญต่างๆที่มักมีรายงานการระบาด ดังนั้นงานวิจัยนี้จึงได้ทำการคัดเลือกเฟจที่มีความสามารถในการทำลายเชื้อ *Salmonella* สายพันธุ์ที่มีรายงานการระบาดมากที่สุดคือ *S.* Enteritidis และ *S.* Typhimurium

การเพิ่มประสิทธิภาพของเฟจในรูปแบบสารควบคุมทางชีวภาพได้มีการพัฒนาให้อยู่ในรูปแบบของ phage cocktail โดยมีการทดสอบด้วยวิธี efficiency of plating (EOP) และ lytic ability assays ชี้ให้เห็นว่าเฟจจำนวน 3 สายพันธุ์คือ KP4 KP5 และ KP50 มีความสามารถในการทำลาย *S.* Enteritidisและ*S.* Typhimurium สูงสุด จากการศึกษาลักษณะทางสัณฐานโดยใช้กล้องจุลทรรศน์แบบส่องผ่าน (TEM) พบว่าเฟจทั้งหมดจัดอยู่ในตระกูล *Caudovirales* และวงศ์ *Siphoviridae* คุณสมบัติทางชีวภาพซึ่งบ่งบอกถึงประสิทธิภาพในการทำลาย *S.* Enteritidis และ *S.* Typhimurium ประกอบด้วย latent period ของเฟจ KP4 KP5 และ KP50 เมื่อเข้าทำลาย*S.* Enteritidis อยู่ในช่วง 5 ถึง 40 นาที และเมื่อเข้าทำลาย *S.* Typhimurium อยู่ในช่วง 5 ถึง 15 นาที ขนาด burst size ของเฟจ KP4 KP5 และ KP50 เมื่อเข้าทำลาย *S.* Enteritidis อยู่ในช่วง 25 ถึง 98 PFU/cell และเมื่อเข้าทำลาย *S.* Typhimurium อยู่ในช่วง 70 ถึง 112 PFU/cell จากการวิเคราะห์เชิงพันธุกรรมพบว่าเฟจ KP4 KP5 และ KP50 ไม่ปรากฏยีนที่เกี่ยวข้องกับ lysogeny module ยีนที่ก่อให้เกิดความรุนแรงหรือความเป็นพิษต่อมนุษย์และสัตว์ รวมถึงยีนที่เกี่ยวข้องกับการต้านยาปฏิชีวนะ ผลการทดลองชี้ให้เห็นว่าเฟจทั้งสามสามพันธุ์เป็น vilurent phage จากการศึกษาประสิทธิภาพของ phage cocktail ในการทำลายเชื้อ *S.* Enteritidis และ *S.* Typhimurium ในหลอดทดลองพบว่า เชื้อทั้งสองถูกลดจำนวนลงมากกว่า 4 log CFU/mL ภายในเวลา 4 ชั่วโมง นอกจากนี้ผลการทดลองชี้ให้เห็นว่าไม่มีการดื้อต่อเฟจของเชื้อทั้งสองสายพันธุ์หลังจากการเข้าทำลายโดย phage cocktail เมื่อทดสอบประสิทธิภาพของ phage cocktail ในการลดเชื้อ*S.* Enteritidis และ *S.* Typhimurium ในเนื้อไก่สด ต้นอ่อนทานตะวัน และอาหารสัตว์ พบว่าเชื้อ *S.* Enteritidis ถูกลดจำนวนลงประมาณ 0.66 log CFU/cm^2 1.27 log CFU/g และ 1.87 log CFU/g ตามลำดับ และเชื้อ *S.* Typhimurium ถูกลดจำนวนลงประมาณ 1.73 log CFU/cm^2 1.17 log CFU/g และ 2.38 log CFU/g ตามลำดับ ดังนั้น phage cocktail ที่พัฒนาขึ้นโดยงานวิจัยนี้มีประสิทธิภาพในการควบคุมเชื้อ *Salmonella* ในอาหารที่มีองค์ประกอบเชิงเคมี ชีวภาพ และกายภาพที่หลากหลาย

เพื่อการลดข้อจำกัดและเพื่อพัฒนารูปแบบการประยุกต์ใช้แบคเทอริโอเฟจ งานวิจัยนี้จึงได้นำเทคนิค microencapsulation มาใช้และมีการพัฒนารูปแบบของเฟจให้อยู่ในรูปผงแห้งเพื่อเป็นการปกป้องเฟจจากสภาวะรุนแรงในกระบวนการทำแห้ง โดยได้ทดสอบหาสัดส่วนที่เหมาะสมที่สุดของ WPI (Whey Protein Isolate) และน้ำตาล trehalose เพื่อทำให้เฟจรอดชีวิตมากที่สุดเมื่อผ่านกระบวนการทำแห้ง ผลการทดลองพบว่าสัดส่วนของ WPI และน้ำตาล trehalose ที่ 3:1 คือสูตรที่ทำให้เฟจรอดชีวิตมากที่สุดคือ 91.9% ผลการวิเคราะห์โครงสร้างภายนอกของเฟจในรูปผงแห้งที่ได้จากสูตรที่เหมาะสมที่สุดโดยเทคนิคการวิเคราะห์ด้วยกล้องอิเล็กตรอนแบบส่องกราด (SEM) พบว่า WPI และน้ำตาล trehalose เกิดการผสานเชิงโครงสร้างซึ่งกันและกัน การวิเคราะห์โดย fourier transform infrared spectroscopy

(FTIR) แสดงให้เห็นว่า WPI และน้ำตาล trehalose ในสูตรสารผสมดังกล่าวจับกันด้วยพันธะไฮโดรเจน ผลของการวิเคราะห์โดย differential scanning calorimetry (DSC) แสดงให้เห็นว่าเฟจในรูปผงแห้งที่ได้จากสูตรที่เหมาะสมที่สุดมีค่า T_g อยู่ที่ 63.43°C สัดส่วนของ WPI และน้ำตาล trehalose จากสูตรที่เหมาะสมที่สุดนี้มีคุณสมบัติพิเศษเชิงโครงสร้างทางเคมีในการปกป้องเฟจจากสภาวะรุนแรงต่างๆ อาทิเช่น ความเป็นกรด-ด่างในช่วงกว้าง (pH 1.5 ถึง pH 9.5) และที่อุณหภูมิในระดับต่างๆ (4°C 25°C และ 50°C)

Phage cocktail ที่ถูกพัฒนาขึ้นยังได้ถูกนำมาเปลี่ยนรูปให้อยู่ในรูปแบบผงแห้งโดยใช้สูตรและวิธีการข้างต้น การศึกษาความคงตัวและสภาวะการเก็บรักษาที่เหมาะสมของ phage cocktail ในรูปแบบผงแห้ง พบว่าการเก็บรักษา phage cocktail ในรูปแบบผงแห้งในบรรจุภัณฑ์แบบ aluminium laminated foil ที่อุณหภูมิ 4°C เป็นสภาวะการเก็บรักษาที่เหมาะสมที่สุด โดยในสภาวะนี้เฟจมีการอยู่รอดในอัตราที่สูง (มีการลดลงของเฟจอยู่ที่เพียง 0.5 log PFU/mL) รวมถึงการคงสภาพทางเคมีกายภาพ (สีและค่า water activity) ของ phage cocktail ในรูปแบบผงแห้งตลอดระยะเวลาการเก็บรักษาเป็นเวลา 12 สัปดาห์

ผลการทดลองพบว่า phage cocktail ในรูปแบบผงแห้งมีประสิทธิภาพในการลดจำนวนเชื้อ*S.* Enteritidis และ *S.* Typhimurium ในหลอดทดลองที่37°C ได้ 1.79 และ 3.63 log CFU/mL ตามลำดับ และที่10°C ได้ 0.43 และ 2.36 log CFU/mL ตามลำดับ ผลการทดสอบประสิทธิภาพของ phage cocktail ในรูปแบบผงแห้งในการลดเชื้อ *S.* Enteritidis และ *S.* Typhimurium ในเนื้อไก่สด ต้นอ่อนทานตะวัน และอาหารสัตว์ พบว่าเชื้อ *S.* Enteritidis ถูกลดจำนวนลงถึง 0.57 CFU/cm^2 0.86 CFU/g และ 1.92 CFU/g ตามลำดับ และ *S.* Typhimurium ถูกลดจำนวนลงถึง 1.78 CFU/cm^2 1.2 CFU/g และ 1.74 CFU/g ตามลำดับ เพื่อศึกษาการยอมรับของผู้บริโภคต่อการประยุกต์ใช้ phage cocktail ในรูปแบบผงแห้งกับอาหารสดและอาหารสัตว์ จึงได้ทำการทดสอบทางประสาทสัมผัสจากผู้ทดสอบจำนวน 30 คน ผลการทดสอบชี้ให้เห็นว่าเนื้อไก่สดและต้นอ่อนทานตะวันที่มีการโรย phage cocktail ในรูปแบบผงแห้งได้รับการยอมรับจากผู้บริโภคเป็นเวลา 3 วันและอาหารสัตว์ที่มีการโรย phage cocktail ในรูปแบบผงแห้งได้รับการยอมรับจากผู้บริโภคเป็นเวลา 2 วันของการเก็บรักษา

นอกจากนี้งานวิจัยนี้ยังได้ศึกษาเพิ่มเติมเกี่ยวกับประสิทธิภาพของการเสริมฤทธิ์กันระหว่างยาปฏิชีวนะและแบคเทอริโอเฟจในการยังยั้งเชื้อ *Salmonella* พบว่าการเสริมฤทธิ์กันระหว่างยาปฏิชีวนะและแบคเทอริโอเฟจมีประสิทธิภาพในการยังยั้งเชื้อ *Salmonella* ได้ดีกว่าการใช้ยาปฏิชีวนะเพียงอย่างเดียว อีกทั้งยังสามารถลดการเกิดการดื้อยาของเชื้อ *Salmonella* ได้ในระดับโมเลกุล จึงสามารถประยุกต์ใช้ทั้งยาปฏิชีวนะและแบคเทอริโอเฟจในการยับยั้งเชื้อ *Salmonella* ได้อย่างมีประสิทธิภาพ

งานวิจัยนี้ชี้ให้เห็นว่าฟาร์มสัตว์ในประเทศไทยเป็นแหล่งที่ส่งผลถึงความชุกและความหลากหลายของเฟจที่มีความจำเพาะต่อเชื้อ *Salmonella* โดยเป็นเฟจที่มีความสามารถในการทำลายเชื้อ *Salmonella* สายพันธุ์ที่หลากหลายจากแหล่งต่างๆ Phage cocktail ทั้งในรูปแบบของเหลวและผงแห้งที่พัฒนาขึ้นจากงานวิจัยนี้มีประสิทธิภาพในการทำลายเชื้อ *S.* Enteritidis และ *S.* Typhimurium ไม่เพียงแค่ในหลอดทดลอง แต่มีประสิทธิภาพในการควบคุมการปนเปื้อนของเชื้อในตัวอย่างอาหารได้จริง อีกทั้ง phage cocktail ในรูปแบบผงแห้งยังมีคุณสมบัติในการทนต่อสภาวะรุนแรงต่างๆ น้ำหนักน้อย และมีอายุการเก็บรักษาที่ยาวนาน ส่งผลดีต่อการนำไปประยุกต์ใช้ในอุตสาหกรรมเพื่อการควบคุมการปนเปื้อนของเชื้ออย่างมีประสิทธิผลและและง่ายต่อการเก็บรักษาและการขนส่ง นอกจากนี้รูปแบบของเฟจที่พัฒนาขึ้นในงานวิจัยนี้ยังสามารถนำไปประยุกต์ได้กับอาหารหลากหลายประเภท รวมถึงในแหล่งต่างๆที่มีความเสี่ยงสูงในการปนเปื้อนเชื้อ *Salmonella* เพื่อการพัฒนาความปลอดภัยตลอดห่วงโซ่อาหาร

Thesis Title Development of Encapsulated *Salmonella* Bacteriophages to Reduce *Salmonella* spp. in Food Matrices

Author Miss Kantiya Petsong

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ABSTRACT

Salmonella Enteritidis and *Salmonella* Typhimurium are the most important serovars that have often been linked to food contamination and foodborne outbreaks. Bacteriophage (phage) has the outstanding properties over antibiotics and other chemical agents with regards to its specificity to kill bacterial pathogens. Phage applications have gained interest as alternative strategy for controlling bacterial pathogens in the food industry. This study aimed to develop microencapsulated phage as dry powder to improve phage applications as an effective strategy to control *Salmonella* serovars. Abundance and diversity of *Salmonella* phages in various animal farms (chicken, swine, goat and bovine) in Songkhla province, Thailand were evaluated upon phage isolation. A total of 36 *Salmonella* phages were obtained, suggesting that animal farms in our study are common sources of abundant *Salmonella* phages. Phenotypic characterization of all isolated phages was determined by phage lysis profiles. Phages were evaluated for their lysis profiles on 47 *Salmonella* strains (28 serovars) isolated from various sources (animal farms, seafood processing plant and humans) in Thailand and the US. Most of isolated phages showed the broader host range on *Salmonella* strains isolated from animal farms in Thailand as compared to other sources. However, a few phages showed a broad spectrum against *Salmonella* strains from different communities and continents. Genotypic characterization by pulsed-field gel electrophoresis (PFGE) analysis revealed the estimated genome sizes ranging from 50 ± 2 kb to 200 ± 2 kb among isolated phages. Phenotypic and genotypic characterizations indicated that our isolated phages could effectively control important *Salmonella* serovars. To improve the antibacterial efficiency of our phages against important *Salmonella* serovars (*S.* Enteritidis and *S.* Typhimurium), we thus selected three

phages which showed the highest efficiency and further develop as a phage cocktail. Based on efficiency of plating (EOP) and lytic ability assays, KP4, KP5 and KP50 were selected. Electron microscopy analysis classified these phages in the order *Caudovirales* and family *Siphoviridae*. One-step growth curve assay revealed the burst size of three phages of approximately 25-98 PFU/cell and latent period of 5-40 min on *S.* Enteritidis. On *S.* Typhimurium the burst size revealed approximately 70-112 PFU/cell and latent period of 10-15 min. Genome sequencing analysis and annotation revealed that phages KP4, KP5 and KP50 presented as virulent phages due to the absence of the lysogeny module, genes associated virulence/toxins and genes associated antibiotic resistance.

Antibacterial efficiency of our developed phage cocktail was investigated in *in-vitro*, foods and also feed model. *In-vitro* study, *S.* Enteritidis and *S.* Typhimurium were decreased by more than 4 log CFU/mL after 4 h of phage cocktail treatment. In addition, phage-resistant development was not found in *Salmonella* after phage cocktail treatment. In chicken meat, *S.* Enteritidis and *S.* Typhimurium were decreased by 0.66 and 1.73 log CFU/cm^2, respectively. In sunflower sprout, *S.* Enteritidis and *S.* Typhimurium were decreased by 1.27 and 1.17 log CFU/g, respectively. In animal feed, *S.* Enteritidis and *S.* Typhimurium were decreased by 1.87 and 2.38 log CFU/g, respectively. Antibacterial efficiency studies indicated that our developed phage cocktail provided high efficiency to control *Salmonella* contamination in food matrices.

A new form of microencapsulated phage as dry powder was developed to improve the limitations and extend the application of phage as traditional lysate form. To protect phage particles from drying method (freeze-drying), the formulations presented the combination of coating materials (whey protein isolate; WPI and trehalose) were optimized. The ratio of WPI/trehalose at 3:1 presented the optimal formulation with the highest encapsulation efficiency (EE) of 91.9%. SEM images indicated the complex physical structure of coating materials. Fourier transform infrared spectroscopy (FTIR) analysis revealed the chemical interaction of coating materials as H−bonding. Differential scanning calorimetry (DSC) analysis indicated T_g of our develop dry phage powder with optimal formulation at 63.43°C. Our dry phage powder with optimal formulation showed

the specific functional properties to protect phage particles as high stability in wide range of pH (1.5 to 9.5) and various temperatures (4°C, 25°C and 50°C).

A developed phage cocktail was transformed as dry powder by the optimized formulation using freeze-drying. Stability and storage conditions studies revealed that dry phage cocktail powder kept in aluminium laminated foil bag at 4°C showed the suitable conditions of dry phage cocktail powder. The phage titer was decreased by only 0.5 log PFU/mL and the physio-chemical properties (color and a_w) of dry phage cocktail powder remained unchanged over 12 weeks of storage. In addition, the study revealed that dry phage cocktail powder showed higher phage survivability as compared to the lysate form. Dry phage cocktail powder showed the desirable antibacterial efficiency to decrease the number of *S.* Enteritidis and *S.* Typhimurium in *in-vitro*, foods and also feed model. *In-vitro* study showed that *S.* Enteritidis and *S.* Typhimurium were decreased by 1.79 and 3.63 log CFU/mL, respectively at 37°C, and 0.43 and 2.36 log CFU/mL, respectively at 10°C. In chicken meat, *S.* Enteritidis and *S.* Typhimurium were decreased by 0.57 and 1.78 log CFU/cm^2, respectively. In sunflower sprout, *S.* Enteritidis and *S.* Typhimurium were decreased by 0.86 and 1.2 log CFU/g, respectively. In animal feed, *S.* Enteritidis and *S.* Typhimurium were decreased by 1.92 and 1.74 log CFU/g, respectively. Sensory evaluation (30 panelists) indicated that the quality of foods (chicken meat and sunflower sprout) and feed applied with dry phage cocktail powder were accepted for 3 days and 2 days of storage, respectively. Results indicated that the developed dry phage cocktail powder provided high antibacterial efficiency to control *Salmonella* serovars in different food matrices without negative effect on the acceptability of consumers.

In addition, this study also investigated the effect of a combination of antibiotic and bacteriophage in controlling *Salmonella*. The results showed that antibiotic and bacteriophage provided the synergistic effect against *Salmonella* compared to using antibiotic alone. Moreover, this effective strategy could lower the mechanism of antibiotic resistance at the molecular level, indicating that this combination can be used as the effective strategy to control *Salmonella*.

Overall, this study indicates that animal farm environments in Thailand provide abundance and diversity of *Salmonella* phages which could effectively eliminate important *Salmonella* serovars isolated from different sources and continents. Our developed phage cocktail in both lysate and dry powder form showed high efficiency to control *Salmonella* serovars in different food matrices. Dry phage cocktail powder developed here showed high efficiency to protect phage particles in the harsh conditions. This study suggests that phage as dry powder is a novel convenient form for applications, storage and transportation. In addition, this developed form can be used in various food matrices and also in high-risk sources of *Salmonella* contamination to improve safety along the food production chain.

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Kantiya Petsong

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ABBREVIATIONS AND SYMBOLS

BLAST	=	Basic local alignment search tool
CDC	=	Centers for disease control and prevention
CFU	=	Colony forming unit
DSC	=	Differential scanning calorimetry
EE	=	Encapsulation efficiency
EFSA	=	European food safety authority
EOP	=	Efficiency of plating
ERS	=	Economic research service
FAO	=	Food and Agriculture Organization of the United Nations
FTIR	=	Fourier transform infrared spectroscopy
g	=	Gram
h	=	Hour
kp	=	Kilobasepair
mm		Millimeter
mM	=	Millimolar
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
M	=	Molar
NCBI	=	The National Center for Biotechnology Information
NIH Thailand	=	National Institute of Health of Thailand
PBS	=	Phosphate buffered saline
PEG	=	Polyethylene glycol
PFGE	=	Pulsed-field gel electrophoresis
PFU	=	Plaque forming unit
rpm	=	Revolutions per minute
RAST	=	Rapid Annotation using Subsystem Technology

ABBREVIATIONS AND SYMBOLS (Continued)

SDS	=	Sodium dodecyl sulfate
SEM	=	Scanning Electron Microscopy
SM buffer	=	Salt-Magnesium buffer
TBE buffer	=	Tris/Borate/EDTA buffer
TE buffer	=	Tris/ EDTA buffer
T_g	=	Glass transition temperatures
TEM	=	Transmission Electron Microscopy
TSA	=	Tryptone Soya Agar
TSB	=	Tryptone Soya Broth
WPI	=	Whey Protein Isolate
XLD	=	Xylose-Lysine-Deoxycholate Agar
°C	=	Degrees Celsius
$\times g$	=	Relative centrifugal force
%	=	Percent
µl	=	Microliter
µm	=	Micrometer

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Salmonella enterica is one of the major bacterial pathogens that can cause a serious public health concern worldwide. This pathogen can be transmitted from animals to humans or humans to humans leading to *Salmonella* infections (non-typhoidal salmonellosis) which is linked to foodborne and gastrointestinal illnesses (Acheson and Hohmann, 2001). Among 2,600 serovars, *S.* Enteritidis and *S.* Typhimurium have been reported as the two most common serovars of *Salmonella enterica* associated with the outbreaks of salmonellosis (García *et al.*, 2018). In the US, the estimated number by the Centers for Disease Control and Prevention (CDC) indicated that 1.3 million human foodborne illnesses were associated with salmonellosis and more than 500 deaths each year (Callaway *et al.*, 2008). The high numbers of cases indicates that *Salmonella* contamination requires better effective strategies for control and reduction.

Bacteriophages (phages) are viruses of bacteria which have the outstanding properties especially the specificity to the bacterial host cells (Sulakvelidze *et al.*, 2001). According to their specificity of infection, the use of bacteriophages has emerged as a promising tool for food preservation and safety (Hagen and Loessner, 2010; Goodridge and Bisha, 2011). Certain phages have been shown to have minimal effects on the change of quality and sensory properties of specific foods (Fister *et al.*, 2016). To improve the antibacterial efficiency and avoid phage-resistant bacteria of phage-based biocontrol, phage cocktail (the combination of effective phages) has gained more of interest. Phage cocktail as bio-control agent has been studied to control *Salmonella* serovars in various food products, including raw meat (Spricigo *et al.*, 2013), meat products (Grant *et al.*, 2017), fresh produce (Kocharunchitt *et al.*, 2009; Ye *et al.*, 2010) and even animal feed (Heyse *et al*., 2015; Soffer *et al*., 2016). However, using of phage as traditional liquid form (lysate) has some limitations with regards to applications for use in various food matrices, the phage stability in harsh conditions and conditions of storage and transportation.

Microencapsulation is a technique which can improve the limitations and extend the application of the traditional lysate form (phage suspension in liquid form). Microencapsulated form of phages has shown several advantages for agricultural and food

applications. For examples, a previous study showed that microencapsulated phages could have a shelf-life of over 3 months of storage (Haddad *et al.,* 2017). Microencapsulation can be defined as a process of surrounding substance as core materials (e.g. compounds, particles) within the protective coating materials on a very small scale (Jeyakumari *et al*., 2016). The products from this process can be called microcapsule or microsphere (Dubey *et al*., 2009). As bio-control in food application, the size of microcapsule or microsphere should be acceptable by the consumer. Among several methods of microencapsulation, spray-drying and freeze-drying provide the microencapsulated phage as dry powder (Gouin, 2004). In order to protect phage particles from the drying conditions, protective coating materials are needed. Whey protein isolate (WPI) and disaccharide trehalose are the interesting coating materials which showed the potential to protect living cell and phages from the process of drying conditions to transform material to be dry powder (Vonasek *et al.,* 2014; Arslan *et al.,* 2015; Puapermpoonsiri *et al.,* 2010).

This study aimed to develop microencapsulated phage as dry powder to improve phage applications as an effective strategy to control *Salmonella* serovars. We first isolated *Salmonella* phages from various animal farm environments in Songkhla province, Thailand to study the abundance and diversity of *Salmonella* phages. Lysis profiles of isolated phages against diverse *Salmonella* strains from various sources (animal farms, food processing plant and human) in Thailand and US was determined to understand the phages'ability in lysing each host strain. Phages which showed the highest efficiency to kill *S.* Enteritidis and *S.* Typhimurium were developed as a "phage cocktail" (combination of multiple effective phages) to improve the antibacterial efficiency. Each phage composed in the cocktail was determined physical morphology, biology (one-step growth curve) and genome sequencing. Efficiency of the phage cocktail in reducing the two important *Salmonella* serovars (*S.* Enteritidis and *S.* Typhimurium) *in-vitro*, foods (chicken meat and sunflower sprout) and animal feed were investigated. The optimal formulation of microencapsulated phage as dry powder was developed by optimizing the combination of WPI and disaccharide trehalose. Specific functional properties of microencapsulated phage formulation to protect phage particles in harsh conditions (pH and temperatures) were investigated. The stability, including survivability and physio-chemical properties during storage of dry phage cocktail powder kept in different packaging (aluminium laminated foil; LF and low density poly ethylene; LDPE) and different temperatures (4°C and 25°C) were also investigated. The efficiency of dry phage cocktail powder in reducing

S. Enteritidis and *S.* Typhimurium *in-vitro*, foods (chicken meat and sunflower sprout) and animal feed were investigated. To access consumers' acceptance on foods and feed applied with phage cocktail dry powder, sensory evaluation using a nine-point Hedonic scale was performed with a group of panelists.

1.2 Literature Review

1.2.1 *Salmonella* spp.

Salmonella species (spp.) are the Gram-negative, rod-shaped, non-spore forming, facultative anaerobic and motile bacterium (by peritrichous flagella). However, there are non-motile *Salmonella* variant include *S.* Gallinarum and *S.* Pullorum. The estimated size of this genus is approximately 2-5 μm long and 0.8-1.5 μm wide, straight rod. Generally the biochemical characteristic of *Salmonella* can be defined into 2 metabolisms (respiratory and fermentative). The optimal growth temperature of *Salmonella* is 37°C. *Salmonella* are positive with catalase, methyl red and Simmons citrate and negative with oxidase, indole and Voges-Proskauer. This pathogen can catabolize D-glucose and other carbohydrates to acid and gas as the products. In addition, H_2S can be produced by this pathogen also (Abulreesh, 2012).

Salmonella is groped into the family Enterobacteriaceae, and divided into two species (*S. enterica* and *S. bongori*). More than 2,600 serovars of *Salmonella* spp. (Issenhuth-Jeanjean *et al.,* 2014), *S. enterica* is divided into six subspecies including *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (VI) (Brenner *et al.,* 2000).

1.2.2 Epidemiology of *Salmonella* spp.

The outbreak associated with *Salmonella* has been reported globally each year. *Salmonella* has been reported as the leading pathogen which cause food-borne outbreaks (Figure 1.1) (EFSA, 2015). The estimation of 93.8 million cases (gastroenteritis) and 155,000 deaths has been reported. Among these numbers, 80.3 million cases were foodborne illness (Majowicz *et al.,* 2010). In the United States, *Salmonella* acts as the leading cause of foodborne illness, with an estimated number of 1.2 million illnesses every year and approximately 360,000 *Salmonella* illnesses attributed to FSIS-regulated products in FY2014 (USDA, 2014). The USDA Economic Research Service (ERS) was estimated

the annual cost of foodborne illnesses for *Salmonella* (non-typhoidal) in the US, the total cost is $3,666,600,031. (Hoffmann *et al.*, 2015). The clinical disease of *Salmonella* infection in humans has been divided into two groups depending on the serovars of *Salmonella*, comprising of Typhoid fever, caused by serovars *S.* Typhi and *S.* Paratyphi A (both are found only in humans) and non-typhoidal salmonellosis, caused by serovars other than *S.* Typhi and *S.* Paratyphi A (Marzel *et al.*, 2016). Non-typhoidal *Salmonella* is the important causes of bacterial foodborne infections and public health concern worldwide. Foodborne disease outbreaks due to non-typhoidal *Salmonella* have been reported continuously every year (CDC, 2018).

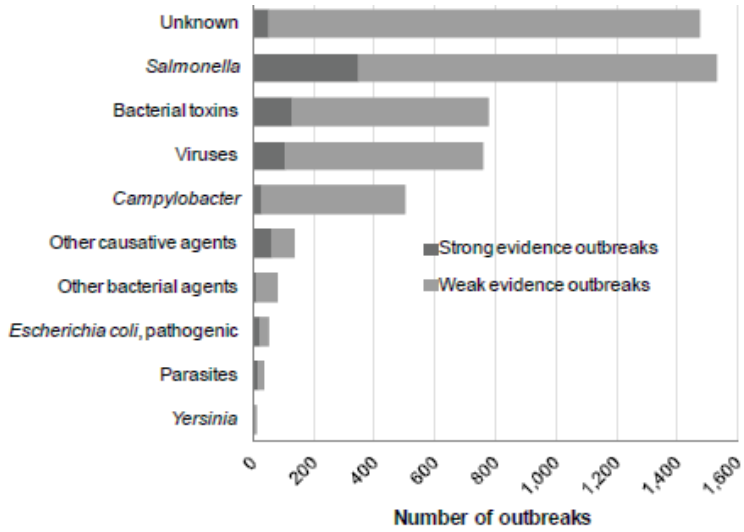

Figure 1.1. Distribution of all food-borne outbreaks per causative agent in the EU.
Source: EFSA, 2015.

1.2.3 Prevalence of *Salmonella* in the environment

Salmonella is the common pathogen normally found along the food environments. The main habitat of *Salmonella* serovars represented by the intestinal tract of humans and animals. Environments related animal farms such as feedstuff, soil, litter, and fecal are generally recognized as the source of *Salmonella* serovars contamination (Andino and Hanning, 2015). As mentioned above, this pathogen alive in gastrointestinal tract of animals, indicated that fecal material is the important issue in this way. When the animals excrete, the pathogen can spread around the farm and can contaminate into the water, soil, grass and plants which growing around the farm area. When the animals roam and consume the plants or water, they can be contaminated with pathogen from those

sources. These animals may get sick or can be the carrier of the foodborne pathogen. However, the prevalence and diversity of *Salmonella* serovars can be transmitted by insects and other animals to the broad location and normally in the polluted water (Andino and Hanning, 2015).

1.2.3.1 Prevalence of *Salmonella* serovars found in humans

Serovars of *Salmonella* predominantly found in humans are different as both geography and environment. These issues are playing a role as the important factors which effect on the differentiation of the prevalence and the diversity of *Salmonella* around each place. For example, in the EU, *S.* Enteritidis and *S.* Typhimurium are the serovars most frequently associated with human illness (EFSA, 2017) (Table 1.1). *S.* Enteritidis contamination cases are frequently associated to eggs and poultry origin. *S.* Typhimurium contamination cases are frequently associated to pork or beef (Hugas and Beloeil, 2014).

Table 1.1. Common serovars (top 5) of *Salmonella* reported in the European Union, Thailand and the United states between 2004 to 2016.

Geography	*Salmonella* serovars	References
EU	Enteritidis	EFSA, 2017
	Typhimurium	
	Monophasic Typhimurium 1.4.[5].12:i:5	
	Infantis	
	Newport	
Thailand	Weltevreden	NIH Thailand, 2004
	Enteritidis	
	Anatum	
	Derby	
	1, 4, 5, 12:i:-ssp.I	
United States	Enteritidis	EFSA, 2017
	Typhimurium	
	Newport	
	I 4,[5],12:i:-	
	Javiana	

1.2.4 Prevalence of *Salmonella* serovars on farms

Salmonella is the pathogen commonly found in the food chain. The shedding in animals and humans diseases related to this pathogen. The highest shedding by animals can be found in the summer season (McEvoy *et al.,* 2003; Fossler *et al.,* 2005). *Salmonella* spp. has been isolated from many types of animal farms such as dairy farms, swine farms and poultry farms. Previous studies reported that *Salmonella* ecology and its diversity related to dairy farm. Roy *et al.* (2001) and Wells *et al.* (2001) isolated *Salmonella* from the feces of healthy dairy cattle (Roy *et al.,* 2001; Wells *et al.,* 2001). Similarly Edrington *et al.* (2004) reported that this pathogen may live as the normal or temporality members of the gastrointestinal microbial population (Edrington *et al.,* 2004). In addition, Funk *et al.* (2001) reported that that *Salmonella* generally found in swine farms (Funk *et al.,* 2001). For poultry farm, CDC always reported salmonellosis linked to live poultry, chicken and duckling (CDC, 2018). The diversity and prevalence of *Salmonella* serovars differ among studies in different regions and types of farms. Serovars which have consistency rates of specific consisting of Heidelberg, Kentucky, Enteritidis, Typhimurium and Montevideo as these are the highest recovered serovars (Andino and Hanning, 2015).

1.2.5 High-risk food products and products commonly contaminated with *Salmonella*

In the basic, high risk food can be defined as the food that can be harmful to humans because it can be supported the growth of pathogenic bacteria. High risk food normally means the food which has not cook before consume. There are many groups of food associated with high-risk food (Table 1.2) such as meat, poultry, eggs, dairy products, shellfish and other seafood as well as ready-to-eat foods such as sandwiches, cooked meats, pates and soft cheeses. The two major genus of bacteria that typically cause chicken food poisoning are *Salmonella* and *Campylobacter*. Eggs and product that made from raw egg are at high-risk for *Salmonella* food poisoning. Raw meat may involve in cross-contamination of cooked meat in the refrigerators. For dairy products, unpasteurized milk can be harmful as this product has not been heat treated, which can support the growth of microorganisms. A number of people normally eat raw shellfish, which may contain harmful toxins or bacteria that can cause food poisoning. Unwashed fruit and vegetables can be contaminated with microorganisms through manure. Generally, fresh produce is consumed fresh and this may cause high-risk of food poisoning. Previously, CDC reported

Salmonella outbreaks, foods which are generally contaminated with *Salmonella* spp. comprise of poultry, meat, fresh vegetables, peanut butter and animal feed (CDC, 2018).

Table 1.2. High risk foods and products commonly contaminated with *Salmonella.*

Food category	Food sample
Cooked meat	Ground beef, pork, chicken, turkey and duck
Cooked meat products	Meat pies and pasties, pate, meat stock and gravy, cook-chill meals, ham
Dairy products	Milk, cream, artificial cream, custards, products containing unpasteurized milk, ripened soft and molded cheeses
Egg products	Mayonnaise, mousse and home-made ice cream
Shellfish and other sea-foods	Frozen raw tuna, cockles, cooked prawns and raw oysters
Fresh fruit, vegetables and their products	Leafy greens, papaya, potato, tomatoes, sprout, cereal grains and peanut butter
Animals and their environments	Amphibians (frogs), birds (baby chicks) and animal feed

Source: CDC, 2012-2018.

1.2.5.1 Poultry meat and products

Poultry meat and products are considered as high-risk food matrices and can commonly be contaminated with *Salmonella* spp. There are many cases of outbreaks reported by CDC which can be indicated poultry meat and products are the major food category that normally contaminated with *Salmonella.*

In 2011, the outbreak of *S.* Hadar was reported to be associated with the turkey burgers. This outbreak involved totally 12 infected persons from 10 states (CDC, 2011). In the same year, multistate outbreak of human infected *S.* Altona and *S.* Johannesburg linked to chicks and ducklings also reported. This outbreak represented the larger outbreak with a total of 68 and 28 individuals infected with *S.* Altona from 20

states and 28 individuals infected with *S.* Johennesburg from 15 states (CDC, 2011). The outbreaks of *S.* Heidelburg probably are the major outbreak of this year. Two products contaminated with this strain have been investigated. The contaminated ground turkey cause a total of 136 infected persons from 34 states and Kosher broiled chicken livers from Schreiber processing operation cause 190 illnesses (CDC, 2012). *Salmonella* infection linked to live poultry in backyard flocks has been reported in 2012, with the number of 195 cases from 27 states 34 hospitalizations and 2 deaths (CDC, 2012). In 2013 several *Salmonella* serovars including Infantis, Lille, Newport, and Mbandaka were linked to the outbreaks in live poultry. Totally 154 ill persons and 29 hospitalizations were reported from 30 states. At the same period multistate outbreak of human *S.* Typhimurium infections linked to live poultry in Backyard flocks was reported. These outbreaks cause the total of 356 ill persons and 62 hospitalizations from 39 states (CDC, 2013). In 2014, Foster farms brand chicken recalled their products because of the outbreak of multidrug-resistant *S.* Heidelberg. The investigations reported 634 ill persons from 29 states with 38% represented hospitalizations (CDC, 2014). The investigations indicated that this outbreak linked to Tyson brand, mechanically separated chicken at a correctional facility. Approximately 33,840 pounds of mechanically separated chicken products that might be contaminated with *S.* Heidelburg are recalled. During this year, the outbreaks of *S.* Enteritidis infections linked to raw, frozen and stuffed chicken entrees produced by Aspen Foods and Barber Foods were reported (CDC, 2014).

1.2.5.2 Fresh produce

Fresh fruits and vegetables are food category which have been reported as *Salmonella* contamination each year (CDC, 2018). Since this food category is normally consume as raw, the practices of cultivation, harvesting and processing are important to protect bacterial (spoilage and pathogenic) contamination (Hanning *et al*., 2009). In the process of produce cultivation, there are several routes that can support *Salmonella* or other microorganism contamination. *Salmonella* is normally found in gastrointestinal tract of domestic and wild animal (Wiedemann *et al.*, 2015). The crop might be contaminated with *Salmonella* by soil related to animal shedding. Water used to grow the crop has high opportunity to contaminate with *Salmonella* by animal feces (Fatica and Schneider, 2011). Since 2008, the outbreaks associated with *Salmonella* contamination in fresh produce have been reported continuously. In September 2008, the outbreak linked to *S.* Typhimurium

contaminated in alfalfa sprouts has been reported with 13 illnesses in Washington and Oregon (FDA, 2008). *S.* Typhimurium has been reported as the most serovars which contaminated in vegetables from Maxico with 23.9% prevalence (Quiroz-Santiago *et al.*, 2009). In 2011, the outbreak of *S.* Agona contaminated in papaya (106 cases) and *S.* Panama contaminated in cantaloupe (20 cases) (CDC, 2011). In 2012, mangoes contaminated with *S.* Braenderup (127 cases), cantaloupe contaminated with *S.* Typhimurium and Newport (261 cases) (CDC, 2012). In 2014, cucumbers contaminated with *S*. Newport (275 cases) (CDC, 2014). In 2015, the outbreak of cucumbers contaminated *Salmonella* occurred again in 907 cases (CDC, 2015). In 2017, *Salmonella* outbreak related to papayas has been reported with 173 cases (CDC, 2017). The number of outbreaks indicating that fresh produce is a food category which should be concerned with *Salmonella* contamination.

1.2.5.3 Animal feed

Animal feed is another source that *Salmonella* can be spread directly to humans. Many reasons can facilitate *Salmonella* to be survived in animal feed. For example, feed composition can influence survivability, as water activity can support the survival of *Salmonella*. However, higher probability to find *Salmonella* in heat treated products than in non-heat treated products (Keerthirathne *et al.*, 2016). Feed is the potential source of hazard to animal and can be transmitted to humans. The control of the quality of feed materials should be emphasized (Kukier *et al.*, 2013). A previous study has shown that *Salmonella* contamination of feed ingredient samples was about 5.3% in corn (19 samples), 100% in cottonseed meal (10 samples), 10% in soybean meal (10 samples) and 4.2% in wheat middling (24 samples) (Jones and Richardson, 2012). Animals feed represent at the beginning of food safety chain in the "farm-to-fork" model (Crump *et al.*, 2002). Previous study indicated that the food-producing animals play the major role as the reservoir of *Salmonella enterica*. The number of 1,412,498 human illnesses, 16,430 hospitalizations, and 582 deaths annually in the US has been reported (Crump *et al.*, 2002). Health Canada has been informed FDA of Canadian outbreaks of human salmonellosis that related to *S.* Infantis in pig- ear dog treats which were manufactured in Canada in 1999, and in 2002 *S*. Newport in beef steak-patty dog treats which were manufactured in Texas. CDC has been reported the outbreak of *S.* Schwarzengrund from January 1, 2006 to October 31, 2008. The contamination of *Salmonella* related to dry pet food caused salmonellosis in 79 infected persons (FDA, 2013). In 2012, the outbreak of *S.* Infantis has been reported.

Multistates outbreak of human *S.* Infantis infections linked to dry dog food is updated with the final report on July 18, 2012. A total of 49 individuals infected with this outbreak strain was reported. The epidemiologic and laboratory investigations indicated that this outbreak linked to dry dog food produced by Diamond Pet Foods at a single production facility in Gaston, South Carolina (CDC, 2012).

1.2.6 Bacteriophages

Bacteriophages or phages are bacterial viruses that multiply themselves inside bacterial host. Phages reproduction require the machinery of bacterial host. Phage can destroy specific bacterial cells via adsorption and entry into the host cell by using the receptors which are very specific for examples; carbohydrates, proteins and lipopolysaccharides on the surface of the bacterial host (Withey *et al.*, 2005). Frederick Twort was the first person who discovered phages in 1915. After two years later, phages were described again by Felix d'Herelle (1917). Bacteriophage offers a number of advantages. For examples phages are exist in natural environment, specific to the bacterial host and nontoxic to natural human or animal microflora population (Sulakvelidze *et al.*, 2001).

1.2.6.1 Biology and life cycles

Bacteriophages have 2 major types of life cycles (Figure 1.2), lytic and lysogenic cycles. Lytic or virulent phages refer to phage that can inject their DNA into the host cell, and then replicate themselves inside the host by using the host DNA machinery. Protein produce and genome will be mixed together to be phage progeny. These phage progeny will eventually cause bacterial cell burst. Lysogenic or temperate phages refer to those phages that can inject their DNA into the genome of host cells and produce prophages. When chromosome of bacteria replicated themselves, prophage is also replicated together as if it belongs to chromosome of bacteria. New cells of bacteria then have prophages hiding in the cell. This system term as lysogenization, and bacteria that have prophage inside are called lysogens or lysogenic bacteria. The host and lysogenic bacteria can be co-evaluated and can change many things to bacteria such as change nonpathogenic strain to be virulent strain. Furthermore, phages can protect the destruction from another phage to the host cell (Brüssow *et al.*, 2004).

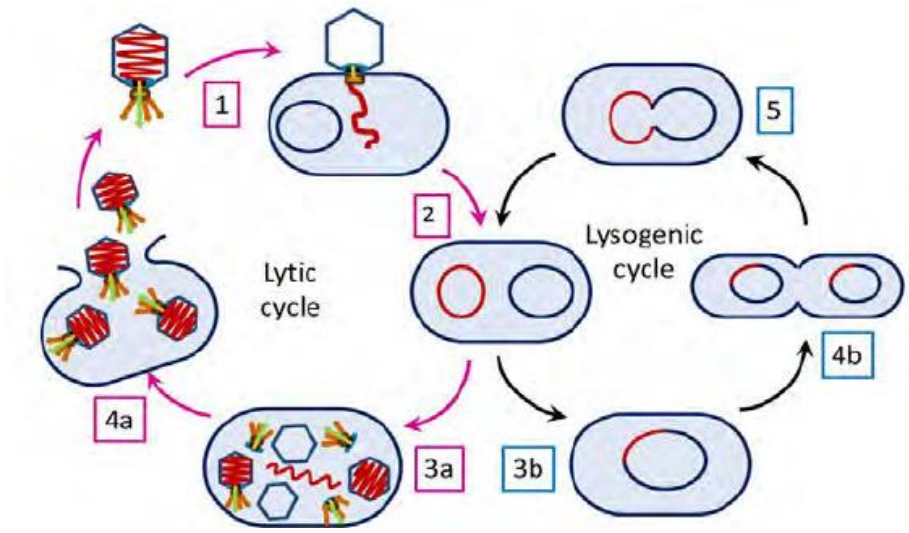

Figure 1.2. Bacteriophage life cycles. (1) phage attach and inject its DNA into the host (2) phage DNA enters lytic or lysogenic cycle (3a) step of phage synthesis (4a) host cell burst; (3b) and (4b) phage genome integrated within the bacterial chromosome (5) prophage excises from the bacterial chromosome and initiates the lytic cycle.
Source: Orlova, 2012.

1.2.6.2 Bacteriophage biology

Bacteriophages have several steps to infected bacterial host cells or replicate themselves. The single-step growth experiment has been recognized as the method to investigate the biology of bacteriophages. Ellis and Delbruck are the researchers who first presented this methodology. These authors devised a method to demonstrate only a single step of the many steps of phage replication.

1.2.6.2.1 One-step growth curve of bacteriophages

The growth mechanism of viruses, including bacteriophages can be divided into sequential steps. (i) phage attach to bacterial host cell, (ii) phage inject their genome into bacterial host (iii) synthesize their proteins and nucleic acid inside the host cell (iv) maturation of new phage particles (progeny), and (v) progeny release step. The biology of bacteriophages can be represented all steps of growth mechanism. One- or single- step growth curve is generally used in order to investigate the biology of bacteriophages (Figure 1.3). Latent or eclipse period is the initial stage of growth curve (this stage similar to the lag phase of general bacteria). Adsorption, infection, and syntheses

step are included during this period. After that maturation or release stage will be occurred (this stage similar to the log phase of general bacteria). In this stage, there are a lot of progeny particles inside bacterial host. These particles are able to release and cause the host cell burst. After this stage, the number of phage particles will be increased and the burst size of particular phage can be determined (Hyman and Abedon, 2009).

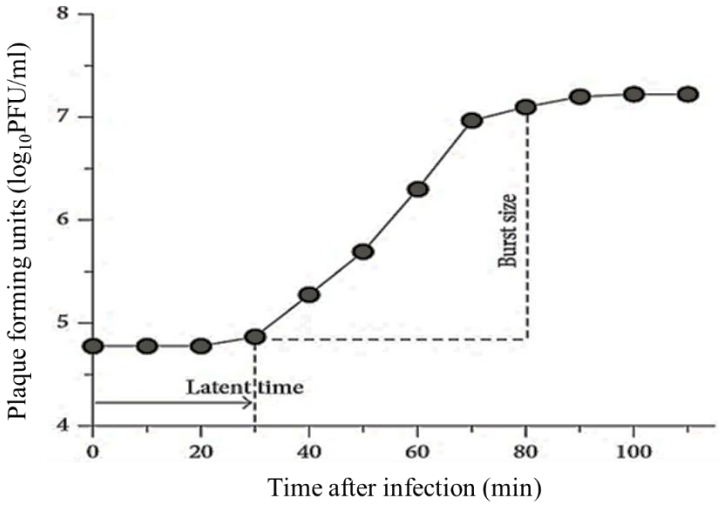

Figure 1.3. One step growth of viruses infecting host cells.
Source: Cao *et al.*, 2015**.**

1.2.6.2.2 Latent or eclipse period and burst size

Latent period can be described as the period between injection steps to lysis step of cells. On another hand, latent period can be defined as constant period as well as the halfway point during the rise period or indeed. Rise period is latent periods of individual cell vary over a range (Wang, 2005). The range of *Salmonella* phage latent period has been summarized with the range of 15-45 min (Calsina *et al*., 2011). Rise period begins at the end of latent period and ending when phage titer is increasing. The term of bacteriophage burst size can define as yield of new phage particles by lysis of an infected host cell. The term of burst size normally refer to the average burst size. Determination of burst size related to comparison of infective cells in the culture prior to phage-induced bacterial cell lysis versus infective centers present in cultures following such lysis. The number of progeny released (burst size) varies from 50 to 200 new phage particles (Wommack and Colwell, 2000).

1.2.6.2.3 Multiplicity of infection (MOI)

Multiplicity of infection (MOI) is the average number of phage particles per bacterial cell. The MOI determination can be calculated by dividing the number of phage phage particles added (PFU/mL) by the number of bacterial cells added (CFU/mL). Even though the value of MOI can indicate the average number of phage per bacterial cell, the actual number of phage that infects any given bacterial cell is a statistical function (Shabram and Aguilar-Cordova, 2012).

1.2.6.3 Concerned genes in phage genome for food application

1.2.6.3.1 Major genes involves in phage lytic and lysogenic life cycle

Lysis and lysogenic module play the major key on the mechanism of phage life cycles. For lysis module, holin and endolysin act as the major proteins which involved the mechanism of host cell lysis (Shin *et al*., 2014). The process of lysis achieved by the collaboration of these protein. Holin damaged cytoplasmic membrane of host cell by creating the holes. Endolysin uses these holes as transport channels to digests the peptidoglycan layer (Krupovič *et al*., 2008). For lysogeny module, Cro, CI, CII, CIII, N and Q are well known as lysogeny control region. Expression of N and Cro proteins resulting in the producing of CII, CIII and Q which activate the production of integrase and CI protein (Shin *et al*., 2014). Integration enzyme such as integrase enzyme and CI play the main role to turn off the repressor, resulting the process forward to the recombination step instead of replication step (Kropinski *et al*., 2007). However, induction by host DNA damage provides RecA protein to inactivate CI and allow lytic genes to process lytic cycle (Atsumi and Little, 2006). In order to determine the life cycle of bacteriophages, lysis or lysogenic module should be focused on. Genomic analysis of *Salmonella* phages have been annotated. Moreno Switt *et al.* (2013) were reported the lysis and lysogenic module which indicated the life cycles of lytic and lysogenic *Salmonella* phages isolated from animal farms (Table 1.3).

Table 1.3. Summary of major genes in the clusters of lysis module and lysogeny module of *Salmonella* phages.

Lysis module	Lysogeny module
Endolysin	Cro/C1 protein
Lysozyme	XRE-family of transcriptional regulators
Lysin	Integrase
LysB	Transcriptional regulator
LysC	Cox protein
LysN	Regulatory protein CII
	Regulatory protein CI
	Regulatory protein Cro
	Excisionase

Source: Moreno Switt *et al.,* 2013.

1.2.6.3.2 Genes associated virulence, toxins and antibiotic resistance

Phages have ability to contribute the virulence, toxin and antibiotic resistance in the process of infection (Wagner and Waldor, 2002; Moreno Switt *et al*., 2013). Genetic exchange transfer of bacterial DNA by phage is well known as transduction (Watson *et al*., 2018). Therefore the presence of these genes is the major of concern. *Salmonella* phages have been reported to carry these concern genes (Table 1.4) e.g. phage Gifsy-1 encodes *gipA* which enhance survival in the Peyer's patch (Stanley *et al*., 2000 cited in Wagner and Waldor, 2002), phage SopEϕ transduces type III secretion system (Mirold *et al*., 1999 cited in Wagner and Waldor, 2002) and phage FSL SP-016 carries bicyclomycin R (Moreno Switt *et al*., 2013).

Table 1.4. Genes associated virulence, toxins and antibiotic resistance in *Salmonella* phages.

Virulence and toxin associated genes	antibiotic resistance associated genes
Agglutinating adhesion (Moreno Switt *et al*., 2013)	Co-transduction: *Amp-cam, Amp-tet, Sul-str* (Schmieger and Schicklmaier, 1999)
Virulence protein MsgA/putative damage inducible protein DinI (Moreno Switt *et al*., 2013)	*amp, cam, tet* (Schmieger and Schicklmaier, 1999)
gipA (Satnley *et al*., 2000)	bla_{CMY-2} (Zhang and Lejeune, 2008)
sitA (Suez *et al*., 2013)	Tellurite resistance (Moreno Switt *et al*., 2013)
avrA (Suez *et al*., 2013)	Biocyclomycin resistance protein (Moreno Switt *et al*., 2013)
sprB (Suez *et al*., 2013)	Plasmid conferring kanamycin resistance native to donor organisms (Bearson and Brunelle, 2015)
orgA (Suez *et al*., 2013)	Cefoxitin (Ross and Topp, 2015)
ttrC (Suez *et al*., 2013)	Sulfamethazine (Ross and Topp, 2015)
ssrB (Suez *et al*., 2013)	

1.2.6.4 Applications of bacteriophages against foodborne pathogens

Phages have been previously evaluated the efficiency against antibiotic resistant bacteria, including the foodborne pathogen *Salmonella* (Carvalho *et al.,* 2012). Phage-based bio-control agent has recognized as an effective strategy to control various pathogens in several food products (Hagens *et al.,* 2010; Goodbridge *et al.,* 2011). Phage application as commercial products has been previously granted for GRAS status such as ListShield™ and SalmoFresh™ (FDA, 2016; FDA, 2013).

Bacteriophage can use to control several pathogens such as *Salmonella* Enteritidis in melon and apple slices (Leverentz *et al.,* 2003), *Listeria monocytogenes* in melon and apple slices (Leverentz *et al.,* 2003), *Escherichia coli* O157:H7 in beef steaks (O'Flynn *et al.,* 2004), *Salmonella* Typhimurium in pigs (Lee and Harris, 2001).

Salmonella bacteriophages have been characterized and shown the diversity among the phages isolated from dairy farms in New York (Moreno Switt *et al.,* 2013). The study reported a number of *Salmonella* phages isolated from manure storage and animal holding areas with 78% of samples collected. They suggested that *Salmonella* phages are common in the dairy pre-harvest environment. With the high diversity of *Salmonella* phages, they can be developed *Salmonella* detection and control as the novel strategies. A number of applications employing *Salmonella* phages have been evaluated. Heringa *et al.* (2010) have developed a bacteriophage mixture that could be effective in reducing *Salmonella* contamination in compost under different environmental conditions. This research study showed that *Salmonella* phages could reduce but not completely eliminated *Salmonella* from compost because environmental factor such as UV radiation, fluctuating temperature and precipitation, could be of influencing factors (Heringa *et al.*, 2010).

1.2.6.5 Use of *Salmonella* bacteriophages in food production chain

Salmonella phages have been studied and evaluated their effectiveness in different food matrices (Table 1.5). For example, a phage cocktail comprising of 2 *Salmonella* phages showed the efficacy in reducing *S.* Oranienburg by 1 log CFU/g (Kocharunchitt *et al.,* 2009). Another study by Guenther *et al.*, 2012 tested the broad host range, virulent phage FO1-E2 for the reduction of *Salmonella* Typhimurium in different RTE foods that were spiked with 1 x 10^3 CFU/mL of *Salmonella* and treated with 3×10^8 PFU/g of phage. Samples were stored for 6 days at 8°C or 15°C. The results showed that, after storage at 8°C, no viable cells remained following phage FO1-E2 application, corresponding to a more than 3 log units reduction. At 15°C, application of phage lowered *Salmonella* Typhimurium count by 5 log units on turkey deli meat and in chocolate milk, and by 3 log unit on hotdogs and in seafood (Guenther *et al.,* 2012). Lim *et al.* (2012) have evaluated the efficacy of a single bacteriophage (phage CJ07) combined in feed additives, *in-vitro*, against *S.* Enteritidis. Treatment with (phage CJ07) could prevent *S.* Enteritidis colonization and horizontal transmission in commercial chicken farms that were later released to the environment. Study showed that *Salmonella* phage which could be survived in gastrointestinal tract of chickens might be infect in kill *S.* Enteritidis and provide protection from horizontal spread of *S.* Enteritidis due to decreased bacterial shedding and environmental contamination (Lim *et al.,* 2012). Reduction of *S.* Enteritidis in raw and smoked salmon by phage cocktail were investigated by Galarce *et al.* (2014). They reported

that the reduction range of 0.75 to 3.19 log CFU/g and 2.82 to 3.12 log CFU/g were observed in raw salmon at 18°C and 4 °C, respectively. Whereas, the reduction of 2.82 to 3.12 log CFU/g and 0.50 to 1.16 log CFU/g were observed at 18°C and 4 °C, respectively in smoked salmon (Galarce *et al.,* 2014). Grant *et al*. were reported < 1 log reduction of *Salmonella* on ground chicken after treated with the commercial *Salmonella* phage cocktail (Salmonelex^{TM}) (Grant *et al.,* 2017). The efficiency of *Salmonella* phage cocktail comprising 5 phages against *S*. Enteritidis and *S*. Typhimurium has been reported with approximately 3 and 2 log reductions at 25°C and 4°C, respectively (Duc *et al.,* 2018). The efficiency of a lytic *Salmonella* phage SE07 against *S.* Enteritidis on various food matrices (fruit juice, fresh eggs, beef and chicken meat) has been reported. The reduction of *Salmonella* approximately 2 log cycles was observed on food tested after 48 h at 4°C (Thung *et al.,* 2017). Controlling *Salmonella* in dry pet food was investigated by Heyse *et al*., 2015. They were observed >1 log reduction of *Salmonella* after treated with phage cocktail comprising 6 *Salmonella* phages for 60 min (Heyse *et al.,* 2015). The commercial *Salmonella* phages (SalmoLyse®) were investigated their efficiency to reduce *Salmonella* in pet food and raw pet food ingredients. Approximately 3 log reduction was observed after tested (Soffer *et al.,* 2016).

Table 1.5. The use of phage-based biocontrol applications for controlling *Salmonella* in food production chain.

Food matrices	Target *Salmonella* serovars	*Salmonella* phages applied and application method[a]	Result	Reference
Chicken	*S.* Enteritidis	Phage ϕCJ07 mixed with feed and was given to one day-old chicks infected with 5×10^7 CFU/bird for 21 days at 3 MOIs* (0.01, 1, and100).	*Salmonella* counts were not detected in 70% of contact chickens treated with phage at MOI 100 at 3 weeks after treatment.	Lim *et al.*, 2012
Chicken breast	*S.* Enteritidis and *S.* Typhimurium	A phage cocktail (three phages) was used to test with chicken breast by a dipping method at MOI of 10^3.	The chicken breast dipped with the phage cocktail and kept at 4°C for 7 days showed a reduction of 2.2 and 0.9 log CFU/g for	Spricigo *et al.*, 2013
Alfalfa seeds	*S.* Oranienburg	Two *Salmonella* phages (SSP5 and SSP6) were used to treat alfalfa seeds (10^7 CFU/mL) at MOI* 70 (12 h at 25°C).	Only a 1-log CFU/g reduction of *Salmonella* was observed after seeds were sprouting within 5 days.	Kocharunchitt *et al.*, 2009

[a] Approximate MOI (multiplicity of infection; ratio of phages to bacterial cells) of phages applied in the study is indicated with (*).

Source: Petsong and Vongkamjan, 2015.

Table 1.5. (Cont.)

Food matrices	Target *Salmonella* serovars	*Salmonella* phages applied and application method[a]	Result	Reference
Manure compost	*S.* Typhimurium	A phage cocktail (five phages) was treated against *S.* Typhimurium at MOIs 1, 10, and 50 on dairy manure compost at different moisture contents (30, 40, 45, and 50%).	A reduction of more than 2 log units within 4 h was observed at all moisture levels compared to the control.	Heringa *et al.*, 2010
Hot dogs, sliced turkey breast, seafood, chocolate milk	*S.* Typhimurium	Phage (FO1-E2) was used to treat foods that were inoculated with 10^3 CFU of *Salmonella* cells and then treated with 3×10^8 PFU/g (MOI* 10^5) for 6 days at 8°C or 15°C.	All phage-treated *Salmonella* cells were completely eliminated in foods at 8°C. At 15°C, a reduction of 5 log units on turkey deli meat, and in chocolate milk was observed. A reduction of 3 log units was observed on hot dogs and in seafood at 15°C.	Guenther *et al.*, 2012

[a] Approximate MOI (multiplicity of infection; ratio of phages to bacterial cells) of phages applied in the study is indicated with (*).
Source: Petsong and Vongkamjan, 2015.

1.2.7 Microencapsulation

Microencapsulation can be defined as the process used to entrap living cells (e.g. probiotics, bacteriophage) or molecules (e.g. essential oil, flavor) in the material capsule, normally called the wall. The products of this process (microcapsule or microsphere) should be very tiny droplets or particles of liquid or solid material which are coated with a continuous film of polymeric material (Bansode *et al.*, 2010).

The morphology of microcapsule or microsphere can be classified into the major 3 types, consisting of (i) mononuclear or continuous core/shell microcapsule which has a single hollow chamber within the capsule (ii) multinuclear or polynuclear microcapsule which has several size of chamber within the capsule and (iii) matrix microcapsule which has the matrix of the core material integrated within the matrix of the coating materials (Figure 1.4) (Raybaudi-Massilia and Mosqueda-Melgar, 2012; Sanjoy *et al.*, 2011).

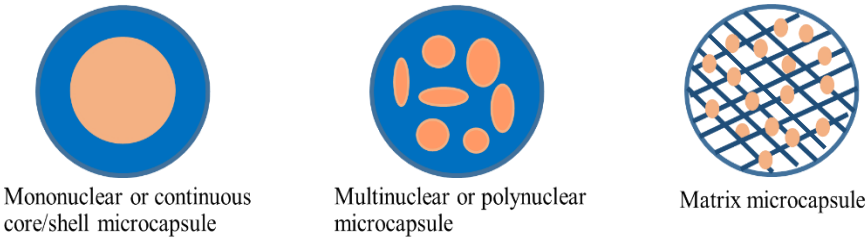

Figure 1.4. Morphology of microcapsules or microspheres

Source: Adapted from Raybaudi-Massilia and Mosqueda-Melgar, 2012

Microencapsulation can be used widely in the food industry. For example, for controlling the oxidative reaction, masking flavors, colors or odors (Burgain *et. al.*, 2011), particularly protecting the active cell (Ma *et al.*, 2008). Living cells or molecular particles are protected by the wall from the environment which can damage or reduce cell survival. Recently, using of phage application to control bacterial pathogen in food industry gained more of interest. According to bacteriophage applications, selection of methods and process conditions to maintain bacteriophage ability and controllable release rate of bacteriophage to the target environments are the main of purpose. The goal of encapsulation is to develop the entrapped phage in the micro-scale with the potential to survive not only in the harsh conditions but also processing and storage conditions. Therefore, several food companies interested on the optimal system which can protects phage particles from these tolerance and technological factors (Choinska-Pulit *et al.*, 2015).

In the food industry, microencapsulation is a very useful tool to extend the shelf life (maintain viability of living cells), decrease the weight of particles in order to support the comfortable of transportation, storage and ease of use, especially, to control the release rate of active cells. Microencapsulation technique can protect the cells from physical barriers and also from the harsh conditions such as acidic conditions, high-thermal condition, shear stress and high pressure. There are many reports that support the efficacy of encapsulation techniques useful for the food industry. For examples, Ma *et al.* (2008) reported that microencapsulation of bacteriophage Felix O1 could protect the phage from simulated intestinal fluid. This result indicated that phage in the microsphere form could survive the low pH range better than free phages (Ma *et al.*, 2008). A study by Saez *et al.* (2011) developed the new microencapsulated phage cocktail. They reported that encapsulated phages were stable at 4°C and 22°C for up to two weeks (Saez *et al.*, 2011). In addition, the efficacy of this microencapsulated phage cocktail was evaluated in feed compared with free-phage cocktail provided via gavage *in-vivo*. The result showed that at 2 h and 4 h, pigs in the phage-treated group were less likely to shed *Salmonella* by 38.1% and 42.9%, respectively. While pigs in the free-phage gavage treated were showed shedding of *Salmonella* by 71.4% and 81.1% at 2 h and 4 h, respectively. Findings suggest that direct feeding of microencapsulated phages is a practical effective means for the reduction of *Salmonella* colonization and shedding in pigs. Permpoonsiri *et al.* (2009) encapsulated selected bacteriophages for *Staphylococcus aureus* or *Pseudomonas aeruginosa* by the freeze-drying method (Permpoonsiri *et al.*, 2009). This formulation technique improved the delivery of bacteriophages and can be an appropriate application for phage therapy.

Important properties of encapsulation techniques for active cells used with controlled agents within a matrix include:

1) Protection ability from biotic and abiotic stress factors by providing a beneficial microenvironment.
2) Slow the release as a function of material properties.
3) Control the release triggered by environmental conditions and material properties.
4) Isolation of active ingredients during applications.

Microencapsulation techniques comprise various methods in order to form a microsphere. Such techniques, including spray-drying, freeze-drying, extrusion and emulsification are implemented to extend the shelf-life, improve convenient handling and transportation and storage. Among several methods which normally used for encapsulation of cells, gel entrapment using natural polysaccharide (generally use extrusion), such as calcium alginate, κ-carrageenan and gellan gum, has been shown to have desirable efficacy in term of increase survivability of active cells (Picot and Lacroix, 2004). However, the microcapsules produced by this technique present a large bead diameter (approximately 2-5 mm) which may be a distinguishable size for consumers, resulting in negative acceptability (Solanki *et al.*, 2013). The techniques commonly used to encapsulate phages are spray drying and freeze drying. The wall material used for this technique must be soluble in water at an acceptable level (Gouin, 2004). The disadvantages from a biological viewpoint are control of the variations in particle shape, size distribution, high temperatures and fast drying rates that normally will not allow for encapsulation of sensitive living cells and bacteriophages (Vemmer and Patel, 2013).

1.2.7.1 Freeze-drying (lyophilization) method

By this technique, a mixture of the active agent and carrier material is dissolved in water. The mixture is frozen at a temperature of approximately -40°C and then dried by sublimation under low pressure and temperature is reduced to between -90 and -50°C. Generally, bacteriophage in a liquid form stored at 4°C has a limitation of 1-year storage period (Merabishvili *et al.*, 2013). Freeze-drying is an alternative formulation technique for bacteriophages. This technique has been shown the ability to extend the stability of bacteriophages for a long-time storage (Ackermann *et al.*, 2004). Ackermann *et al.* (2004) investigated the stability of several bacteriophages by various methods, lyophilization, +4°C, and deep-freezing at -80°C or in liquid nitrogen). The results found that phages stabilized by freeze-drying (50% glycerol) showed the best condition and the phage stability can be extended up to 21 years (Ackermann *et al.*, 2004). However, the disadvantages of freeze-drying have been reported that the protein damage from freezing and drying stresses may be occurring (Merabishvili *et al.*, 2013; Wang, 2000). Freeze-dried bacteriophages can be destabilized by these stresses. Thus the special stabilizers must be added in order to protect the organism from freezing stress (cryo-

protectant) or drying stress (lyo-protectant) and also to increase its stability upon storage (Merabishvili *et al.*, 2013; Abdelwahed *et al.*, 2006).

1.2.7.2 Materials

There are various materials which have been suggested to be a potential wall (materials which can be coated the particles i.e., cells or molecules). For instance, natural polysaccharides (alginates, carrageenans, agar/agarose, gellan gum, guar gum, starch, starch-based materials, cellulose, pectin and chitosan), saccharide (glucrose, sucrose, trehalose), polypeptide (poly-L-lysin), protein (gelatin, whey), lipid (waxes) and biopolymers. Among these materials whey protein and trehalose are materials of interest as a wall which can improve the application for target cells as microcapsules.

1.2.7.2.1 Whey protein

Whey proteins are useful by-products of cheese production. The physicochemical properties of whey protein suggest that they may be suitable for novel food applications (Gunasekaran *et al.*, 2007). Whey is a globular protein which represents approximately 20 percent of the total protein content of milk. The composition of whey proteins consists of β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulin and protease peptones (Santos *et al.*, 2018). β-lactoglobulin and α-lactalbumin act as the major components of the whey protein fraction. Whey proteins have multifunctional properties which are desirable in microencapsulation such as gelation, water binding, solubility, foaming, viscosity and emulsification for foods (Burrington, 2012). The good solubility is the most important property of whey proteins which is the main requirement of dehydration method. Whey protein can be dissolved in water with over a broad pH range (2 to 9) (Xiong, 1992). The ability to absorb water of whey protein is due to the presence of their hydrophilic group (-OH, -CONH-, -$CONH_2$, -COOH and –SO_3H) (Gunasekaran *et al.*, 2007). Moreover, several studies indicate that whey proteins are suitable for use as the wall in coating active cells in microcapsules. For example, Arslan *et al.* (2015) investigated the ability of different materials as the wall for the purpose of probiotic encapsulation by spray-drying (Arslan *et al.*, 2015). Among gelatin, whey protein concentrate, modified starch, maltodextrin, pea protein isolate and gum arabic, whey protein was the best material as the wall in order to protect the active cells from high thermal conditions (Arslan *et al.*, 2015). This material provides the survival probiotics after

treated by spray-drying with a survivability rate 91.81% (Arslan *et al.*, 2015). The denaturation of whey protein by high temperature explained by the unfolding of the protein followed by aggregation, which includes protein-protein interactions that are covalent (not reversible) and non-covalent (possibly reversible) (Ryan *et al.*, 2012). Previous studies demonstrated the desirable methods necessary for the development of encapsulation techniques for cells, including bacteriophages. For instance, Picot and Lacroix (2004) encapsulated bifidobacteria (a probiotic) in a whey protein-based microcapsules using spray-drying. The viable cell counts entrapped in whey protein microcapsules using this method were significantly higher than free cells after 28 days in yoghurt stored at 4°C (+2.6 log cycles) (Picot and Lacroix, 2004). The encapsulation of bacteriophages in whey protein film was studied by Vonasek *et al*. (2014), this study showed that whey protein isolate can stabilize phages at 22°C and 4°C without significant loss in phage viability for more than one month. They also compared the release of phages from phage encapsulating whey protein isolate films upon exposure to an aqueous environment and lettuce leaf surface as model aqueous and food surface systems. They reported that within 3 h, approximately 3.3% of the encapsulated phages were released into an aqueous system. While, 0.37% of encapsulated phages were released to the leaf surface. However, films made from this material are able to release significant concentration of phages in an aqueous environment within 6 h. While with another surface, less phage was release to the surface (Vonasek *et al.*, 2014). A previous study investigated the use of whey proteins as a coating material for microencapsulation and controlled delivery applications. They reported that whey proteins can be used as the nanoparticles systems which controlled the release rate of the active cells and demonstrated that preheating of whey proteins at 60°C can improve the particle size uniformity (Gunasekaran *et al.*, 2007).

1.2.7.2.2 Trehalose

Trehalose is a non-reducing disaccharide consisting of two glucose moieties joined by an α-1,1 glucosidic bond (Albertorio *et al.*, 2007). Trehalose has been shown to provide superior stabilization of proteins and biological systems, protecting against damage caused by freezing. There are several desirable functionalities attributes of trehalose, this saccharide can be a material suitable for use as the wall of microcapsules. The advantages of trehalose for use as a coating material of bioactive compounds can be described. For examples; (i) process stability; trehalose remains stable under low

temperature conditions and will not take part in Maillard reactions with amino acid and proteins, (ii) solubility; trehalose can solubilize rapidly in water, (iii) low hygroscopicity (iv) high glass transition temperature and (v) depression of freezing point. The ability of trehalose to act as a thermal and dehydration protection of living cells has been explained via the water-replacement hypothesis and the verification hypothesis. The demonstration described that the chemical structure of trehalose provides the conformational stability to proteins structure by hydrogen bond. This phenomenon hence limiting the protein's mobility. The glassy state (glass transition temperature (T_g), between 79°C and 115°C) provides trehalose to be a stable glassy matrix at room temperature (Willart *et al.*, 2002). All the functional properties of trehalose support its use as a coating material for microencapsulation of bioactive compounds, including bacteriophages. Several studies presented the efficiency of trehalose to protect bacteriophages from very low or high temperatures experienced by the drying method. For example, Vandenheuvel *et al.* (2013) used several saccharide solutions (dextran 35, lactose, and trehalose) as the wall material in order to encapsulated bacteriophage by spray-drying. They demonstrated that trehalose is a promising excipient for spray-drying phages. Their results showed that these powders are stable at low temperatures (4°C) and relative humidity (0%). They suggested that high humidity causes crystallization of the amorphous matrix which could destroy the encapsulated phages. Storage at a higher temperature (25°C) causes thermal instability which could destroy the embedded phages. In addition, they indicated that storage conditions are important parameters to take into account in phage therapy development (Vandenheuvel *et al.*, 2013). A form of bacteriophages needs to be more desirable for the food industry such that it provides certain properties, i.e., extended shelf-life, decrease the weight of particles in order to support the comfortable of transportation, storage, ease of use, and can be applied to various food products. The development of microspheres containing living cells are thus extremely of interest. Not only as dried powder microspheres for using with dried food products, microencapsulated phages can be applied in a liquid form via mixing with water in order to use with high moisture content foods.

RESEARCH OBJECTIVES

1. To isolate *Salmonella* bacteriophages from samples collected from various animal farms, including poultry, swine, goat, and bovine farms.
2. To characterize *Salmonella* bacteriophages phenotypically by the host lysis profile determination, morphology analysis by Transmission Electron Microscopy (TEM) and genotypically by (i) the phage genome size estimation using Pulsed-Field Gel Electrophoresis (PFGE) analysis and (ii) whole genome sequencing analysis of phages included in the phage cocktail.
3. To develop a phage cocktail targeting the two most common serovars of *Salmonella*: *S.* Enteritidis and *S.* Typhimurium.
4. To develop and evaluate specific functional properties of a new form of phage-based control by using encapsulation technique.
5. To evaluate the stability, storage conditions and physio-chemical properties of the developed encapsulated phage cocktail, and investigate its effectiveness in reducing *S.* Enteritidis and *S.* Typhimurium *in-vitro* and in different food matrices.
6. To evaluate the overall acceptability of consumers on foods with/without microencapsulated phage cocktail.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *SALMONELLA* BACTERIOPHAGES

2.1 Abstract

Bacteriophage shows outstanding specific property to lyse target bacterial host and has gained more of interest as bio-control agent for use in the food industry. *Salmonella* is normally diverse in animal farm environments. This study isolated *Salmonella* phages from several animal farms (chicken, swine, goat and bovine) in Thailand. The recovery of high number of phages indicates that free range farms represented the major source of *Salmonella* phages. All 36 isolated phages were evaluated their lysis profiles on 47 *Salmonella* strains (28 serovars) from various sources, including animal farms, seafood processing plant and human in Thailand and the US. Lysis profiles revealed that phages isolated in this study showed broader host range on host strains isolated from Thailand compared to those strains isolated from US. A number of our phages also showed the broad spectrum against *Salmonella* isolates from the same and different communities and even continents. Estimated genome sizes ranging from 50 $\pm$ 2 kb to 200 $\pm$ 2 kb were observed among isolated phages, indicating phage genomic diversity in this region. Findings suggest that animal farms provided an abundance of *Salmonella* phages presenting the potential lysis ability on hosts from various environments.

2.2 Introduction

Non-typhoidal *Salmonella enterica* is the most common bacterial pathogen which causes the serious public concern worldwide (Acheson and Hohmann, 2001). The US Centers for Disease Control and Prevention (CDC) reported an estimated 1 million domestically acquired salmonellosis cases with 19,000 hospitalizations and 380 deaths (Centers for Disease Control and Prevention, 2018). Increased numbers of salmonellosis cases have also been reported by the European Food Safety Authority (European Food Safety Authority, 2015). Distribution of *Salmonella* on farms leads to the likelihood of *Salmonella* contamination in various food of animal origin and produce. *Salmonella* serovars predominantly present in food can be differed, for example, serovars Enteritidis and Typhimurium are common in eggs (Shahzad *et al*., 2012), serovars Kentucky and Enteritidis are common in poultry (USDA, 2014), serovars Newport, Infantis and Javiana are common in fruits and vegetables (Reddy *et al*., 2016). The environments of food processing plant have been linked to occurrence of diverse *Salmonella* serovars. For example, serovars Weltevreden (Santos *et al*., 2014; Ponce *et al*., 2008), Anatum, Enteritidis, Newport and Typhimurium have been previously detected in wastewater from the plant (Espigares *et al*., 2006). *Salmonella* serovars predominant in human are also of crucial concern. These include *Salmonella* serovars Weltevreden, Enteritidis and Anatum which have been reported as the most common serovars isolated from human in Thailand (Bangtrakulnonth *et al*., 2004). The occurrence of *Salmonella* contaminated in poultry meat has been often reported (CDC, 2018). Fresh produce such as sprout has also been reported as the high risk food that is commonly been contaminated by *Salmonella* (CDC, 2018; Grant *et al*., 2009). Control of *Salmonella* in both food categories is thus crucial.

Bacteriophages are viruses of bacteria which are very specific to bacterial hosts (Clokie *et al.*, 2011). Habitat of phages is normally related to their hosts as predator and prey (Díaz-Muñoz and Koskella, 2014). Interesting of phage therapy is increasing continuously, since this natural antibacterial has outstanding property over the others (Sulakvelidze *et al.*, 2001). According to their specificity of infection, the use of bacteriophages has emerged as a promising tool for food safety (Hagen and Loessner, 2010; Goodridge and Bisha, 2011). This study isolated *Salmonella* phages from various animal farms in Thailand including free range and commercial farms. Lytic ability of isolated *Salmonella* bacteriophages on *Salmonella* strains isolated from various different sources (animal farms, processing plant and human) and geography (Thailand and US) was

investigated. The estimated genome sizes of representative *Salmonella* phages isolated from different sources were also determined.

2.3 Materials and methods

2.3.1 Sample collection

A total of 12 samples were collected from six animal farms, including poultry, swine, goat and bovine farms in Songkhla province, Thailand between January 2014 and October 2016. Of 6 farms, three of which was commercial farms and three other was free range farms. Approximately 500 g of each randomly selected sample (feces, feed, soil and drinking water) was collected using a sterile spatula and transferred to a sterile bag. Feces samples were collected from the floor inside the pens of the commercial farms where animals resided. For free range farms, feces samples were collected from random open areas where animals resided. Feed samples were taken from the storage containers freshly prepared for feeding animals. Soil samples were taken from the free-range farms only and from random open areas where animals resided. Drinking water in farms were also collected. Each sample was kept in a cooler box containing ice packs and transferred to a laboratory. Collected samples were stored at 4°C until analysis in the laboratory.

2.3.2 Bacterial strains used in the study

Salmonella strains used in this study consisted of 47 *Salmonella* strains presenting 28 common serovars (Table 2.1). All *Salmonella* strains tested are the representative of predominant serovars isolated from various sources (including animal farms, food processing plant and human) in Thailand and the United States. A total of 23 isolates of *Salmonella* strains from animal farms and human in the United States were obtained from the Food Safety Laboratory, Cornell University (indicated with a prefix of "FSL"). Other 24 strains of *Salmonella* were previously isolated from animal farms, food processing plant, and human in Thailand. These were obtained from the Faculty of Microbiology, Mahidol University and the Faculty of Agro-Industry, Prince of Songkla University, Thailand (indicated with a prefix of "PSU-BS-"). *Salmonella* strains were kept in 15% glycerol at -80°C as working stocks. For overnight cultures, an isolated colony of *Salmonella* from Tryptone Soya Agar (TSA; Oxoid, Hampshire, UK) was transferred in 5 mL of Tryptone Soya Broth (TSB; Oxoid, Hampshire, UK) and incubated at 37°C for 16-18 h.

Table 2.1. *Salmonella* strains used for phage isolation and determination of phage lysis profiles.

Serovars	Strains ID	Source (Country)
Agona	H2-016*	Pig slaughterhouse (Thailand)
	FSL S5-667	Bovine (US)
Anatum	PSU-BS-104SL	Seafood processing plant (Thailand)
	FSL A4-525*	Bovine (US)
Braenderup	FSL S5-373	Human (US)
Cerro	FSL R8-242	Bovine (US)
Corvolis	PSU-BS-092SL	Animal farm (Thailand)
	FSL R8-092	Human (US)
Dublin	W1-016	Dairy farm (Thailand)
	FSL S5-368	Bovine (US)
Enteritidis	FSL S5-371	Human (US)
Give	PSU-BS-181SL	Animal farm (Thailand)
	H2-018*	Dairy farm (Thailand)
Heidelberg	FSL S5-455	Human (US)
Infantis	FSL S5-506	Human (US)
Javiana	FSL S5-406	Human (US)
Kedougou	H2-061	Pig slaughterhouse (Thailand)
Kentucky	W1-010*	Dairy farm (Thailand)
	PSU-BS-058SL	Chicken farm (Thailand)
	PSU-BS-116SL	Seafood processing plant (Thailand)
	FSL S5-431	Bovine (US)
Krefeld	PSU-BS-111SL	Seafood processing plant (Thailand)
Mbandaka	W1-015	Dairy farm (Thailand)
	PSU-BS-035SL	Chicken farm (Thailand)
	FSL A4-793	Bovine (US)

(***)** represents strains of *Salmonella* used as hosts for *Salmonella* phage isolation.

Table 2.1. (Cont.)

Serovars	Strains ID	Source (Country)
Montevideo	FSL S5-474	Bovine (US)
	FSL S5-403	Bovine (US)
Muennster	FSL S5-917	Bovine (US)
Newport	FSL S5-548	Bovine (US)
Oranienburg	FSL R8-376	Human (US)
Ordonez	PSU-BS-181SL	Seafood processing plant (Thailand)
Panama	FSL S5-454	Human (US)
Rissen	H2-015	Pig slaughterhouse (Thailand)
Saintpaul	FSL S5-369	Human (US)
Stanley	PSU-BS-001SL	Swine farm (Thailand)
	H2-002*	Pig slaughterhouse (Thailand)
	PSU-BS-041SALX	Seafood processing plant (Thailand)
	FSL S5-464	Human (US)
Tennessee	PSU-BS-183SL	Seafood processing plant (Thailand)
Typhimurium	H2-001*	Pig slaughterhouse (Thailand)
	PSU-BS-014SL	Chicken farm (Thailand)
	FSL S5-370	Human (US)
Virchow	H2-117*	Human (Thailand)
	FSL S5-961	Human (US)
Weltevreden	H2-031	Pig slaughterhouse (Thailand)
	PSU-BS-013SL	Chicken farm (Thailand)
	FSL R8-798	Human (US)

(***)** represents strains of *Salmonella* used as hosts for *Salmonella* phage isolation.

2.3.3 Isolation and purification of *Salmonella* phages

Salmonella phages were isolated using enrichment isolation with a multi-strains of *Salmonella* cocktail whose serovars are shown to be predominant in Thai animal farms (including Agona H2-016, Anatum A4-525, Give H2-018, Kentucky W1-010, Stanley H2-002, Typhimurium H2-001 and Virchow H2-117), following the procedures of Moreno Switt *et al*. (2013). Briefly, 25 g of each sample was enriched with 225 mL of TSB

and 2.5 mL of host mixture. After filtration of the mixture through 0.45-µm and 0.22-µm syringe filters, filtrate (100 µl) was used to prepare the double layer by mixing with each host isolate previously grown in TSB for 16-18 h at 37°C. For each overlay, 300 µl of the 1:10 dilution of the overnight host isolate was mixed with 4 mL of 0.7% TSA followed by incubation at 37°C for 18-24 h. Plaques were observed on each host lawn. A distinct isolated plaque was selected and suspended in 300 µl of Phosphate Buffered Saline (PBS, pH 7.4) for purification. Serial dilutions were performed and appropriate dilution was subjected to three passages with a specific host that showed positive result, using double layer agar technique (Vongkamjan *et al*., 2012).

2.3.4 Lysate preparation and titer determination of *Salmonella* phages

An isolated plaque from the third purification passage was used to prepare 10-fold serial dilutions in PBS. Appropriate dilutions were used to prepare the overlay with a given host to yield semi-confluent lysis. Overlay was harvested with 10 mL of Salt-Magnesium buffer (SM buffer, pH 7.5), followed by centrifugation at 3213×*g* for 15 min at 4°C. Supernatant was filtered through a 0.22-µm syringe filter and phage lysates were kept at 4°C. Each phage lysate was serially diluted in PBS and 100 µl of each dilution was mixed with 300 µl of host, then the mixture was poured on bottom agar (Vongkamjan *et al*., 2012). Phage titers were determined after incubation at room temperature for 16-18 h by counting plaques present on each plate of a given dilution (Vongkamjan *et al*., 2012).

2.3.5 Determination of lysis profiles of *Salmonella* phages

Lysis profile for each phage was determined by a spot test on bacterial lawn of a given *Salmonella* strain in the collection included in this study. Briefly, 5 µl of each phage lysate representing 10^8 PFU/mL were spotted on the bacterial host lawn prepared as mentioned above but without filtrate. Phage lysis patterns were determined after 18-24 h of incubation at 25°C. Experiment was performed in independent triplicates. Phage lysis patterns were analyzed by converting a positive lysis (zone of lysis on a spot) to score 1 and negative results were converted to score 0. A heatmap representing lysis groups was generated by cluster analysis, following Vongkamjan *et al*. (2012) with Ward's method of binary distance, using the R software program (version 2.14.2; R development Core Team 2012 [https://www.r-project.org/]).

2.3.6 Genome size determination of *Salmonella* phages

Representative *Salmonella* phages from each kind of samples were selected for genome size determination. A total of 17 phage isolates were included for Pulsed-Field Gel Electrophoresis (PFGE) analysis as described by Moreno Switt *et al.* (2013) and Vongkamjan *et al.* (2012) with modifications. Briefly, agarose plugs for PFGE analysis were prepared by mixing equal volume (55 µl) of a given phage lysate (approximately 10^6-10^8 PFU/mL) with and 1.3% low melting point agarose. Plugs were kept at low temperature (4°C) in order to solidify for 1 h. Plugs were loaded into 1% agarose gel and electrophoresis was performed in 0.5X TBE buffer using CHEF-DR III system (Bio-Rad, Hercules, CA). PFGE was performed for 20 h with 0.5-5 s of switch time. Two size markers were included; CHEF DNA size standard of 8-48 kb ladder and CHEF DNA size standard lambda λ ladder 0.05-1 Mb (both from Bio-Rad, Hercules, CA).

2.4 Results and discussion

2.4.1 Recovery of *Salmonella* phages from various animal farms in Thailand

A total of 12 samples was collected from 3 sampling visits to various animal farm environments in Songkhla province, Thailand. Three samples from sampling 1 collected from commercial farms represented 2 phages. The vast number of phages (34 phages) was obtained from the other 9 samples from two samplings at free range farms (Table 2.2). The distribution of *Salmonella* phages can be varied depending on farm types or abundance of hosts which is typically associated with presence of *Salmonella* serovars on particular farms (Moreno Switt *et al*., 2013). Overall, we recovered 36 *Salmonella* phages from poultry, swine, bovine and goat farms. The finding suggested that animals and environments of these animal farms are likely to have *Salmonella* hosts and their phages presented. Recovery of *Salmonella* phages have been reported from several animal farms such as poultry farm (Bao *et al*., 2011; Hungaro *et al*., 2013), swine farm (Callaway *et al*., 2010; McLaughlin *et al*., 2006), bovine farm (Moreno Switt *et al*., 2013; Wongsuntornpoj *et al*., 2014) and goat farm (Shukla *et al*., 2014). Our study suggested that free range animal farms represented a major source of phages. Similar to previously reported sources of phages, Wongsuntornpoj *et al*. (2014) isolated a number of *Salmonella* phages from small-scale free range cattle farms in Thailand (Wongsuntornpoj *et al*., 2014). The presence of bacteriophage is normally related to bacterial host population (Chibani-Chennoufi *et al*., 2004). For free range animal farm, soil in an open land may have high opportunity for

exposure to wild birds, insects, rodent droppings, and other carriers of *Salmonella* (Bailey and Cosby, 2005), thus exhibiting a major source of *Salmonella* host and *Salmonella* phages.

Table 2.2. Recovery of *Salmonella* phages from various animal farms.

Animal farms (sampling date [mo/yr])	Number of sample (number of phages)
Sampling 1; 04/2014	
Poultry farm 1; commercial	1 (1)
Swine farm; commercial	1 (0)
Goat farm; commercial	1 (1)
Sampling 2; 08/2015	
Bovine farm; free range	1 (3)
Poultry farm 2; free range	4 (15)
Sampling 3; 10/2016	
Poultry farm 3; free range	4 (16)
Total	12 (36)

2.4.2 Lysis profiles of *Salmonella* phages from animal farms in Thailand on *Salmonella* strains from various sources in Thailand and the US

All 36 *Salmonella* phages obtained in this study was tested on 47 *Salmonella* strains representing 28 serovars. These phages were classified into three groups based on the host range, including (A) broad, (B) narrow and (A*) special broad host range (Figure 2.1). In group A, 14 phages showed strong lysis ability to *Salmonella* strains from Thailand but lower weaker lysis ability to *Salmonella* strains from the US. However, several phages showed ability to lyse *Salmonella* strains from both continents and different sources. In group B, 21 phages showed the ability to lyse *Salmonella* strains mostly isolated from Thailand, especially *Salmonella* isolated from animal farms. One unique phage (KP34) isolated from a commercial poultry farm was classified into the special broad host range group. This phage showed the broadest host range among isolated phages with the ability to lyse over 60% of *Salmonella* strains from Thailand and 48% of *Salmonella* strains from the US. Overall, phages isolated in this study presented different spectrum to lyse host strains from both continents and different sources.

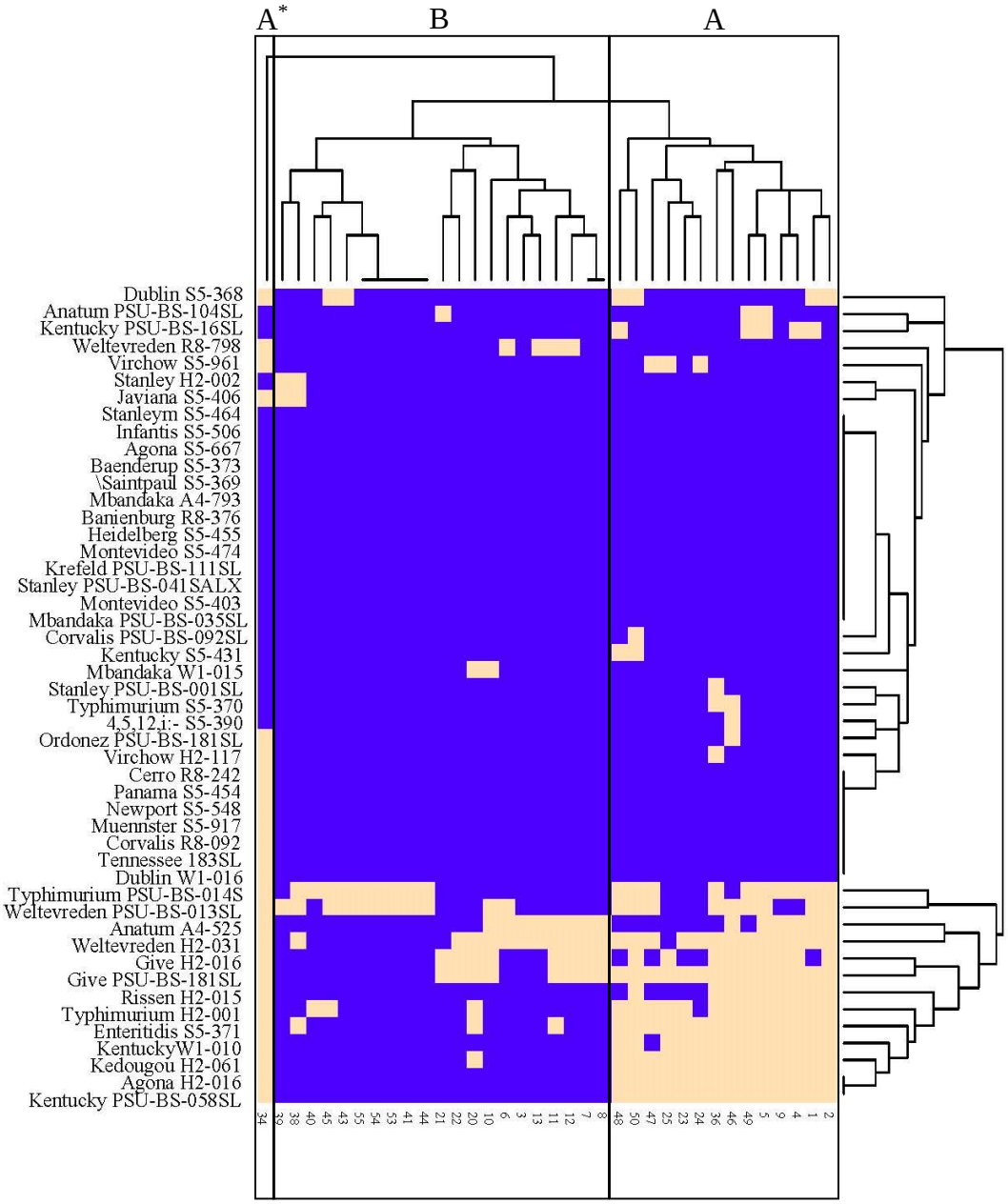

Figure 2.1. Heatmap representation of lysis profiles of 36 *Salmonella* phages tested (vertical axis) on 47 *Salmonella* host strains (horizontal axis) from Thailand and the US. Phages were classified into three groups based on the host range, including (A) broad, (B) narrow and (A*) special broad host range. Beige areas indicate lysis and dark areas indicate no lysis.

2.4.3 Phage susceptibility of different *Salmonella* serovars from various sources

All 36 *Salmonella* phages obtained in this study was tested on 47 *Salmonella* strains of different major serovars previously isolated from the environments related to animal farms, animal slaughterhouse and food processing plant and from humans. High susceptibility to phage lysis was observed among *Salmonella* strains mostly isolated from animal farms and slaughterhouse in Thailand (Table 2.3), including serovars Agona, Give, Kedougou, Kentucky, Typhimurium and Weltevreden. Two additional strains showing high susceptibility to phage lysis included serovars Anatum and Enteritidis. Other five serovars, including Kentucky, Rissen, Dublin, Virchow and Weltevreden from diverse sources from both Thailand and the US presented medium susceptibility to phage lysis. Several serovars showed low susceptibility to phage lysis. However, among serovars classified in this group, most *Salmonella* strains were isolated from the US. These included serovars Agona, Braenderup, Heidelberg, Infantis, Javiana, Kentucky, Mbandaka, Montevideo, Muenster, Newport, Oranienburg, Panama, Saintpaul, Stanley and Typhimurium.

Lysis profiles indicated that our isolated phages showed better ability to lyse *Salmonella* strains from animal farms in Thailand as compared to strains from the US. Finding indicates the relationship between phages and *Salmonella* hosts to the source of isolation. This phenomenon can be explained by the mechanisms of the phage-host receptors which are related to the evolution as pray-predator (Chaturongakul and Ounjai, 2014). However, the spectrum of phage lysis can be extended across different sources or regions of host habitats. In this study, our isolated phages showed lysis on *Salmonella* strains from diverse sources including environments related to animal farms, animal slaughterhouse and food processing plant and from humans. Phages could also lyse bacterial hosts from different continents. Overall, phages isolated from animal farms in this study could lyse most serovars of *Salmonella* that have been reported as the predominant and prevalent serovars in human, foods and animal farms in Thailand including *Salmonella* serovars Agona, Anatun, Give, Enteritidis, Kedougou, Kentucky, Typhimurium and Weltevreden (Bangtrakulnonth *et al*., 2003; O'Flynn *et al*., 2004; Saengthongpinit *et al*., 2014; Vaeteewootacharn *et al*., 2005).

Table 2.3. Phage susceptibility of different *Salmonella* serovars recovered from various sources.

Serovars	Source	% susceptibility(level)[a]	
		Thailand	US
Agona	Pig slaughterhouse	41.7 (H)	-
Anatum	Bovine	-	41.7 (H)
Enteritidis	Human	-	50.0 (H)
Give	Animal farm	63.9 (H)	-
	Dairy farm	50.0 (H)	-
Kedougou	Pig slaughterhouse	44.4 (H)	-
Kentucky	Dairy farm	38.9 (H)	-
	Chicken farm	41.7 (H)	-
Typhimurium	Pig slaughterhouse	47.2 (H)	-
	Chicken farm	55.6 (H)	-
Weltevreden	Pig slaughterhouse	69.4 (H)	-
	Chicken farm	58.3 (H)	-
Dublin	Bovine	-	19.4 (M)
Kentucky	Seafood processing plant	13.9 (M)	-
Rissen	Pig slaughterhouse	27.8 (M)	-
Virchow	Human	-	11.1 (M)
Weltevreden	Human	-	13.9 (M)
Agona	Bovine	-	No lysis
Anatum	Seafood processing plant	8.3 (L)	-
Braenderup	Human	-	No lysis
Cerro	Bovine	2.8 (L)	-
Corvalis	Animal farm	2.8 (L)	-
	Human	2.8 (L)	-
Dublin	Dairy farm	2.8 (L)	-
Heidelberg	Human	-	No lysis

[a] Phage susceptibility level defined by % of total phages that could lyse each host strain (n = 36): <10% (Low; L); 11-30% (Medium; M); >31% (High; H). If no strain tested, '-' is shown in table.

Table 2.3. (Cont.)

Serovars	Source	% susceptibility(level)[a]	
		Thailand	US
Infantis	Human	-	No lysis
Javiana	Human	-	8.3 (L)
Kentucky	Bovine	-	5.6 (L)
Krefeld	Seafood processing plant	No lysis	-
Mbandaka	Dairy farm	5.6 (L)	-
	Chicken farm	No lysis	-
	Bovine	-	No lysis
Montevideo	Bovine	-	No lysis
	Bovine	-	No lysis
Muennster	Bovine	-	2.8 (L)
Newport	Bovine	-	2.8 (L)
Oranienburg	Human	-	No lysis
Ordonez	Seafood processing plant	5.6 (L)	-
Panama	Human	-	2.8 (L)
Saintpaul	Human	-	No lysis
Stanley	Swine farm	2.8 (L)	-
	Pig slaughterhouse	5.6 (L)	-
	Seafood processing plant	No lysis	-
	Human	-	No lysis
Tennessee	Seafood processing plant	2.8 (L)	-
Typhimurium	Human	-	5.6 (L)
Virchow	Human	5.6 (L)	-

[a] Phage susceptibility level defined by % of total phages that could lyse each host strain (n = 36): <10% (Low; L); 11-30% (Medium; M); >31% (High; H). If no strain tested, '-' is shown in table.

2.4.3 Estimation of phage genome sizes

Genome sizes of representative phages from different sources were estimated. Of 17 representative phages, the genome size range between 50 ± 2 kb and 200 ± 2 kb was observed (Table 2.4 and 2.5). Variations in the genome sizes of *Salmonella* phages obtained from animal farms in Thailand suggest diverse groups of phages were found from these environments. Previous studies have reported various genome sizes among *Salmonella* phages isolated from dairy farms in the US, ranging from 22 kb to 156 kb (Moreno Switt *et al*., 2013), while some phages from dairy farms in Thailand exhibited the genome sizes of 40 kb to 200 kb (Wongsuntornpoj *et al*., 2014). Phages ΦSH17, ΦSH18 and ΦSH19 isolated from sewage effluent in UK showed the estimate genome sizes with 40, 48.5 and 155 kb, respectively (Hooton *et al*., 2011). Phage SPN3US isolated from chicken feces in Korea revealed a complete genome size at a length of 240 kb (Lee *et al*., 2011). In addition, some identical genome sizes observed among phages from different farms, suggesting presence of common phage type from these animal farm-related sources.

Table 2.4. Estimated genome sizes of *Salmonella* phages isolated from animal farm environments in this study and from various samples in previous studies.

Source of isolation	Country	Estimated genome size (kb)	Reference
Goat feces	Thailand	200 ± 2	This study
Bovine feces	Thailand	50 ± 2, 60 ±2	This study
Chicken feed	Thailand	60 ± 2, 72 ± 2	This study
Soil (poultry farm)	Thailand	50 ± 2, 60 ± 2, 105 ± 2	This study
Drinking water (poultry farm)	Thailand	97 ± 2	This study
Chicken feces	Thailand	50 ± 2, 60 ± 2, 103 ± 2	This study
Dairy farms	US	22 to 156	Moreno Switt *et al*., 2013
Dairy farms	Thailand	40 to 200	Wongsuntornpoj *et al*., 2014
Sewage effluent	UK	40, 48.5, 155	Hooton *et al*., 2011
Chicken feces	Korea	240	Lee *et al*., 2011

Table 2.4. (Cont.)

Source of isolation	Country	Estimated genome size (kb)	Reference
Sewage (poultry farm)	Korea	40	Kim *et al*., 2012
Water buffalo feces	southern Italy	39	Paradiso *et al*., 2016
Swine lagoon effluent	UK	42	Turner *et al*., 2012

Table 2.5. Genome size of representative *Salmonella* phage isolates from various animal farms in this study.

Phage ID	Source	Estimated genome size (kb)
KP3	Goat feces	200 ± 2
KP4	Soil (poultry farm 2)	105 ± 2
KP5	Chicken feces (poultry farm 3)	60 ± 2
KP6	Chicken feed (poultry farm 2)	72 ± 2
KP7	Soil (poultry farm 2)	60 ± 2
KP10	Chicken feed (poultry farm 2)	60 ± 2
KP11	Soil (poultry farm 2)	60 ± 2
KP34	Chicken feces (poultry farm 1)	60 ± 2
KP38	Bovine feces	60 ± 2
KP39	Bovine feces	50 ±2
KP41	Drinking water (poultry farm 3)	97 ± 2
KP43	Chicken feces (poultry farm 3)	60 ± 2
KP49	Soil (poultry farm 3)	60 ± 2
KP50	Chicken feces (poultry farm 3)	103 ± 2
KP53	Chicken feces (poultry farm 3)	50 ± 2
KP54	Soil (poultry farm 3)	50 ± 2
KP55	Chicken feed (poultry farm 3)	60 ± 2

2.5 Conclusion

This study aims to understand the abundance and diversity of *Salmonella* phages in Songkhla province, Thailand in order to use the obtained phages for further study in the development of phage-based biocontrol. *Salmonella* phages could be isolated from various animal farms environments that have been related to sources of *Salmonella*. We have obtained over 36 phages in our phage collection and further characterized these isolated phages. The number of isolated phages indicated that animal farms included in this study provided high abundance of *Salmonella* phages. Lysis profiles and wide range of estimated genome sizes of isolated phages indicated that our phages are diverse and common among *Salmonella* phages which have been reported. In addition, a few phages isolated here showed the broad spectrum to lyse various *Salmonella* strains from different sources and regions. Overall, most of isolated phages showed the broader host range on *Salmonella* strains isolated from animal farms in Thailand compared to the other sources. Remarkably, a number of phages in our collection showed high ability to lyse several important serovars which predominant in various crucial sources (human, foods and animal farms) in Thailand such as *Salmonella* serovars Agona, Anatun, Give, Enteritidis, Kedougou, Kentucky, Typhimurium and Weltevreden. In addition, our phages also showed the high ability to lyse the most common *Salmonella* serovars which is concerned worldwide (*S.* Enteritidis and *S.* Typhimurium). Data here suggests potential development of our phages as an effective strategy to combat the occurrence of predominant *Salmonella* serovars outbreaks in various crucial sources not only in Thailand, but also to develop the alternative effective strategy to control the concerned *Salmonella* serovars worldwide.

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CHAPTER 3

DEVELOPMENT OF *SALMONELLA* PHAGE COCKTAIL

3.1 Abstract

S. Enteritidis and *S.* Typhimurium are the most important serovars that have often linked to food contamination and foodborne outbreaks. Phages have been approved by the GRAS status and gained more interest for use as bio-control agent in the food industry. This study developed the effective *Salmonella* phage cocktail from our phage collection. The morphology, biology and whole genome of phages composed in the cocktail were studied. Efficiency of a developed phage cocktail against *S.* Enteritidis and *S.* Typhimurium *in-vitro*, in high-risk foods including chicken meat and sunflower sprout and in animal feed were also evaluated. Of all isolated phages, nine phages showed strong lysis ability on *S.* Enteritidis and *S.* Typhimurium. Efficiency of plating (EOP) and lytic ability assays revealed that three phages (KP4, KP5 and KP50) showed the highest ability to lysed *S.* Enteritidis and *S.* Typhimurium. Electron microscopy analysis revealed that all three phages were classified into order of *Caudovirales* and family of *Siphoviridae*. One-step growth curve indicated that at MOI 100, phages KP4, KP5 and KP50 showed a burst size of 25.1, 30.1 and 97.7 PFU/cell, respectively, with latent period of 5, 15 and 40 min, respectively on *S.* Enteritidis. On *S.* Typhimurium a burst size revealed as 70.8, 173.7 and 112.2 PFU/cell, respectively with latent period of 15, 10 and 15 min, respectively. Whole genome sequencing analysis revealed that all three phages KP4, KP5 and KP50 showed the absence of lysogeny modules, virulence and toxin associated genes and also antibiotic resistance associated genes, indicating that all three phages are virulent phages. *In-vitro* study, phage cocktail showed high efficiency to reduce *S.* Enteritidis and *S.* Typhimurium by approximately 4 log CFU/mL after 4 h of treatment. In addition, phage cocktail showed the efficiency to reduce *S.* Enteritidis and *S.* Typhimurium in chicken meat, sunflower sprout and animal feed by approximately 0.66 log CFU/cm^2, 1.27 log CFU/g and 1.87 log CFU/g, respectively of *S.* Enteritidis, and by approximately 1.73 log CFU/cm^2, 1.17 log CFU/g and 2.38 log CFU/g, respectively of *S.* Typhimurium. Overall results suggest that our developed phage cocktail provided

high efficiency to control *Salmonella* in foods and feed that have been related to high risk of *Salmonella* contamination.

3.2 Introduction

Salmonella spp. is the major foodborne pathogen which can cause serious public health concern worldwide (CDC, 2012; EFSA, 2015). Among over 2,600 serovars of *Salmonella* that have been identified, *Salmonella enterica* serovars Enteritidis and Typhimurium have been reported as the top common causes of salmonellosis (García *et al.*, 2018). The main habitat of *Salmonella* serovars is the intestinal tract of humans and farm animals. This pathogen can be distributed to farm environments by several routes such as water, soil and feed stuff (Andino and Hanning, 2015). Poultry origins, fresh produce and feed have been reported as the high risk foods that are commonly contaminated with *Salmonella* (Hanning, 2009; Hugas *et al*., 2014; Antunes *et al*., 2016; CDC, 2018). Common outbreaks associated foods have also been reported in 2012 to 2018 (CDC, 2018). Therefore, the effective strategies to control *Salmonella* in these food categories should be focused on.

Application of bacteriophage (phage) as a bio-control agent against pathogens has provided outstanding properties over antibiotic (e.g. specificity to target hosts) (Clokie *et al.*, 2011) or chemical agents commonly used in meat products which have some negative effects on human health (Kazi and Annapure, 2016). Developed phage preparation against *Salmonella* has been approved by the Generally Recognized as Safe (GRAS) status such as a commercial product by Phagelux (FDA, 2016). Nowadays, phage cocktail gained more of interest to improve the efficiency of phage application. Phage cocktail (mixing of several effective phages) has been reported as an effective application to control pathogens in food products, therapeutic and even animal models (Bao *et al.*, 2015; Mendes *et al.*, 2014; Yen *et al.*, 2017). Bao *et al.* (2011) developed two *Salmonella* phages which showed the high efficiency to control *S.* Enteritidis as a cocktail. Their developed phage cocktail showed the efficiency to reduce the number of *S.* Enteritidis up to 4 log CFU/sample in various foods (chicken breast, pasteurized whole milk and Chinese cabbage) (Bao *et al.*, 2011). Mendes *et al.* (2014) reported the therapeutic potential of five phages developed as a cocktail to control the organisms causing diabetic foot infections (Mendes *et al.*, 2014).

The impact of phage biology, including phage burst size and latent period plays the major role to the efficiency of phages against the bacterial host cells. Bacteriophages which present short latent period and large burst size will be replicated more quickly and the progeny particles could be released more efficiently (Bao *et al*., 2011). For safety concern, phages which will be used as bio-control agent are needed to ensure the absence of virulence or toxin associated genes, antibiotic resistance genes and transduction tested (Moreno Switt *et al*., 2013).

In this study, *Salmonella* phages from our collection were screened for the top three phages which showed the strong lysis ability to lyse *S.* Enteritidis and *S*. Typhimurium. Selected three phages with the highest lysis ability as indicated by EOP (Efficiency of plating) were used to develop a phage cocktail. In order to classify the family of selected phages, physical morphology were determined by transmission electron microscopy (TEM) analysis. To further evaluate the cell lysis efficiency, one-step growth curve of each phage was investigated. To confirm the suitability and safety for use of these phages in the cocktail, whole genome sequencing analysis was performed. The efficiency of developed phage cocktail was evaluated against *S.* Enteritidis and *S*. Typhimurium *in-vitro* and in representative high-risk foods, including raw chicken meat and sunflower sprout and also in animal feed.

3.3 Materials and methods

3.3.1 Development of a phage cocktail targeting two major serovars of *Salmonella*

To develop a *Salmonella* phage cocktail against *S.* Enteritidis and *S*. Typhimurium, nine phages which showed the strong ability to lyse targeted serovars (including phages KP1, KP 2, KP4, KP5, KP9, KP34, KP36, KP49 and KP50) were investigated for their lytic activity by a spotting test and efficiency of plating (EOP) using method modified from Mirzaei and Nilsson, 2015. In this study, *S.* Enteritidis FSL S5-371 and *S.* Typhimurium H2-001 were used as the target hosts. *S.* Anatum FSL A4-525 was used as the reference host of phage KP1, KP2, KP5, and KP9. *S.* Virchow H2-117 was used as the reference host of phage KP34 and KP036. *S.* Agona H2-016 was used as the reference host of phage KP49 and KP50. Three *Salmonella* phages

which showed the highest lytic ability and EOP were selected to prepare a cocktail using a ratio of 1:1:1 for each phage.

3.3.1.1 Spotting assay

Each *Salmonella* phage dilutions ranging in concentration from 10^3 to 10^7 PFU/mL were spotted on bacterial lawn of *S.* Enteritidis and *S.* Typhimurium prepared following a procedure mentioned in 2.3.3 immediately after spotting, the plates were incubated at room temperature for 16-24 h. The clear zone or visible plaques formed by serial dilutions on the plate were determined as +++, confluent lysis (clear spot); ++, semi-confluent lysis (semi-clear); +, turbidity without plaque formation; −, no lysis. The experiment was repeated three times for each phage.

3.3.1.2 Efficiency of plating (EOP) assay

Similar to a spotting assay, each *Salmonella* phage dilutions ranging in concentration from 10^3 to 10^7 PFU/mL were spotted on bacterial lawn of *S.* Enteritidis and *S.* Typhimurium prepared following a procedure mentioned in 2.3.3. EOP assay replicates for a particular phage were done in parallel on target and referent hosts tested. The EOP was calculated by the ratio of the average PFU on target host to the average PFU on a corresponding reference host. EOP values were presented as 3 levels; high production ($EOP \geq 0.5$), medium production ($0.01 \leq EOP < 0.5$), and low production ($0.0001 < EOP < 0.01$).

3.3.2 Transmission electron microscopy (TEM) analysis of *Salmonella* phages

Individual phages composed in phage cocktail was identified the morphology by TEM analysis. A small drop of phage suspension was spotted onto a carbon-coated copper mesh grid and allowed to sit for 2-3 min. Excess phage suspension was then removed with filter paper. For negative staining one drop of phosphotungstic acid (pH 7.4) was added to each grid, and excess stain was removed 1 min later with filter paper. Each grid was then covered and allowed to dry for 15 min. Transmission electron microscope JEM-100CX II (Jeol, Japan) was operated at 80kV and instrumental magnification of 100,000x.

3.3.3 One-step growth curve study

A one-step growth curve for three phages included in the phage cocktail was investigated following a protocol of Bao *et al*. (2011) with modifications. The bacterial hosts *S.* Enteritidis and *S*. Typhimurium grown overnight (10^7 CFU/mL) in TSB as mentioned in 2.3.2 were mixed with 10^8 and 10^9 PFU/mL of phage to a final volume of 30 mL to represent the multiplicity of infection (MOI) of 100 and 10. The co-culture was incubated at 37°C (220 rpm) for an initial attachment for 20 min, followed by centrifugation of the sample at 6000×*g* for 10 min at 4°C to remove the excess phage as the supernatant. Cell pellets were re-suspended with the same volume (30 mL) as pre-centrifugation with TSB and resumed to incubation for additional 60 min. Lysate (1 mL) was taken every 5 min for the standard plaque count assay (in triplicates) to determine the number of phages obtained from each period. Latent period was defined as the time interval between the adsorption (not including 20 min of pre-incubation) and the beginning of the first burst, as indicated by the initial rise in the phage titer. Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period.

3.3.4 Whole genome sequencing analysis of phages composed in phage cocktail

3.3.4.1 *Salmonella* phages DNA extraction

Nucleic acid of *Salmonella* phages was isolated using a phenol/chloroform procedures based on the Lambda phage DNA isolation protocol previously described (Sambrook and Russell, 2001) with minor modifications. Phages in the concentration of approximately 10^8 PFU/mL were precipitate using Polyethylene glycol (PEG 8000) (PanReac AppliChem, USA) in the presence of salt. DNase I (Promega, Madison, WI) (5 µg/ml) and RNase A (Sigma, St. Louis, MO) (30 µg/ml) were used to remove bacterial nucleic acids. After phenol/chloroform (VWR/AMRESCO, USA) extraction and ethanol precipitation, nucleic acid was dissolved in 50-100 µl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). A ratio of $OD_{260/280}$ values was quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific Inc, USA).

3.3.4.2 Phage genome sequencing, assembly and annotation

DNA of all three *Salmonella* phages were submitted for whole genome sequencing by Illumina HiSeq2500 platform through a 100-base pair end reads service provided by Macrogen Inc. (Seoul, South Korea). Phage whole genome assembly, annotation and comparison were performed. The *de novo* genome assembly was carried out with the aid of SOAPdenovo2 (Luo *et al*., 2012). Genome annotation was performed using RAST annotation server (Rapid Annotation using Subsystem Technology) (Aziz *et al*., 2008), NCBI-BLAST (Basic local alignment search tool) (Altschul *et al*., 1990). Nucleotide sequence alignment was performed using the EasyFig 2.2.3 software (Sullivan *et al*., 2011).

3.3.5 Efficiency of phage cocktail to reduce *S.* Enteritidis and *S.* Typhimurium *in-vitro* and evaluation of phage-resistance in *Salmonella* after treated with phage cocktail

Overnight culture of *S.* Enteritidis and *S.* Typhimurium prepared as mentioned in 2.3.2 (10 mL) were centrifuged at 6000×*g* for 10 min at 4°C. To wash cell pellets, 5 mL of PBS were added and centrifuged at the same conditions three times. Washed *Salmonella* pellets were suspended in TSB and diluted to approximately 10^5 CFU/mL. Phage cocktail stock was diluted with TSB to achieve phage concentration at 10^7 PFU/mL. Each *Salmonella* isolate suspension and phage cocktail preparation were mixed at a ratio of 1:1 by volume and incubated at 37°C in a shaking incubator (ThermoStableTM IS-30model, DAIHAN Scientific, Korea) at 220 rpm for 12 h. Controls included in the study were only *S.* Enteritidis and *S.* Typhimurium cultured in TSB. The cell numbers of *S.* Enteritidis and *S.* Typhimurium from the treatments and controls were enumerated every 4 h interval by a spread plate on TSA. After 12 h of experiment, samples were collected for the analyses of phage resistance.

The changes in resistance phenotype of *S.* Enteritidis and *S.* Typhimurium after treated with a phage cocktail were evaluated by a spotting test. Five colonies of *S.* Enteritidis and *S.* Typhimurium from controls and treatments of phage cocktail recovered from TSA after 12 h of experiment above were re-cultured in TSB, followed by incubation at 37°C for 16-18 h. Each culture was used to prepare an overlay for a spotting test as mentioned above (see 2.3.3). Serial dilutions (10^3-10^7

PFU/mL) of phage cocktail and each individual phage mixed in a cocktail were spotted on each bacterial lawn. Phage lysis patterns were determined after 18-24 h of incubation.

3.3.6 Efficiency of phage cocktail to reduce *S.* Enteritidis and *S.* Typhimurium in foods and animal feed

Two types of fresh foods and animal feed used in this study are chicken meat, sunflower sprout and animal feed. Samples were purchased from the supermarkets. To eliminate *Salmonella* that may be present, chicken meat and sunflower sprout were soaked in 50 ppm free chlorine concentration solution (Sigma-Aldrich, St. Louis, Mo, USA) for approximately 5 min (FAO, 2000). Subsequently, the samples were soaked and washed in sterile distilled water for approximately 5 min for 3 times. For animal feed, samples were eliminated the initial microbial contamination by autoclaving. Chicken breast was aseptically cut into pieces of approximately 5 x 5 cm^2. Sunflower sprout and animal feed were aseptically weight approximately 5 g. Washed cell pellets of *S.* Enteritidis and *S.* Typhimurium were diluted to 10^5 CFU/mL in PBS. One milliliter of each *Salmonella* strain was evenly transferred to the surface of each piece of chicken meat, sunflower sprout, and animal feed in a sterile Whirl-Pak bag to achieve a inoculation level of approximately 10^5 CFU/piece or 10^5 CFU/5 g. Samples were left in clean bench for 10 min to allow the cells to adapt to the conditions on food samples tested. *Salmonella* phage cocktails 1 mL at 10^7 PFU/mL was transferred to each piece of chicken and sunflower sprout in a sterile Whirl-pak bag. PBS buffer 1 mL was added to samples inoculated with *Salmonella* as controls. Treatments and controls were stored at 4°C (chicken meat and sunflower sprout) and 30°C (animal feed), number of *Salmonella* was enumerated at day 0, 1, 2, 3 and 4 on Xylose-Lysine-Deoxycholate agar (XLD; Oxoid, Hampshire, UK).

3.4 Results and discussion

3.4.1 Morphology of phages composed in a cocktail

Transmission electron microscopy analysis revealed that the morphology of all three phages (KP4, KP5 and KP50) included in a cocktail presented

a long and contractile tail with approximately 50 nm of capsid head (Figure 3.1). Data suggest that they were classified into the order *Caudovirales* and the family *Siphoviridae*. Since 1959, the morphology of >5500 bacteriophages were determined under electron microscope. More than 96% of them are tail and classified into the order *Caudovirales* and three families, i.e., *Myoviridae, Podoviridae* and *Siphoviridae* (Ackerman, 2007). Most of effective *Salmonella* phages are tailed-phages. Lappe *et al*. (2009) were isolated and characterized 16 *Salmonella* phages. All are tailed-phages, belonged to three families *Myoviridae* (4 phages), *Podoviridae* (6 phages) and *Siphoviridae* (6 phages) (Lappe *et al*., 2009). Bao *et al*. (2011) were reported two lytic *Salmonella* phages which belonged to family of *Siphoviridae* in 2011 (Bao *et al*., 2011) and two lytic *Salmonella* phages which belonged to family of *Myoviridae* in 2015 (Bao *et al*., 2015). In 2017, *Salmonella* phages belonged to families of *Siphoviridae* (Choi *et al*., 2017) and *Podoviridae* were reported (Thung *et al*., 2017). In the recent year, *Salmonella* phages belonged to families of *Myoviridae* and *Siphoviridae* were reported (Duc *et al*., 2018). Tailed-phages have been reported as the most stable for storage and showed the longest survivability (Ackerman *et al*., 2004).

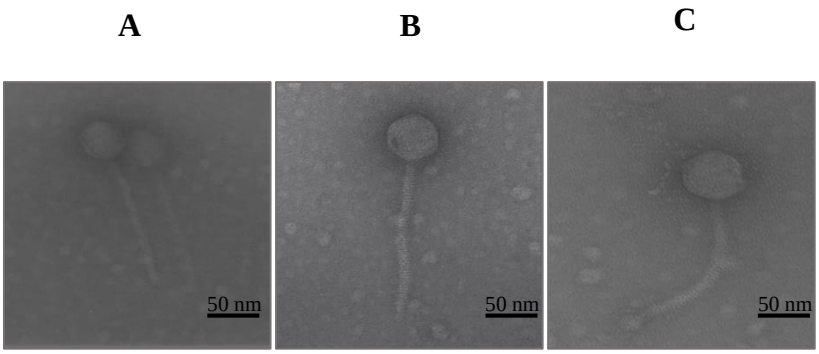

Figure 3.1. TEM images of individual phage composed in phage cocktail preparation. Magnification 100000x. Letter A, B and C represent morphology of phage KP4, KP5 and KP50, respectively.

3.4.2 Biology of phages composed in the cocktail

Among 36 isolated *Salmonella* phages, nine phages showed the high ability to lyse *S.* Enteritidis and *S*. Typhimurium. Of these phages, KP4, KP5 and KP50 were selected for the development as phage cocktail due to their highest lysis activity on *S.* Enteritidis and *S.* Typhimurium (Table 3.1). Each phage composed in a phage

cocktail preparation showed the latent period on both *S.* Enteritidis as 5, 15 and 40 min at MOI 100, and 10, 15 and 10 min at MOI 10 for phages KP4, KP5 and KP50, respectively (Table 3.2; Figure 3.2). For *S.* Typhimurium host, these phages showed the latent period as 10, 10 and 15 min at MOI 100, and 15, 15 and 10 min at MOI 10 for KP4, KP5 and KP50, respectively. Here, we reported relatively short latent period on both *Salmonella* hosts for three phages included in a phage cocktail preparation. The range of *Salmonella* phage latent period has been summarized with the range of 15-45 min (Calsina *et al*., 2011). The latent period can be defined as a random period between the moment of being infected and the moment of releasing the infecting agent (Calsina *et al*., 2011). However, Abedon *et al*. (2003) have reported that shorter phage latent periods could be provided by higher bacterial densities and suggested that page exhibiting very short latent periods may be viewed as specialists for propagation when bacteria within cultures are highly prevalent (Abedon *et al*., 2003).

The three phages included in a phage cocktail preparation showed a large burst sizes observed at 25.1, 30.1 and 97.7 PFU/cell on *S.* Enteritidis at MOI 100, and 16.6, 6.6 and 37.1 PFU/cell at MOI 10 for KP4, KP5 and KP50, respectively. For *S.* Typhimurium host, the average burst size of phages KP4, KP5 and KP50 was 70.8, 173.7 and 112.2 PFU/cell at MOI 100, and 19.1, 19.1 and 28.8 PFU/cell at MOI 10, respectively. *Salmonella* phage PSPu-95 and PSPu-4-116 reported by Bao *et al*. (2011) showed burst size as 77.5 and 86 PFU/cell, respectively. Both phages showed a latent period as 20 min (Bao *et al*., 2011). *Salmonella* phage vB_SenS-Ent1 revealed the latent as 25 min and the burst size at 35 PFU/cell (Turner *et al*., 2012). The findings suggest a rapid replication and effective release of new phage particles from both *S.* Enteritidis and *S.* Typhimurium hosts as short latent period and larger burst size were observed among the three pages included in a phage cocktail preparation. Phage latent period and burst size are the major parameters which play the important role in the host lysis system (Santos *et al*., 2014). Bacteriophages which present short latent period and large burst size will be replicated more quickly and the progeny particles could be released more efficiently (Bao *et al*., 2011). Phage burst size has been reported with the wide variation (5-250 PFU/cell) depending on strain infected. This may also suggest a potential use of phages with short latent period and large burst size for controlling

bacterial hosts as reported by previous other studies (Cao *et al*., 2015; Kalatzis *et al*., 2016).

Table 3.1. Lysis ability and efficiency of plating (EOP) of *Salmonella* phages on *S.* Enteritidis and *S.* Typhimurium.

Phage	Reference *Salmonella* serovar	Target *Salmonella* serovar	Lysis ability[a] Phage titer (PFU/mL) 10^7	10^6	10^5	10^4	10^3	Level of EOP[b]
KP1	Anatum	Enteritidis	+++	++	-	-	-	Medium
	(A4-525)	Typhimurium	++	-	-	-	-	Medium
KP2	Anatum	Enteritidis	+++	+++	++	-	-	Medium
	(A4-525)	Typhimurium	+++	++	-	-	-	Low
KP4	Anatum	Enteritidis	+++	+++	+++	+	-	Medium
	(A4-525)	Typhimurium	+++	+++	+++	+	-	Medium
KP5	Anatum	Enteritidis	+++	+++	+++	+	-	Medium
	(A4-525)	Typhimurium	+++	+++	++	-	-	Medium
KP9	Anatum	Enteritidis	+++	+++	+++	++	-	High
	(A4-525)	Typhimurium	+++	+	-	-	-	Low
KP34	Virchow	Enteritidis	+++	+++	++	-	-	Medium
	(H2-117)	Typhimurium	++	-	-	-	-	Low
KP36	Virchow	Enteritidis	+++	+++	+++	++	-	Medium
	(H2-117)	Typhimurium	++	-	-	-	-	Low
KP49	Agona	Enteritidis	++	-	-	-	-	Low
	(H2-016)	Typhimurium	++	-	-	-	-	Low
KP50	Agona	Enteritidis	+++	+++	+++	+++	+	High
	(H2-016)	Typhimurium	+++	+++	+++	+	-	Medium

[a]Clear zone or visible plaques were observed as +++, confluent lysis (clear spot); ++, semi-confluent lysis (semi-clear); +, turbidity without plaque formation**;** −, no lysis.

[b]EOP values were presented in 3 levels: high production (EOP ≥ 0.5), medium production (0.01 ≤ EOP < 0.5) and low production (0.0001 < EOP < 0.01).

Table 3.2. Latent period and burst size of *Salmonella* phages included in a phage cocktail preparation on *S.* Enteritidis and *S.* Typhimurium.

Phage ID	Latent period (min)				Burst size (PFU/cell)			
	S. Enteritidis		*S.* Typhimurium		*S.* Enteritidis		*S.* Typhimurium	
	MOI 100	MOI 10	MOI 100	MOI 10	MOI 100	MOI 10	MOI 100	MOI 10
KP4	5	10	15	10	25.1	16.6	70.8	19.1
KP5	15	15	10	15	30.1	6.6	173.7	19.1
KP50	40	10	15	10	97.7	37.2	112.2	28.8

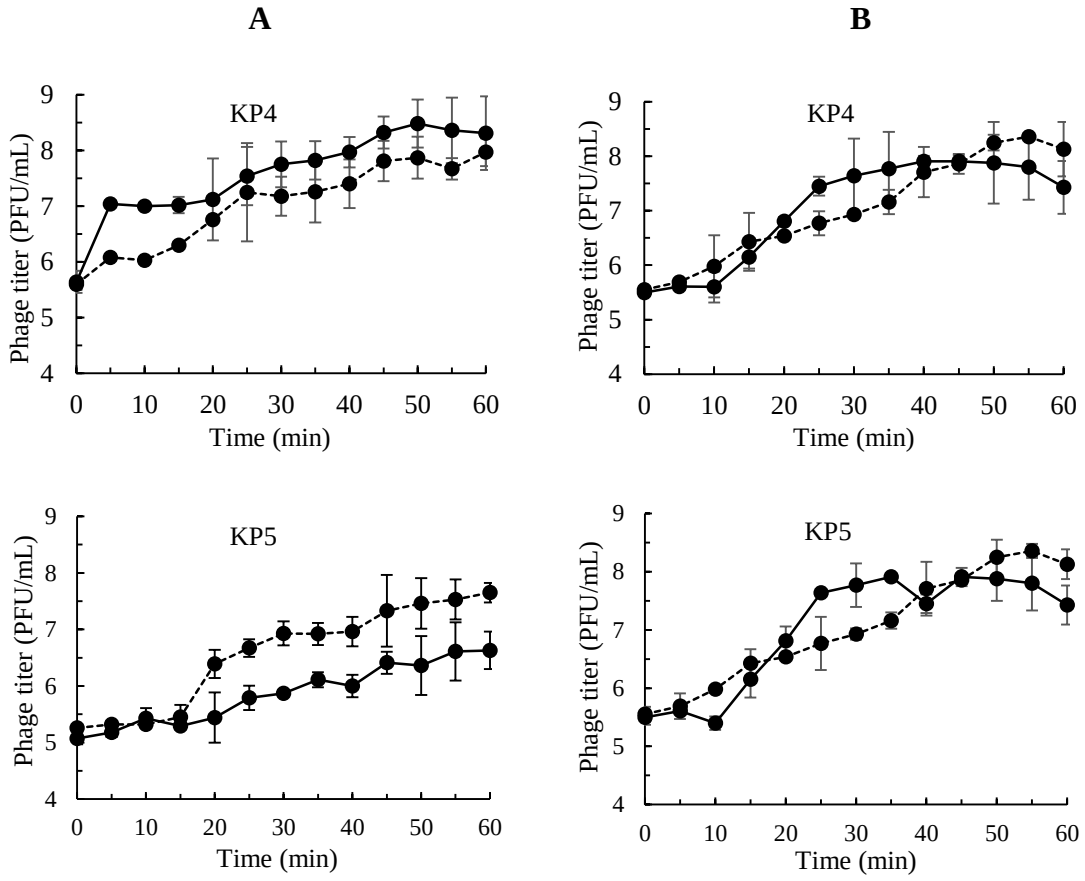

Figure 3.2. One-step growth curve of phage KP4, KP5 and KP50 on *S.* Enteritidis (A) and *S.* Typhimurium (B). MOI 100 presented by —●— and MOI 10 presented by --●--. Bars represent the mean standard deviation (n = 3).

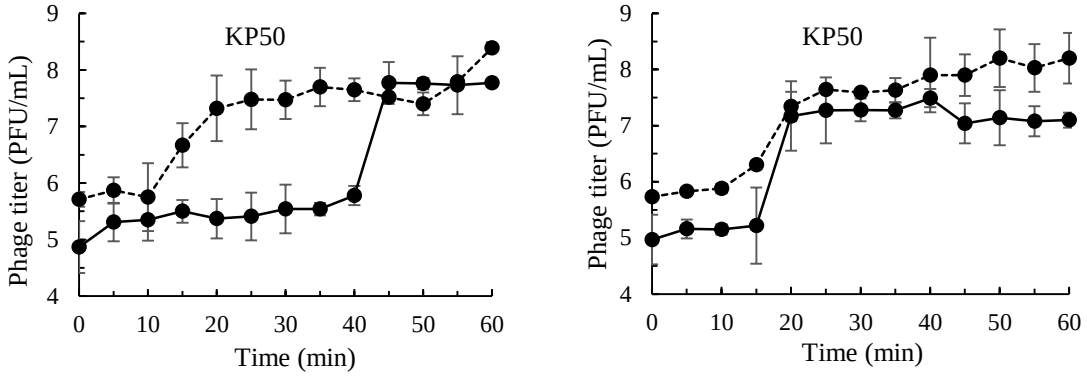

Figure 3.2. One-step growth curve of phage KP4, KP5 and KP50 on *S.* Enteritidis (A) and *S.* Typhimurium (B). MOI 100 presented by —●— and MOI 10 presented by --●--. Bars represent the mean standard deviation (n = 3).

3.4.3. Whole genome sequencing analysis of phages composed in a phage cocktail

3.4.3.1. Sequence information of *Salmonella* phage genomes

Whole genome sequencing analysis and detailed genomic comparison of all three *Salmonella* phages (KP4, KP5 and KP50) composed in cocktail were generated through RAST annotation server (Table 3.3). Genome sequencing of these phages revealed that they are linear double-stranded DNA genome. KP4, KP5 and KP50 presented genome size of 90,164 bp (GC content of 44.1%), 59,958 bp (GC content of 56.3%) and 90,100 bp (GC content of 44.1%), respectively. The number of coding sequences (CDS) is proportion to the size of the phage genome, presenting 152, 74 and 155 for KP4, KP5 and KP50, respectively. A number of eight and nine tRNAs were detected in the genome of KP4 and KP50, respectively. Whereas none of tRNA was detected in the genome of KP5. BLASTX was used for annotation and identification of possible role of these CDSs in phage life cycles. Gene function of KP4 was assigned to 33/152 CDSs and other 119 CDSs were hypothetical proteins. Gene function of KP5 was assigned to 50/74 CDSs and other 24 CDSs were hypothetical protein. For KP50, gene function was assigned to 30/155 CDSs and other 125 CDSs were hypothetical proteins.

Table 3.3. Sequence information of genomes of *Salmonella* phages composed in phage cocktail**.**

Phage ID	Size (bp)	GC content (%)	No. of contig	No. of tRNAs	No. coding Sequences (CDS)	No. of gene functions/hypothetical proteins
KP4	90,164	44.1	1	8	152	33/119
KP5	59,958	56.3	1	0	74	50/24
KP50	90,100	44.1	1	9	155	30/125

3.4.3.2 Annotation and analysis of phage genome sequences

All three phages KP4, KP5 and KP50 (Supplemental table 2, 3 and 4) were found to encode with the functional modules comprised of (i) phage structural protein (ii) phage replication and combination (iii) phage DNA metabolism and (iv) phage lysis modules. In term of phage application, lytic and lysogenic cycles are important to understand. The central repressor plays the major key of phage life cycle in either lytic or lysogenic. Integration enzyme such as integrase enzyme produced by phage in lysogenic cycle play the main role to turn off the repressor, resulting the process forward to the recombination step instead of replication step (Kropinski *et al*., 2007). For lytic cycle, phage lysis module plays the important role as bacterial host lysis. Holin and endolysin are well known as the major proteins which involved the mechanism of host cell lysis (Shin *et al*., 2013). The process of lysis achieved by the collaboration of these protein. Holin damaged cytoplasmic membrane of host cell by creating the holes. Endolysin uses these holes as transport channels to digests the peptidoglycan layer (Krupovič *et al*., 2008). In the lysogenic cycle, CI is a gene which plays the major role to control the repression of lytic gene. Resulting the stable lysogenic state. However, induction by host DNA damage provides *Rec*A protein to inactivate CI and allow lytic genes to process lytic cycle (Atsumi and Little, 2006). Genes associated virulence, toxins and antibiotic resistance are the major of concern in phage application. Phages are well known as a driving force of bacterial evolution through the occurrence of gene transfer. On the other hand, phages can also contribute the virulence, toxin and antibiotic resistance in the process of infection (Wagner and Waldor, 2002; Moreno Switt *et al*., 2013). *Salmonella* phages have been reported to

carry these concern genes e.g. phage Gifsy-1 encodes *gipA* which enhance survival in the Peyer's patch (Stanley *et al*., 2000; Wagner and Waldor, 2002), phage SopEϕ transduces type III secretion system (Mirold *et al*., 1999; Wagner and Waldor, 2002), phage FSL SP-016 carries bicyclomycin R (Moreno Switt *et al*., 2013). However, our analysis showed that all three phage genomes in a cocktail showed the absence of genes associated with the lysogeny module, genes associated with virulence/toxins and genes associated with antibiotic resistance. These three phages thus represent putative virulent phages.

BLAST analysis revealed that some of the newly sequenced phages were distinct from the available phages in databases (Supplemental table 3, 4 and 5). Comparative genomic analysis showed that phages KP4 and KP50 were related to phage 7-11 (unknown source) and phage SE131 (isolated from feces, South Korea) (Table 3.4; Figure 3.3). Genome of phage KP5 showed to be related to several phages from various continents, including India (phage 35 and phage 37 isolated from sewage/water) and the US (phage FSL SP-088, phage FSL SP-124 isolated from dairy farms and phage Siskin isolated from mixed waste water). Results indicate that our phages composed in the cocktail are similar to some previously described phages, but some unique gene contents are also observed. Our phages are also common as these can be found in various geographical regions.

In summary, our phages composed in the cocktail showed the evidence of being virulent phages. All phages showed the absence of lysogeny module associated genes, virulence or toxin associated genes and also antibiotic resistance associated genes. In addition, these phages related to various *Salmonella* phages isolated from different countries and continents.

Table 3.4. Sequence information of other closely related *Salmonella* phages for comparison.

Related organism	Size (bp)	Accession number	Number of tRNAs	Source, Country
phage 7-11	89916	HM997019.1	6	unknown
phage SE 131	89910	MG873442.1	7	feces, South Korea

Table 3.4. (Cont.)

Related organism	Size (bp)	Accession number	Number of tRNAs	Source, Country
phage 35	55391	KR296689.1	0	sewage/water, India
phage 37	60216	KR296691.1	0	sewage/water India
phage FSL SP-088	59454	KC139512.1	0	dairy farms, US
Phage FSL SP-124	59245	KC139515.1	0	dairy farms, US
Phage Siskin	58476	MH631453.1	0	mixed waste water, US

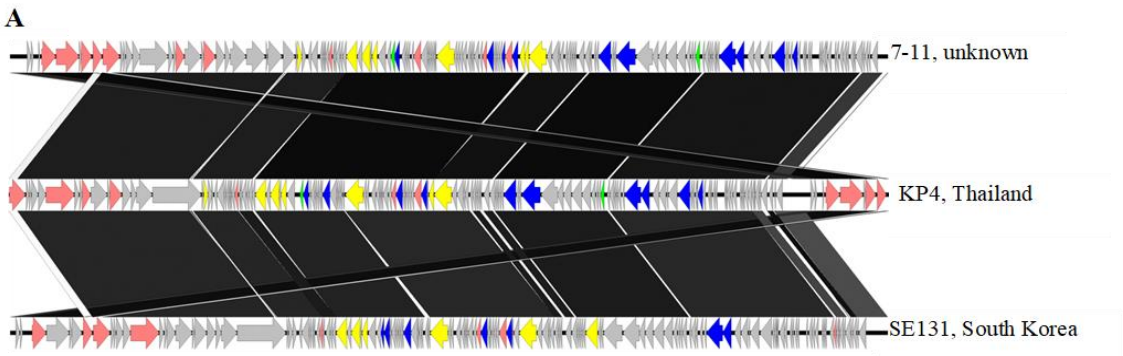

Figure 3.3. Linear representation of phage genome comparison of phages KP4 (A), KP5 (B) and KP50 (C) to the related phages. Functional modules comprise of phage structural protein (pink arrows), phage replication and combination (yellow arrows), phage DNA metabolism (blue arrows), phage lysis modules (green arrows) and phage lysogeny modules (red arrow).

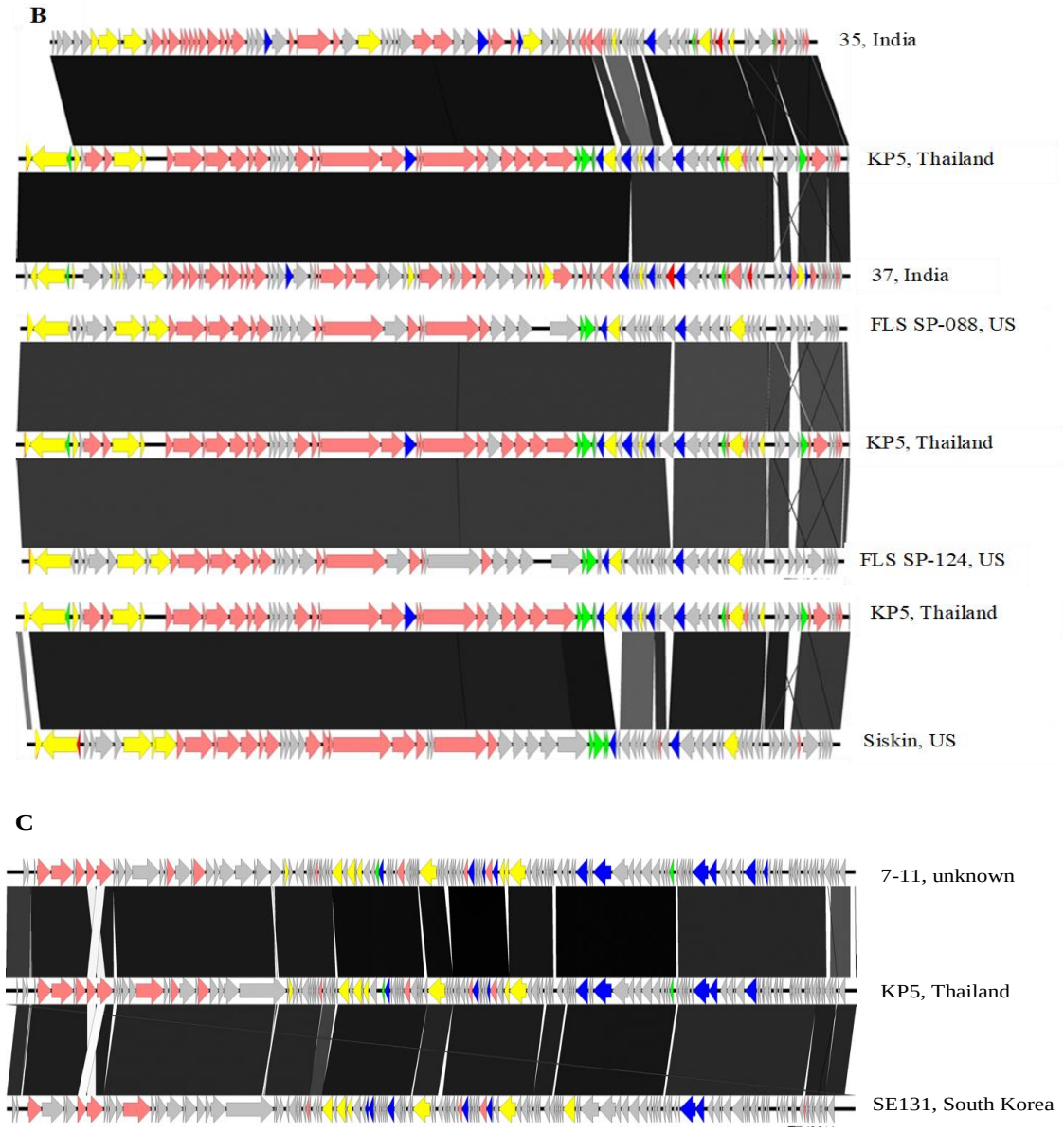

Figure 3.3. Linear representation of phage genome comparison of phages KP4 (A), KP5 (B) and KP50 (C) to the related phages. Functional modules comprise of phage structural protein (pink arrows), phage replication and combination (yellow arrows), phage DNA metabolism (blue arrows), phage lysis modules (green arrows) and phage lysogeny modules (red arrow).

3.4.4 Efficiency of *Salmonella* phage cocktail to reduce *S.* Enteritidis and *S.* Typhimurium *in-vitro* and different fresh foods and animal feed

In the *in-vitro* study, a *Salmonella* phage cocktail could reduce the number of *S.* Enteritidis and *S.* Typhimurium continuously after treatment at MOI 100 as indicated by the highest reduction of both serovars of more than 4 log CFU/mL after 4 h of a phage cocktail treatment (Figure 3.4). During 8 h of treatment, the overall reduction of more than 3 log CFU/mL was observed for both *S.* Enteritidis and *S.* Typhimurium.

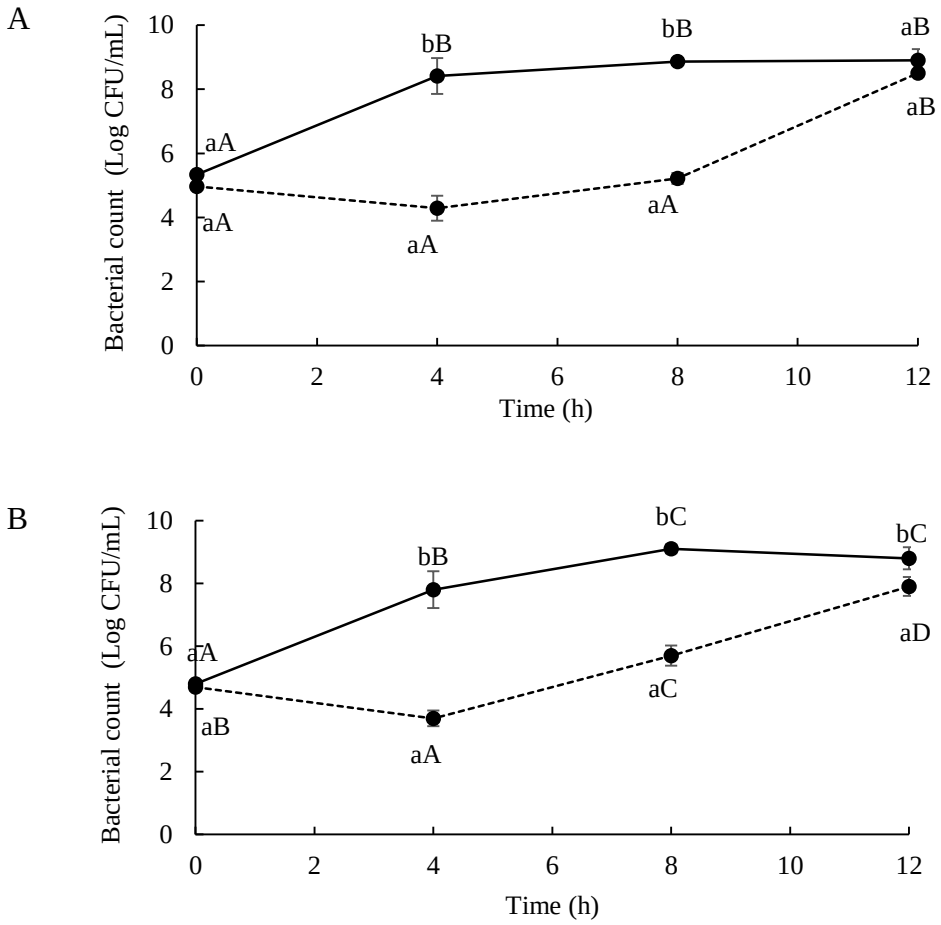

Figure 3.4. Survival of (A) *S.* Enteritidis and (B) *S.* Typhimurium treated with a phage cocktail at 37°C for 12 h. Control (non-phage cocktail treated) presented by —●— and treatment of a phage cocktail presented by ---●---. Bars represent the mean standard deviation (n = 3). Different lowercase letters on the bars indicate significant differences ($p < 0.05$) of bacterial reduction between control and treatment. Different uppercase letters on the bars within the same treatment indicate significant differences ($p < 0.05$) of bacterial reduction during storage time.

The occurrence of *Salmonella* contaminated in healthy poultry animals is reported as the main risk factor which provides easy transmission in table eggs and poultry meat to humans. *S.* Enteritidis and *S.* Typhimurium are considered as the particular serovars public health concern in poultry meat and egg production (Hugas *et al*., 2014; Antunes *et al*., 2015). Fresh produce such as sprout has been reported as the high risk food contaminated *Salmonella* in the continuous period (Hanning, 2009; CDC, 2018). Several studies reported that animal feed is an important product which related to *Salmonella* contamination (CDC, 2012; FDA, 2013; Kukier *et al*., 2013). Although, this product is not human food, it is present at the beginning of the food chain which provides opportunity to animal infected *Salmonella* (Crump *et al*., 2002). Thus, control of *Salmonella* in these three food categories is crucial. In this study, chicken meat, sunflower sprout and animal feed were selected for testing with a phage cocktail preparation. During 4 days at 4°C, the numbers of *S.* Enteritidis and *S.* Typhimurium in chicken meat were decreased by 0.66 and 1.73 log CFU/cm^2, respectively. The numbers of both *S.* Enteritidis and *S.* Typhimurium in sunflower sprout were decreased by 1.27 and 1.17 log CFU/g, respectively. For animal feed, the experiment was investigated at 30°C for 4 days. The numbers of both *S.* Enteritidis and *S.* Typhimurium in animal feed were decreased by 1.87 and 2.38 log CFU/g, respectively (Figure 3.5). Results indicate a potential use of this phage cocktail in chicken meat and sunflower sprout during a storage condition at 4°C and animal feed at 30°C for 4 days. Several studies evaluated the efficiency of *Salmonella* phage cocktail in similar food categories, including raw meat, fresh produce, RTE food and even in animal feed. The efficiency of a lytic *Salmonella* phage SE07 against *S*. Enteritidis on various food matrices (fruit juice, fresh eggs, beef and chicken meat) has been reported. The reduction of *Salmonella* approximately 2 log cycles was observed on food tested after 48 h at 4°C (Thung *et al*., 2017). *S.* Oranienburg inoculated on alfalfa sprouts was treated with a phage cocktail (mixture of 2 phages) and a reduction of 1 log CFU/g of *Salmonella* was observed within 5 days (Kocharunchitt *et al*., 2009). The reduction of *S.* Enteritidis and *S.* Typhimurium on chicken breast was observed at 0.9 and 2.2 log CFU/g, respectively, after treated with a phage cocktail (mixture of three phages) within 7 days at 4°C (Spricigo *et al*., 2013). Reductions of *S.* Enteritidis in raw and smoked salmon by phage cocktail were investigated by Galarce *et al*., 2014. They reported that the reduction range of 0.75 to 3.19 log CFU/g and 2.82 to 3.12 log CFU/g were observed in raw salmon at 18°C and 4 °C, respectively. Whereas, the reduction of 2.82 to 3.12 log CFU/g and 0.50 to 1.16 log CFU/g

were observed at 18°C and 4 °C, respectively in smoked salmon (Galarce *et al*., 2014). Grant *et al*. were reported < 1 log reduction of *Salmonella* on ground chicken after treated with the commercial *Salmonella* phage cocktail (Salmonelex^{TM}) (Grant *et al*., 2017). Controlling *Salmonella* in dry pet food was investigated by Heyse *et al*. (2015). They were observed >1 log reduction of *Salmonella* after treated with phage cocktail comprising 6 *Salmonella* phages for 60 min (Heyse *et al*., 2015). The commercial *Salmonella* phages (SalmoLyse$^{®}$) were investigated their efficiency to reduce *Salmonella* in pet food and raw pet food ingredients. Approximately 3 log reduction was observed after tested (Soffer *et al*., 2016).

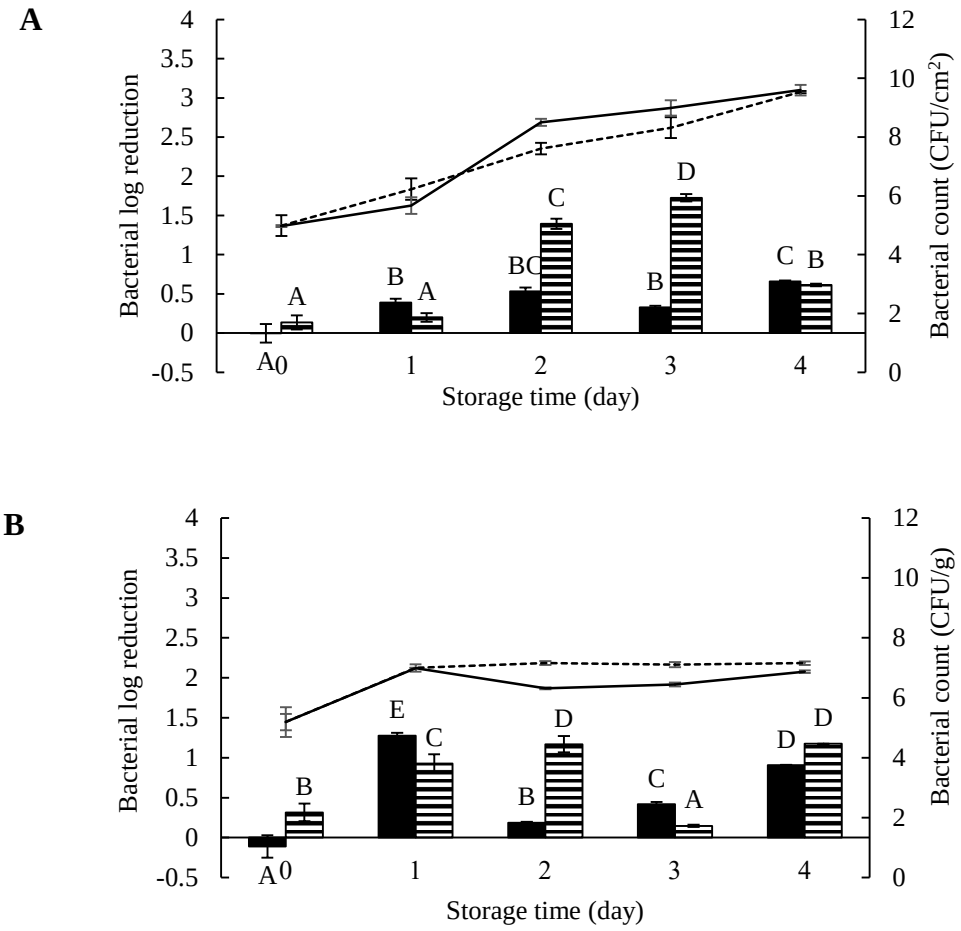

Figure 3.5. Log reduction of *S.* Enteritidis (■) and *S.* Typhimurium (▤) inoculated in (A) chicken meat and (B) sunflower sprout and animal feed (C) treated with a phage cocktail and stored at 4°C for 4 days. Bars represent the mean standard deviation (n = 3). Different uppercase letters within the same serovar on the bars indicate significant differences ($p < 0.05$) of bacterial reduction during storage time.

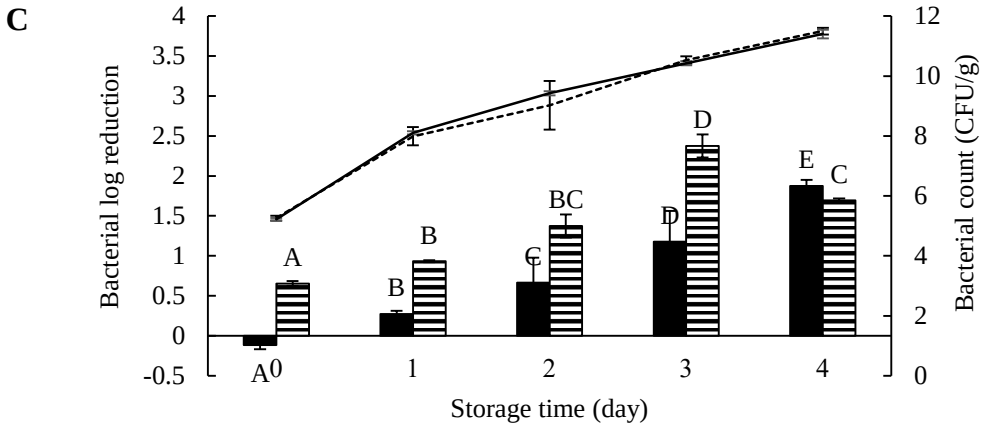

Figure 3.5. Log reduction of *S.* Enteritidis (■) and *S.* Typhimurium (▤) inoculated in (A) chicken meat (B) sunflower sprout and animal feed (C) treated with a phage cocktail and stored at 4°C for 4 days. Bars represent the mean standard deviation (n = 3). Different uppercase letters within the same serovar on the bars indicate significant differences ($p < 0.05$) of bacterial reduction during storage time.

3.4.5 Evidence of phage-resistant development in *Salmonella*

Phage-resistant development in *Salmonella* was investigated after a treatment with our phage cocktail preparation. A phage cocktail and individual phages included in phage cocktail preparation were re-tested on re-cultured *S.* Enteritidis and *S.* Typhimurium. Lysis ability of *Salmonella* isolates treated with a phage cocktail and individual phages presented similar results to the re-cultured *S.* Enteritidis and *S.* Typhimurium from non-phage treatment (Table 3.7). Finding suggests that no mutation of both serovars of *Salmonella* was observed after a phage cocktail treatment. The short period of time to kill the bacterial host has been reported as a major key to avoid bacteria resistant to phage after treated. This phenomenon related to the bacterial immune system (Calsina *et al*., 2011). In this study, all phages composed in a cocktail revealed the short latent period. This may be related to the less resistance of bacterial hosts after treated with phage cocktail.

Table 3.5. Lysis ability of a phage cocktail and individual phages included in a phage cocktail preparation on *S.* Enteritidis (SE) and *S.* Typhimurium (ST) after treated with a phage cocktail.

Treatment	Lysis ability[a]							
	Cocktail		KP4		KP5		KP50	
	Phage titer (PFU/mL)							
	10^7	10^6	10^7	10^6	10^7	10^6	10^7	10^6
Control SE (non-phage treatment)	++	++	++	++	++	++	++	+
Control ST (non-phage treatment)	++	+	++	+	++	−	+	−
Phage-treated SE	++	+	++	−	++	−	++	−
Phage-treated ST	++	+	++	+	++	−	+	−

[a]Clear zone or visible plaques were observed as +++, confluent lysis (clear spot); ++, semi-confluent lysis (semi-clear); +, turbidity without plaque formation; −, no lysis.

3.5 Conclusion

Among 36 *Salmonella* phages from our phage collection that were tested for phage lysis ability, phages KP4, KP5 and KP50 showed the highest efficiency to lyse *S.* Enteritidis and *S.* Typhimurium. These three phages showed a rapid replication and effective release of new phage particles from both *S.* Enteritidis and *S.* Typhimurium hosts as indicated by a short latent period and large burst size. All three phages were classified into order *Caudovirales* and family *Siphoviridae*. Whole genome sequencing analysis revealed that all three phages (KP4, KP5 and KP50) are virulent phage due to the absence of lysogeny module, virulence- and toxin-associated genes and also antibiotic resistance genes. A phage cocktail was developed by combining these three phages at equal ratio. Our developed phage cocktail showed high efficiency in reducing the number of *S.* Enteritidis and *S.* Typhimurium *in-vitro*. Phage cocktail developed here also showed the desirable efficiency to reduce the number of both *S.* Enteritidis and *S.* Typhimurium in fresh food, including raw chicken meat and sunflower sprout. This phage cocktail also showed great reduction of *S.* Enteritidis and *S.* Typhimurium in feed which presented the complex food matrix.

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CHAPTER 4

DEVELOPMENT OF OPTIMAL FORMULA OF MICROENCAPSULATED *SALMONELLA* PHAGE AS DRY POWDER

4.1 Abstract

S. Enteritidis and *S.* Typhimurium are common serovars of *Salmonella* associated with foodborne illnesses. Control approach to prevent food contamination with *S.* Enteritidis and *S.* Typhimurium is of interest. Bacteriophages have been shown a potential usage to control foodborne pathogens. Microencapsulated phage as dry powder is a newly form that provides convenient usage and protects phage from harsh conditions. In this study, seven formulations of wall material used for phage encapsulation were optimized by altering ratios of whey protein isolate (WPI) and trehalose prior to freeze-drying. Encapsulation efficiency (EE) ranged from 57.1% to 91.1%. Formulation representing trehalose alone showed the lowest EE (57.1%), while formulation with combination of WPI/trehalose at ratio of 3:1 (w/w) showed the highest EE (91.9%), thus presenting the optimal formulation. Fourier transform infrared spectroscopy (FTIR) analysis results showed H−bonding in the mixture system of optimal formulation and T_g showed at 63.43°C was revealed by differential scanning calorimetry (DSC) analysis. Microencapsulated phage with optimal formulation showed phage survival for >95% after 3 h, and >90% after 5 h of exposure to pH 1.5 to pH 9.5. In addition, microencapsulated phage showed effectiveness in decreasing the numbers of *S.* Enteritidis and *S.* Typhimurium at the multicity of infections (MOIs) of 100 and 1 by approximately 1 log CFU/mL at 10ºC and 30ºC for both serovars. This study indicates that the formulation with a combination of WPI/trehalose at a ratio of 3:1 (w/w) showed a potential in protecting phages from freeze-drying conditions, while providing a newly developed form of the encapsulated phage for further use against *Salmonella* spp.

4.2 Introduction

Bacteriophages (phages) are viruses of bacteria that are obligate intracellular parasites and host-specific as indicated by their ability to infect and kill specific genus or species of a bacterial pathogen (Petsong and Vongkamjan, 2015). With this outstanding characteristic, phages have been applied as a bio-control agent for controlling a variety of foodborne pathogens, including *Salmonella* spp. (Duc *et al.,* 2018). *Salmonella* is a leading cause of hospitalization and considered the top pathogen responsible for the most foodborne-related deaths in the United States (Scallan *et al.,* 2011). The current use of *Salmonella* phages, mostly in suspension (liquid) has been reported as a desirable approach for lowering the number of *Salmonella* in several food products as well as in feed (Kocharunchitt *et al.*, 2009; Ye *et al.,* 2010; Guenther *et al.*, 2012). However, utilization of phage suspension may have been limited due to its loss which could be affected by the buffering capacity of the storage media, storage conditions or chemical instability in physiological acidic or alkaline pH of the foods or environments where phages are applied.

Different encapsulation methods have been shown the ability to protect bioactive compounds and active cells, e.g., probiotics from destructive environmental and physical factors (El-Salam and El-Shibiny, 2015). However, microencapsulation of phage is not well studied. Microencapsulation becomes an interesting application which can improve, particularly properties of phages (Ma *et al.,* 2008), e.g., survival rates as compared to the phage suspension, the current available form which is not encapsulated. In addition, microencapsulated phages can be applied in low-moisture content or dry food products to maintain the quality and properties over the shelf-life of food. In this study, the free-phage suspension was immobilized using whey protein as a wall material and trehalose as a protective agent and transformed into a powder by lyophilization (freeze-drying). Overall, lyophilization has been reported as an effective method which can improve the stability of bacteriophages up to 21-year period (Ackermann *et al.,* 2004).

Natural biopolymers, such as proteins and polysaccharides are the most important types of surface-active materials that are preferably used for production of food-grade dispersions in the food industry (Jones and McClements, 2010). While several wall materials for encapsulation of active cells were evaluated, whey protein was found as a suitable wall material for probiotic encapsulation by spray-drying (Arslan *et al.,* 2015). This survivability rate of probiotic in whey protein was 91.81% as compared to gelatin, whey protein concentrate, modified starch, maltodextrin, pea protein isolate, and gum

arabic (Arslan *et al.,* 2015). A previous study showed that bacteriophage encapsulated in edible whey protein films could be stabilized at both room and refrigerated temperatures without significant loss in phage infectivity more than one month (Vonasek *et al.,* 2014). An addition of protecting agent is needed during lyophilization to increase the survivability of active cells (Bagad *et al.,* 2017). Disaccharide trehalose acts as a membrane-protecting agent for biological cells during environmental stress conditions such as dehydration and freezing (Zayed and Roos, 2004). A previous study reported the ability of trehalose in protecting phages from freeze drying as indicated by a slight decrease of the titer of freeze dried phage with trehalose after storage for 37 months at 4°C (Puapermpoonsiri *et al.,* 2010). After drying, this sugar forms an amorphous matrix, protecting embedded proteins via direct interaction or by providing structural support via the glass formation (Vandenheuvel *et al.,* 2013). Trehalose is thus an excellent choice as it showed unique combination of water replacement properties, a high glass transition temperature and chemical inertness (Schebor *et al.,* 2010).

The aim of this study was to develop microencapsulated phage as dry powder by altering the ratios of whey protein isolate (WPI) and trehalose used as wall material for the phage, followed by transforming the mixture into a dried powder via freeze drying. A broad host range phage from previous study was used as a core for the encapsulation optimization model. Phage powder was characterized for encapsulation efficiency (EE), morphology, and pH stability. Protein-saccharide interaction and glass transition temperature were studied by Fourier transform infrared spectroscopy (FTIR) analysis and differential scanning calorimetry (DSC) analysis, respectively. Antimicrobial efficacy of microencapsulated phage against *S.* Enteritidis and *S.* Typhimurium at different multicity of infections (MOIs) was investigated.

4.3 Materials and methods

4.3.1 Bacteriophage and phage lysate preparation

Salmonella phage SPT 015, previously isolated from a dairy farm in Thailand and characterized as broad host range (Wongsuntornpoj *et al.,* 2014), was used as a core material in this study. Phage was grown to increase phage titer following a procedure mentioned in 2.3.4. For phage enumeration, the overlay lawn was prepared following a procedure mentioned in 2.3.3. Ten-fold serial dilutions of phage suspension in PBS were spotted onto the host lawn, and incubated at room temperature overnight. Number of plaque

forming units presented on the bacterial lawn was counted. Phage lysate was kept in the conical tube at 4°C with SM buffer until use for study.

4.3.2 *Salmonella* strains

S. Typhimurium (FSL S5-370) was used as a propagating host for *Salmonella* phage SPT 015. *S.* Typhimurium (FSL S5-370) and *S.* Enteritidis (FSL S5-371) (Table 2.1) were used in the antimicrobial assays. For each use, overnight cultures for the study were prepared following a procedure mentioned in 2.3.2.

4.3.3 Encapsulation of *Salmonella* phage and freeze drying

Whey Protein Isolate (WPI) containing >90% (w/w) protein (Davisco Foods International, Le Sueur, MN, USA) was rehydrated in sterilized distilled water (90 mL). The solution was kept overnight at 4°C to ensure complete hydration of protein, followed by heating at 80°C for 30 min under agitation and subsequently cooled to room temperature. Trehalose dihydrate (Wako Pure Chemical Industries, Ltd, Japan) was added directly to each prepared whey protein solution to achieve 10% (w/v) total solid for various proportions as shown in table 4.1. Phage lysate (10^9 PFU/mL) was added into the mixture to achieve 10% (w/v). Mixture was kept at -40°C for 12 h. Frozen mixture (100 mL) was tempered at -50°C using a laboratory scale freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) under vacuum approximately 30 psi (Welch, 8912 Vacuum pump, Gardner Denver Thomas, Inc, Welch Vacuum Technology Niles, IL, USA) for 48-72 h. Size of freeze-dried samples was sorted through 100 mesh sieve, in order to achieve the dry powder with approximately 150 µm in size. Dry powder was weighted to obtain the estimated phage titer per gram.

4.3.4 Characterization of microencapsulated phage and stability assays

4.3.4.1. Bacteriophage survivability assay (plaque forming unit enumeration)

Phage power 1 g was mixed with 10 mL of PBS and left in a shaking incubator (ThermoStableTM IS-30 model, DAIHAN Sciencific, Korea) at room temperature, 220 rpm for 1 h. Suspension of phage powder was used to prepare 10−fold serial dilutions for determination of phage survivability by plaque forming unit counting following a procedure mentioned in 2.3.4. The host lawn was prepared following a procedure mentioned in 2.3.3 and each dilution (100 µl) was spotted onto a prepared host

lawn, and incubated at room temperature overnight. Number of plaque forming units was counted. Survivability of phages after freeze-drying or encapsulation efficiency (EE) was calculated, following: EE (%) = (RP/TP) x 100, where TP is total phage titer initially added for phage encapsulation and freeze-drying into powder and RP is phage titer recovered from the powder.

4.3.4.2 Morphology and surface characterization of microencapsulated phage

Morphology and surface characterization of microencapsulated phage were examined using a Quanta 250 Scanning Electron Microscope (SEM) (Quanta 250, Netherland). The freeze-dried phage powder were fixed on aluminum stubs with double adhesive tape and vacuum coated with a fine layer of gold before viewing under magnification of 500x. Observations were carried out with voltage acceleration of 20 kV.

4.3.4.3 Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR technique was used IR analysis as described by Malferrari *et al.* (2014) and van der Ven *et al.* (2002) with modifications. FTIR spectra in the absorbance mode were obtained using an FTIR spectrometer (EQUINOX 55, Bruker, Germany), with method of mid-infrared spectroscopy. Spectroscopic measurements were performed using 1:10 dilution of freeze-dried phage powder to potassium bromide (KBr). Diffusive reflectance of the IR was recorded with average of 32 scans at a resolution of 4 cm^{-1} for freeze-dried phage powder from a formula using trehalose alone and an optimal formulation, and with average of 64 scans at a resolution of 8 cm^{-1} for freeze-dried phage powder from a formula using WPI alone. Background noise was corrected with pure KBr data.

4.3.4.4 Differential Scanning Calorimetry (DSC) analysis

Glass transition temperatures (T_g) was determined using a differential scanning calorimeter (DSC-7, Perkin Elmer, Norwalk, CT, USA) as described by Schuck *et al.* (2005) with modifications. Temperature calibration was performed using the Indium thermogram. Approximately 5 mg of samples were accurately weighed into aluminum pans, hermetically sealed, and twice scanned over the temperature range of 0 to 250°C with a heating rate of 5°C/min and cooled rate 10°C/min. Liquid nitrogen was used as the cooling medium. The empty aluminum pan was used as a reference.

4.3.4.5 Stability of dry phage powder at various temperatures compared to non-encapsulated phage

Dry phage powder 1 g or non-encapsulated phage 1 ml was mixed with 10 ml of PBS, followed by incubation separately at various temperatures: 4°C, room temperature (25°C) and 50°C. Suspension from each temperature was taken after 1, 8, 16 and 24 h and diluted as 10−fold serial dilution for determination of phage survivability by plaque forming unit counting in duplicates following a procedure mentioned in 2.3.4.

4.3.4.6 Stability of dry phage powder at various pH levels compared to non-encapsulated phage

Dry phage powder and non-encapsulated phage were tested with five pH levels (1.5, 3.5, 5.5, 7.5 and 9.5). To prepare solution with a given pH, 0.5−1M HCl and NaOH were used to adjust to lower/higher pH. Dry phage powder 1 g or non-encapsulated phage 1 ml was mixed individually with 10 ml of each prepared solution, followed by incubation at room temperature for 5 h. Suspension from each pH was taken after 1, 3 and 5 h and diluted as 10−fold serial dilution for determination of phage survivability by plaque forming unit counting following a procedure mentioned in 2.3.4 in duplicates.

4.3.4.7 Antimicrobial efficacy of microencapsulated phage *in-vitro*

Overnight cultures of *S.* Enteritidis and *S.* Typhimurium were prepared following a procedure mentioned in 2.3.2, followed by centrifugation at 6000×*g* for 10 min. Buffer PBS (5 mL) was added for washing cell pellets and centrifuged at the same condition three times. Washed *Salmonella* pellets were suspended and diluted in TSB to achieve the level of 10^4 CFU/mL. Phage powder 0.5 g was mixed with 5 mL of TSB to obtain the final phage titers of 10^6 PFU/mL and 10^4 PFU/mL. Each overnight culture (5 mL) was mixed with a suspension of phage powder to achieve the multiplicity of infection (MOI) of 100 and 1, respectively. All phage co-culture samples were incubated at 10°C and 30°C with shaking at 220 rpm. Each phage co-culture sample was taken after 1, 8, 16 and 24 h of incubation and 10-fold serial dilution was performed in PBS. Appropriate dilutions were used for enumeration of surviving *Salmonella* cells by TSA.

4.3.5 Experimental design and statistical analysis

Matrix of experiments was designed based on two materials, resulting in seven combinations (9 formulations) as listed in table 4.1. Design was generated by using Design-Expert®7 trial version software (Stat-Ease Inc., Minneapolis, USA). The simplex lattice mixture design (SLMD) was used to evaluate the effect of the ratio of wall material (WPI) and trehalose on the highest phage recovery in the dry powder form. A percentage of EE was considered as a response in this design. Mean comparison of EE from each dry phage powder formulation was carried out by Duncan's multiple range tests (DMRT). Significance was declared at $p < 0.05$ using the statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL, USA). Optimized formulation batches were prepared in duplicate. For each batch, EE was evaluated in triplicate.

4.4 Results and discussion

4.4.1 Effects of WPI and trehalose on phage survival and phage release after freeze-drying

Structure of phages is known to have phage genome surrounded by the protective coating (capsid) which is the encoded protein (Drulis-Kawa, 2015). Freeze-drying conditions (freezing and drying) can be a critical stress that can lead to irreversible structural changes and protein aggregation, resulting in destabilization of phages (Merabishvili *et al.,* 2013). Addition of cryo-protectant and lyo-protectant is necessary to provide protective agents for increasing phage survivability from those harsh conditions (Abdelwahed *et al.,* 2006; Merabishvili *et al.,* 2013). In this study, WPI and disaccharide trehalose were combined as wall material and the ratios were altered as 9 formulations for testing. The encapsulation efficiency based on phage survivability after freeze-drying was compared among the formulations and showed the range from 57.1% and 91.1% (Figure 4.1; table 4.2). Phages encapsulated with only a protectant (trehalose) showed the lowest encapsulation efficiency, whereas the highest encapsulation efficiency was observed in a formulation with a ratio of 3:1 (w/w) for WPI to trehalose. This ratio indicates an optimal formulation as indicated by the highest phage survivability after freeze-drying. Overall, this study reported for the first time for the optimal formulation using a combination of WPI and trehalose to maintain the phage survivability in a dry phage powder form.

Component analysis by ANOVA revealed the final equation in terms of encapsulation efficiency (EE) as: +88.7610* A+57.51162 * B+35.69431* A * B, where A is WPI and B is trehalose. This equation suggests that EE was influenced by all factors (WPI, trehalose and the interaction between WPI and trehalose). However, WPI represented the most influence factor as indicated by its higher co-efficient factor which can positively affect the encapsulation efficiency. This can be hypothesized that WPI plays more important role to protect phage from freeze-drying as compared to trehalose. Protein possess the ability to interact, protect and reverse binding with a wide range of active compounds through their functional groups (Chen *et al.,* 2006). In addition, denatured whey protein has been reported to enhance living cells survivability though microencapsulation technique compared to using of nature whey protein (Doherty *et al.,* 2011). Trehalose could play an important role in anhydrobiotic protection, avoiding water crystallization during dehydration–rehydration cycles that could damage phage particles (Malferrari *et al*., 2014). Trehalose has been shown the ability to protect other living cells, including probiotics or mammalian cells through spray-drying or freeze-drying conditions (Broeckx *et al*., 2017; Zhang *et al*., 2017). Our study suggests that WPI plays the major role in protecting phages from freeze-drying conditions, thus maintaining survivability. However, trehalose provides the solubility property for facilitating the release of phage particles from encapsulated form. Overall, the combination of these two wall materials presented the synergistic effect on phage survival and release after freeze-drying. The chemical interaction among the dry phage powder components (phage, WPT and trehalose) could be explained by their chemical structures. Phages exhibit the amphipathic structural protein, consisting of a negatively charge of the phage head (capsid) and a positively charge of the tail fiber (Anany *et al*., 2011). The charge−charge interaction of WPI and phage particles is thus speculated to occur. In the mixture consisting of WPI, trehalose and phage suspension, the networks of H−bonds under a simultaneous H−bonding can connect surface protein groups and disaccharide molecules of trehalose and thus forming the protein–matrix interface (Malferrari *et al*., 2014).

Table 4.1. Ratios and amount of whey protein isolate (WPI) to trehalose (g) based on matrix experimental design.

Formulation	WPI:trehalose (w/w)	WPI:trehalose (g)
1	1:0	10:0
2	0:1	0:10
3	1:1	5:5
4	1:2	3.3:6.7
5	1:3	2.5:7.5
6	2:1	6.7:3.3
7	3:1	7.5:2.5
8	0:1	0:10
9	1:0	10:0

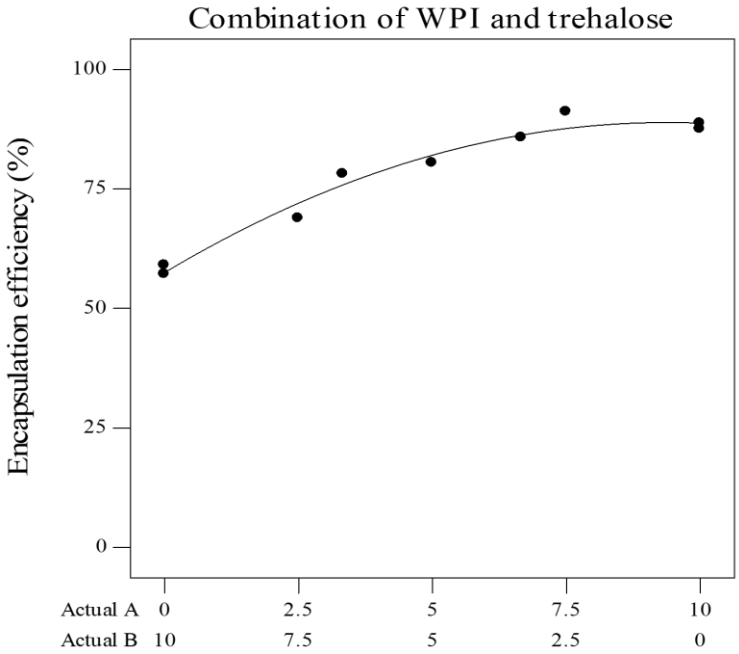


Figure 4.1. Encapsulation efficiency of dry phage powder at various treatments. A and B represent WPI and trehalose, respectively. Model; significant ($p < 0.0001$). Lack of fit; not significance ($p > 0.1414$). R-Squared; 0.9740.

Table 4.2. Phage survivability recovered from microencapsulated phage from various formulations.

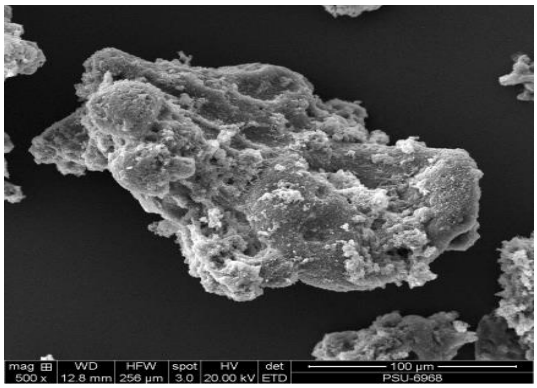
Formulation	Survivability[a](%)
1	88.07 ± 0.07^{f}
2	58.04 ± 0.12^{a}
3	80.39 ± 0.06^{cd}
4	78.12 ± 0.14^{c}
5	68.78 ± 0.11^{b}
6	85.7 ± 0.06^{ef}
7	91.91 ± 0.05^{g}
8	57.10 ± 0.03^{a}
9	84.78 ± 0.00^{ef}

[a] Different lowercase letters indicate significant difference ($p < 0.05$).

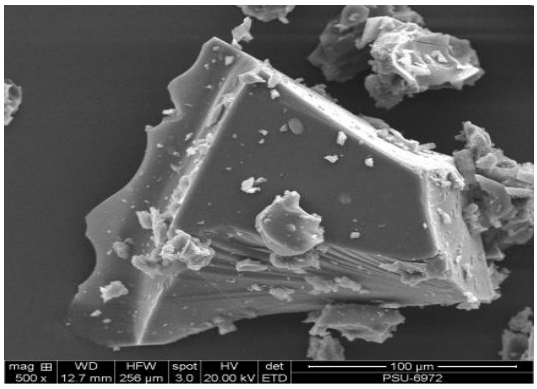
4.4.2 Structure and surface characteristics of dry phage powder

Figure 4.2 shows the microscopic images of dry phage powder. In dry phage powder sample from WPI alone (Figure 4.2−A), rough surface with pores and cracks of various sizes was observed. By using trehalose alone (Figure 4.2−B), dry phage powder showed smooth crystal surface as present in a normal form of saccharides. The phage powder made from a combination of WPI and trehalose showed the smaller pore size and less cracks (Figure 4.2−C) as compared to powder made from only WPI. The surface of the powder was smooth and more compact as WPI and trehalose are soluble and can form a thin layer after the water evaporates. Once a layer is formed, a crust can form under this layer which causes some roughness on the surface due to loss of water during the drying process. Our microencapsulated phage formulation showed similar morphology though SEM with spray-freeze dried WPI/fructooligosaccharide (FOS) (Rajam and Anandharamakrishnan, 2015), indicating the compacted structure of protein and saccharide.

(A)



(B)



(C)

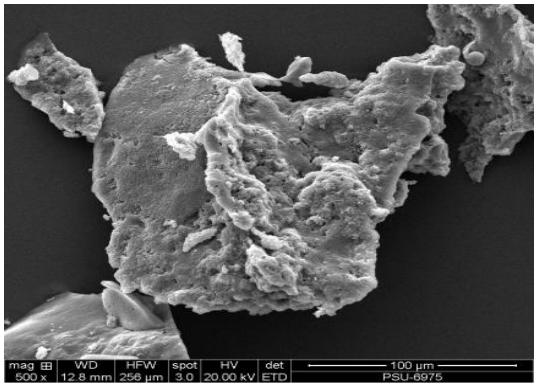


Figure 4.2. SEM images of dry phage powder samples prepared from: (A) WPI alone, (B) trehalose alone and (C) optimal formulation. Magnification of 500x.

4.4.3 Infrared spectra of phage powder

Infrared spectroscopy was used to evaluate preservation of phage proteins in dry phage powder form. Dry phage powder made of WPI alone showed typical bands of amide A (3300 cm^{-1}), amide B (3100 cm^{-1}), amide I (1650 cm^{-1}), amide II (1550 cm^{-1}), amide III (1200−1300 cm^{-1}) and amide VI (620−700 cm^{-1}) (Figure 4.3), these bands were reported by a previous study (Garidel *et al.*, 2006). Distinct bands at 980 cm^{-1} and 900 cm^{-1} were observed in dry phage powder made from trehalose alone. Bands at 983 cm^{-1} and 931

cm^{-1} may correspond to the two vibrational modes (antisymmetric and symmetric stretching) of α-(1↔1)-glycosidic bond or C−O−C vibration (Akao *et al.*, 2001). Moreover, this formulation showed a specific band at 3400 cm^{-1} which is associated with the H−O−H bending motion (Akao *et al.,* 2001). For dry phage powder made from an optimal formulation using a combination of WPI and trehalose, expected bands at 3300−3500 cm^{-1} and 1750−1800 cm^{-1} were observed. The stretching of these bands might be representative of interaction between WPI and trehalose as area at 3300−3500 cm^{-1} is associated with the O−H stretching vibration of the H−bonding (Akao *et al.,* 2001) and area at 1750−1800 cm^{-1} is associated with H−bonding between sugar−protein (Wang *et al.,* 2009). In a previous study, the interaction between saccharide and protein-based material has been studied. The H−bond interaction between the carboxyl group of sugar and the amide group of protein after freeze-drying was observed in dried lysozyme with sugar (Carpenter *et al.,* 1989). Similar interaction could be speculated in our study regarding the interaction of the carboxyl group of trehalose and the amide group of WPI by H−bonding in a mixture system.

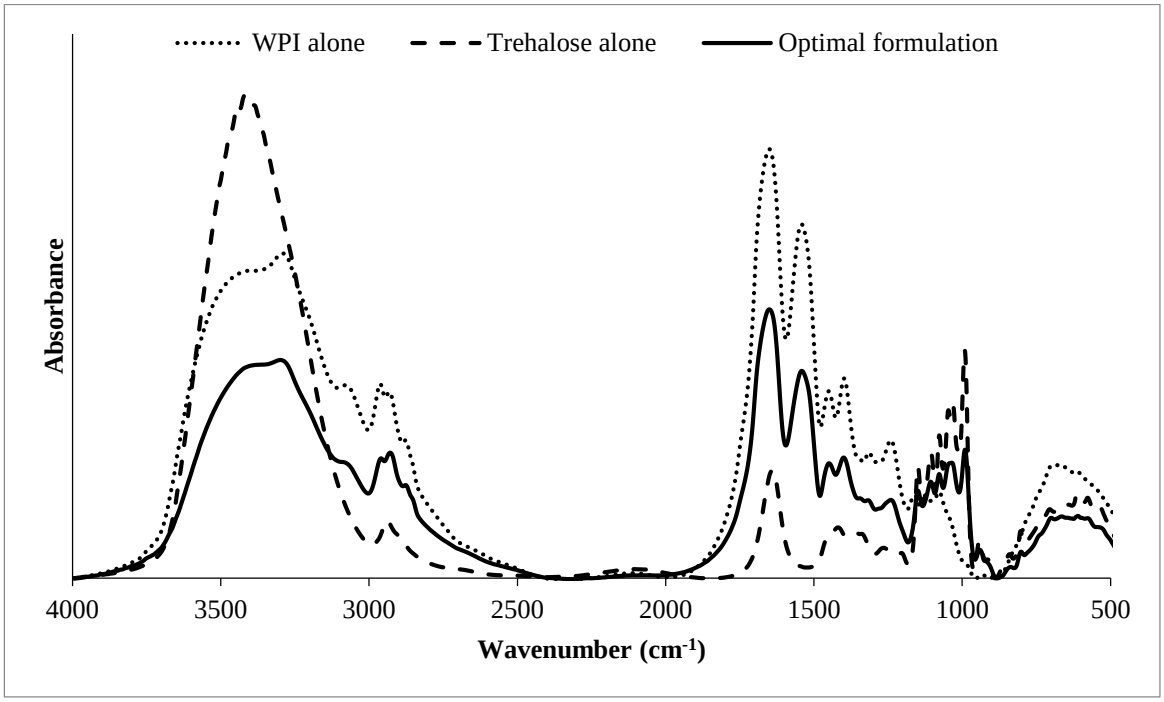


Figure 4.3. Fourier transform infrared spectra of dry phage powder prepared from different formulations.

4.4.4 Glass transition temperatures of dry phage powder

The stability of dry phage powder of three formulations was presented by the glass transition temperatures (T_g). In this study, T_g values of dry phage powder made from WPI alone and trehalose alone were found at 54.08°C and 95.39°C, respectively (Figure 4.4). Dry phage powder made of the optimal formulation with WPI and trehalose showed T_g value of 63.43°C. A previous study has reported T_g of non- and crystallized whey powder at various % relative humidity. Non-crystallized whey powder showed T_g at 51 ± 1 to 21 ± 2°C, while crystallized whey powder showed T_g at 44 ± 1 to 2 ± 1°C when tested at 11-39% of relative humidity (Schuck *et al.*, 2005). The T_g of pure dry trehalose was found to be 106°C (Roe and Labuza, 2005). Overall, WPI/trehalose combination for phage powder suggests an improved T_g as compared to the control formulation of WPI alone. This can prevent the glassy state of materials and reduce crystallization rates, thus providing a longer shelf-life of the encapsulated phage powder during storage. The T_g of formulation is a faction of the relative proportion of glass-forming components and moisture content (Duddu and Dal Monte, 1997). Duddu and Dal Monte (1997) reported that sucrose formulation (T_g ~ 59°C) was found to undergo significant aggregation when stored at 60°C. While, trehalose formulation (T_g ~ 80°C) was stable at the same temperature. Data suggest that during the shelf-life, the formulation should exist in the glassy state in order to avoid the aggregation of formulation. Moreover they found that increasing of protein/sugar ratio could increase the T_gs of the formulation containing sucrose and trehalose (Duddu and Dal Monte, 1997).

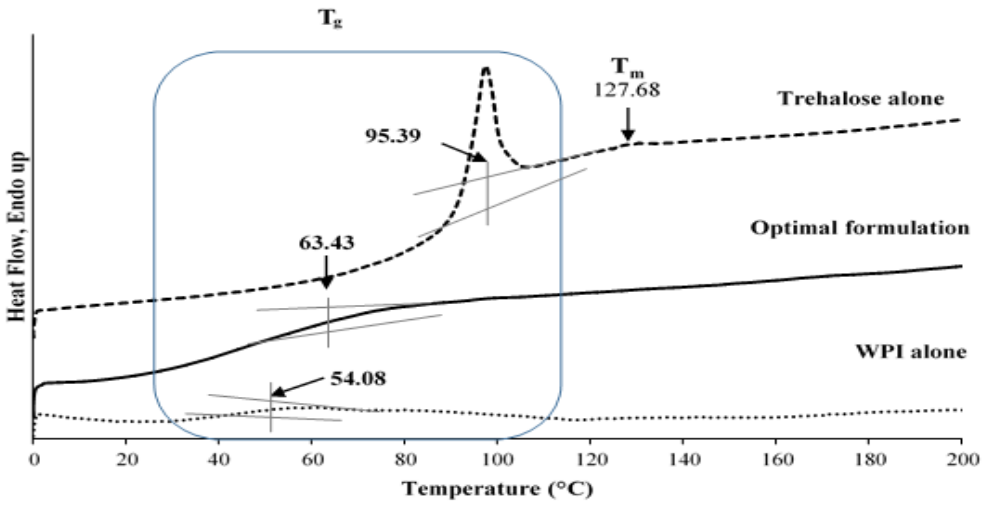


Figure 4.4. Differential scanning calorimetry analysis of dry phage powder prepared from different formulations.

4.4.5 Stability of microencapsulated phage at various temperatures and pH values

The stability in the solution at various temperatures (4°C, 25°C and 50°C) (Figure 4.5) and pH levels (1.5, 3.5, 5.5, 7.5 and 9.5) (Figure 4.6) of dry phage powder with optimal formulation developed in this study was evaluated as compared to dry phage powder with WPI alone, trehalose alone and also non-encapsulated phage. At 4°C, the optimal formulation, WPI alone and trehalose alone could protect phage particles in the solutions up to 95-100% for 24 h, while the non-encapsulated phage could survive up to 100% at this temperature. At 25°C, the optimal formulation, WPI alone and trehalose alone could protect phage particles in the solutions up to 100% for 24 h, while the non-encapsulated phage could survive up to 98% at this temperature. At 50°C, the optimal formulation, WPI alone and trehalose alone could protect phage particles in the solutions up to 100% for 24 h, while the non-encapsulated phage could survive for 63% for 1 h. For the study of stability in various pH levels, at pH 1.5, the optimal formulation and WPI alone showed the desirable properties which could protect phage particles up to 90% after 5 h of exposure. While, trehalose alone could not protect phage particles from this acidic condition. Similary, the non-encapsulated phage showed no survival after 1 of exposure. At pH 3.5, the optimal formulation, WPI and trehalose alone could protect phage particles up to 90% after 5 h of exposure, while the non-encapsulated phage remained the titer of approximately 85% after 3 h of exposure. At pH 5.5, the optimal formulation, WPI and trehalose alone could protect phage particles up to 95% after 5 h of exposure, while the non-encapsulated phage remained the titer of approximately 73% after 5 h. At pH 9.5, the optimal formulation, WPI and trehalose alone could protect phage particles up to 90% after 5 h of exposure, while the non-encapsulated phage remained the titer of approximately 86% after 3 h and no phage particles could survived after 5 h of exposure.

Our finding is in agreement with several previous studies which suggested the application of encapsulated phage. Encapsulation has been reported as the technique which improve phage stability in the harsh conditions (Vonasek *et al*., 2014; Ma *et al*., 2010). A previous study reported that encapsulated phage by whey protein as edible films could be stabilized at both ambient (22°C) and refrigerated (4°C) temperatures more than one month (Vonasek *et al*., 2014). Our data also present similar results to a previous study which reported that encapsulated *Salmonella* phages Felix O1 in the system of chitosan-alginate showed a high survival rate after exposure to simulated gastric fluid (pH 2.4) for 90 mins of incubation at 37°C (Ma *et al*., 2008). Overall, our encapsulated phage could

remain in a system over various temperatures and wide range of pH. Findings here suggest a potential use of this newly developed encapsulated phage powder in food application presenting various pH or in environments with lower pH.

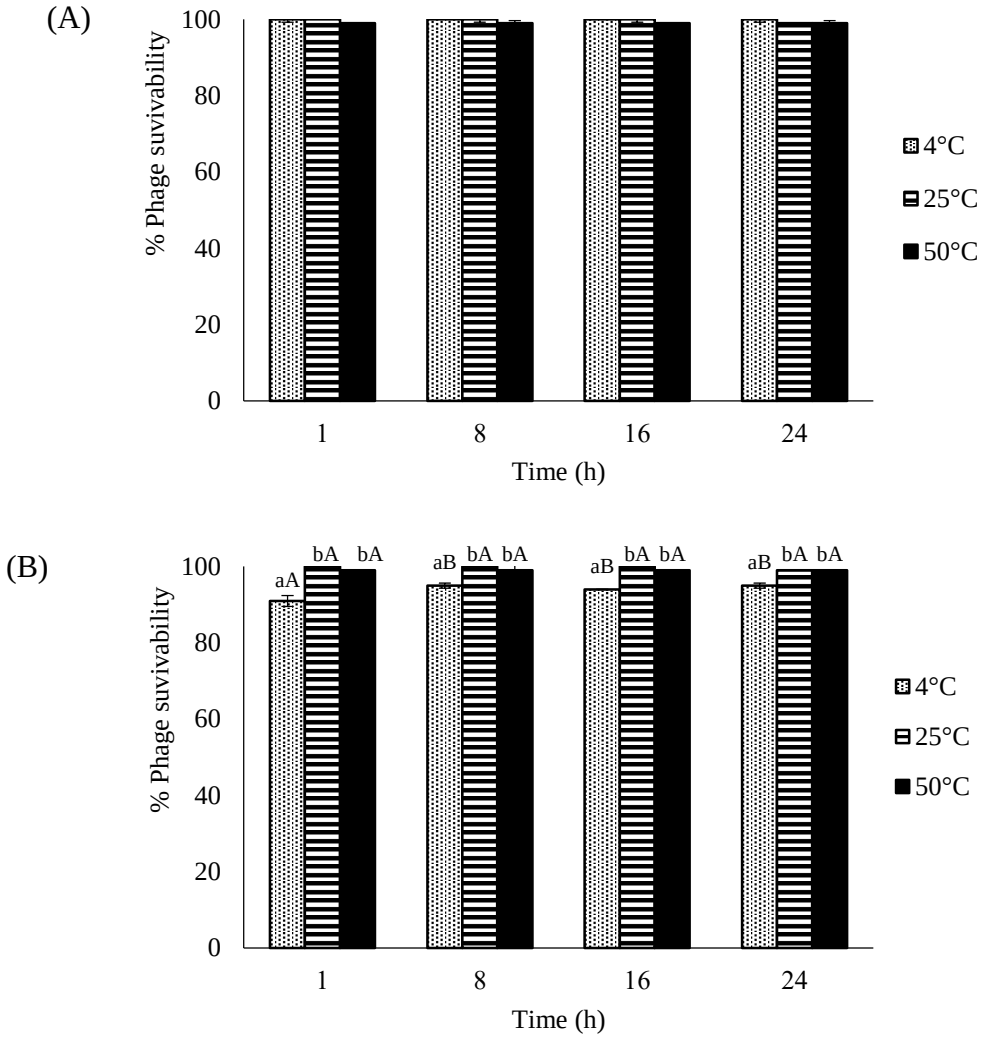


Figure 4.5. Stability and solubility of dry phage powder at 4°C, room temperature (25°C) and 50°C, for 1, 8, 16 and 24 h. Different formulations: (A) WPI alone, (B) trehalose alone, (C) optimal formulation and (D) non-encapsulated phage. Bars represent the mean standard deviation (n = 3). Different uppercase letters on the bars within the same temperature indicate significant differences ($p < 0.05$) between periods of time. Different lowercase letters on the bars within the same time period indicate significant differences ($p < 0.05$) between temperatures. Non-significant differences between temperature and time period of (B) and (C) ($p > 0.05$).

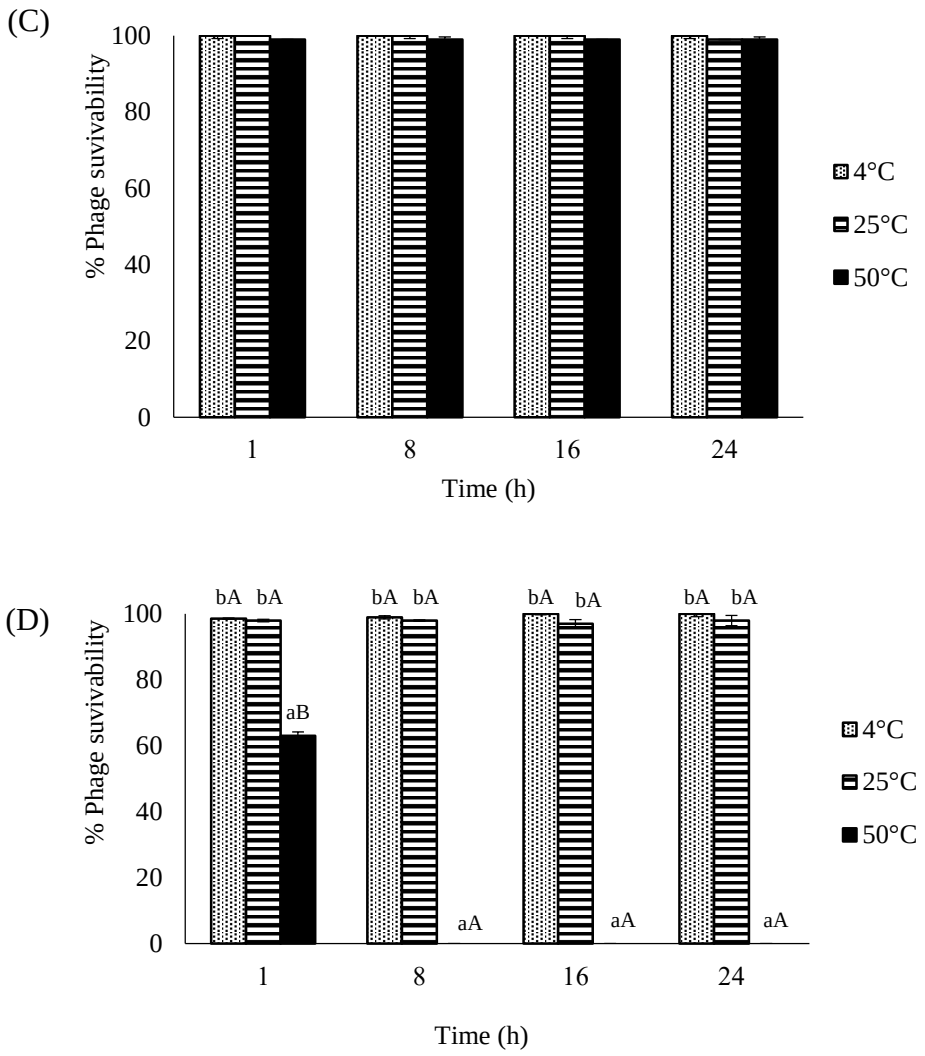


Figure 4.5. Stability and solubility of dry phage powder at 4°C, room temperature (25°C) and 50°C, for 1, 8, 16 and 24 h. Different formulations: (A) WPI alone, (B) trehalose alone, (C) optimal formulation and (D) non-encapsulated phage. Bars represent the mean standard deviation (n = 3). Different uppercase letters on the bars within the same temperature indicate significant differences ($p < 0.05$) between periods of time. Different lowercase letters on the bars within the same time period indicate significant differences ($p < 0.05$) between temperatures. Non-significant differences between temperature and time period of (B) and (C) ($p > 0.05$).

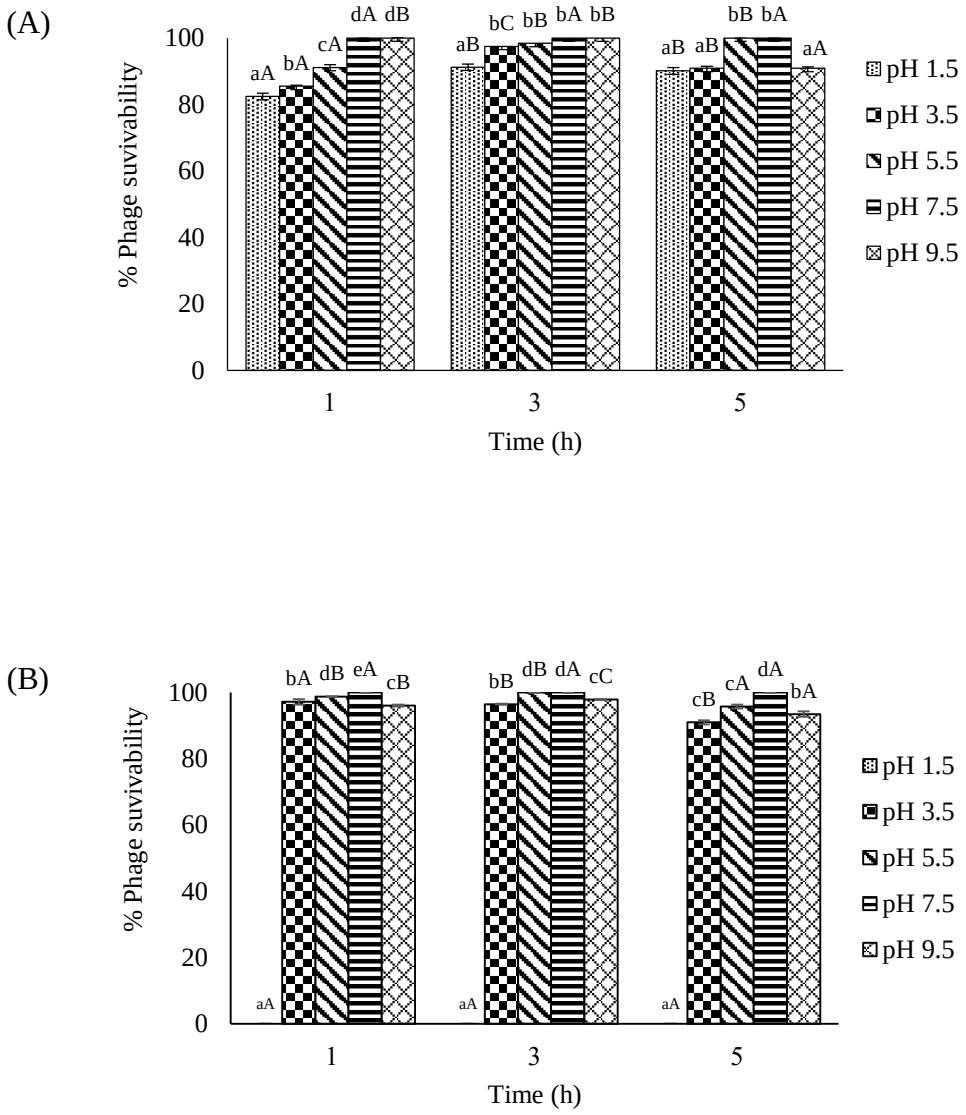


Figure 4.6. Stability of dry phage powder at pH 1.5, 3.5, 5.5, 7.5 and 9.5 for 1, 3 and 5 h. Different formulations: (A) WPI alone, (B) trehalose alone, (C) optimal formulation and (D) non-encapsulated phage. Bars represent the mean standard deviation (n = 3). Different uppercase letters on the bars within the same pH indicate significant differences ($p < 0.05$) between periods of time. Different lowercase letters on the bars within the same time period indicate significant differences ($p < 0.05$) between levels of pH.

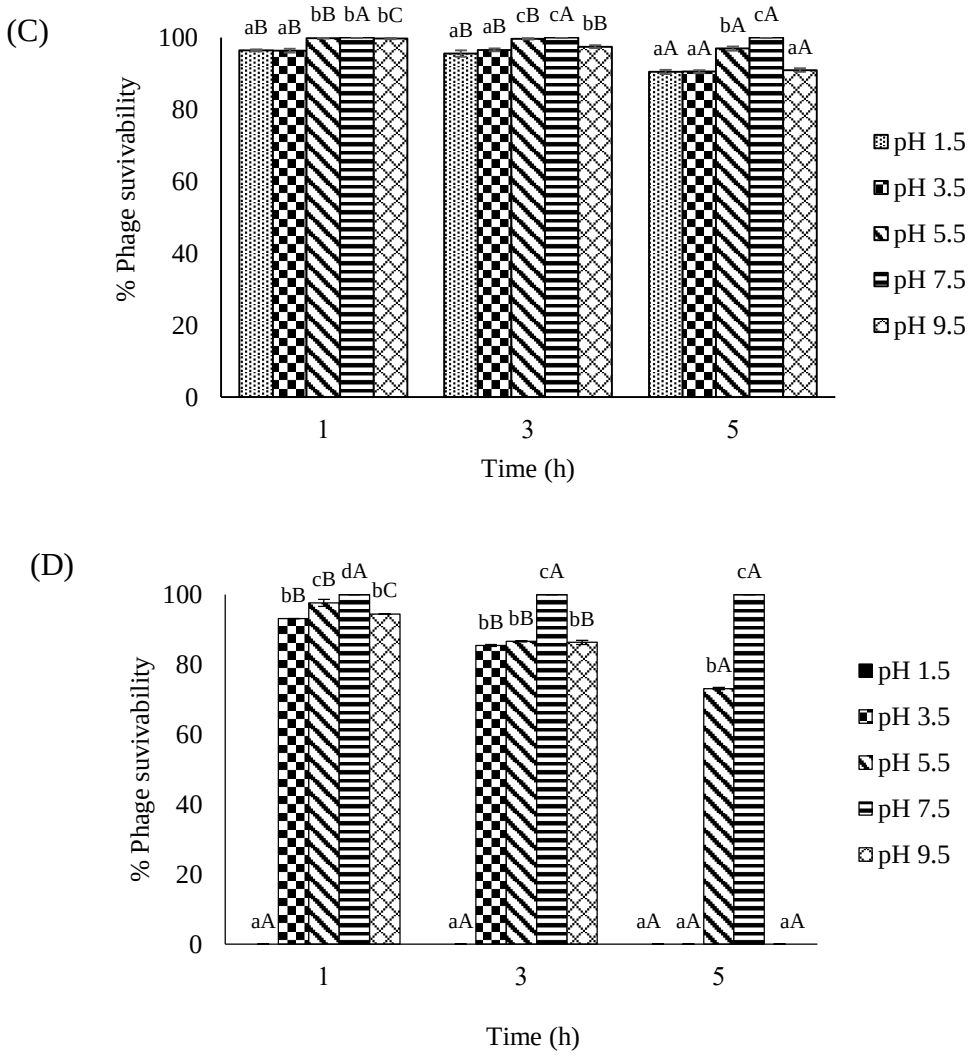


Figure 4.6. Stability of dry phage powder at pH 1.5, 3.5, 5.5, 7.5 and 9.5 for 1, 3 and 5 h. Different formulations: (A) WPI alone, (B) trehalose alone, (C) optimal formulation and (D) non-encapsulated phage. Bars represent the mean standard deviation (n = 3). Different uppercase letters on the bars within the same pH indicate significant differences ($p < 0.05$) between periods of time. Different lowercase letters on the bars within the same time period indicate significant differences ($p < 0.05$) between levels of pH.

4.4.6 Efficacy of microencapsulated phage against *S.* Enteritidis and *S.* Typhimurium *in-vitro*

Efficacy of dry phage powder made from the optimal formulation using WPI/trehalose combination against *S.* Enteritidis and *S.* Typhimurium *in-vitro* at 10°C and 30°C was investigated (Figure 4.7). At 10°C, the cell numbers of *S.* Enteritidis were decreased by approximately 1 log CFU/mL for the MOI 100 within 24 h. Similarly, the cell numbers of *S.* Typhimurium were decreased by approximately 1 log CFU/mL within 24 h for both MOIs (100 and 1). At 30°C, the cell numbers of both *S.* Enteritidis and *S.* Typhimurium were decreased by approximately 1 log CFU/mL within 24 h for both MOIs. This study is the first study that introduces a new form of phage-based bio-control as a powder (lysate-free form) for use to control a bacterial pathogen. The MOIs included in this study that were lower as compared to those used in previous studies may have limited the overall cell reductions. Previous studies have been investigated the efficacy of *Salmonella* phage lysate in the reduction of *S.* Enteritidis and *S.* Typhimurium at various temperatures. Spricigo *et al*. (2013) reported the decrease of *S.* Enteritidis and *S.* Typhimurium in processed food (packaged lettuce) by 2.2 and 3.9 log CFU/g, respectively from phage cocktail treatment at the MOI of 104 at room temperature. On chicken breast treated with a phage cocktail at MOI of 1000 at 4 °C, up to 2.2 log CFU/g reduction was observed (Spricigo *et al.,* 2013). Zinno *et al*. (2014) reported a reduction of *S.* Typhimurium in liquid food samples treated by phage P22 at MOI 10000 by up to 4 log units reduction after 48 h at 4°C (Zinno *et al.,* 2014). Overall, results indicated that dry phage powder made from the optimal formulation using WPI/trehalose combination could reduce *S.* Enteritidis and *S.* Typhimurium.

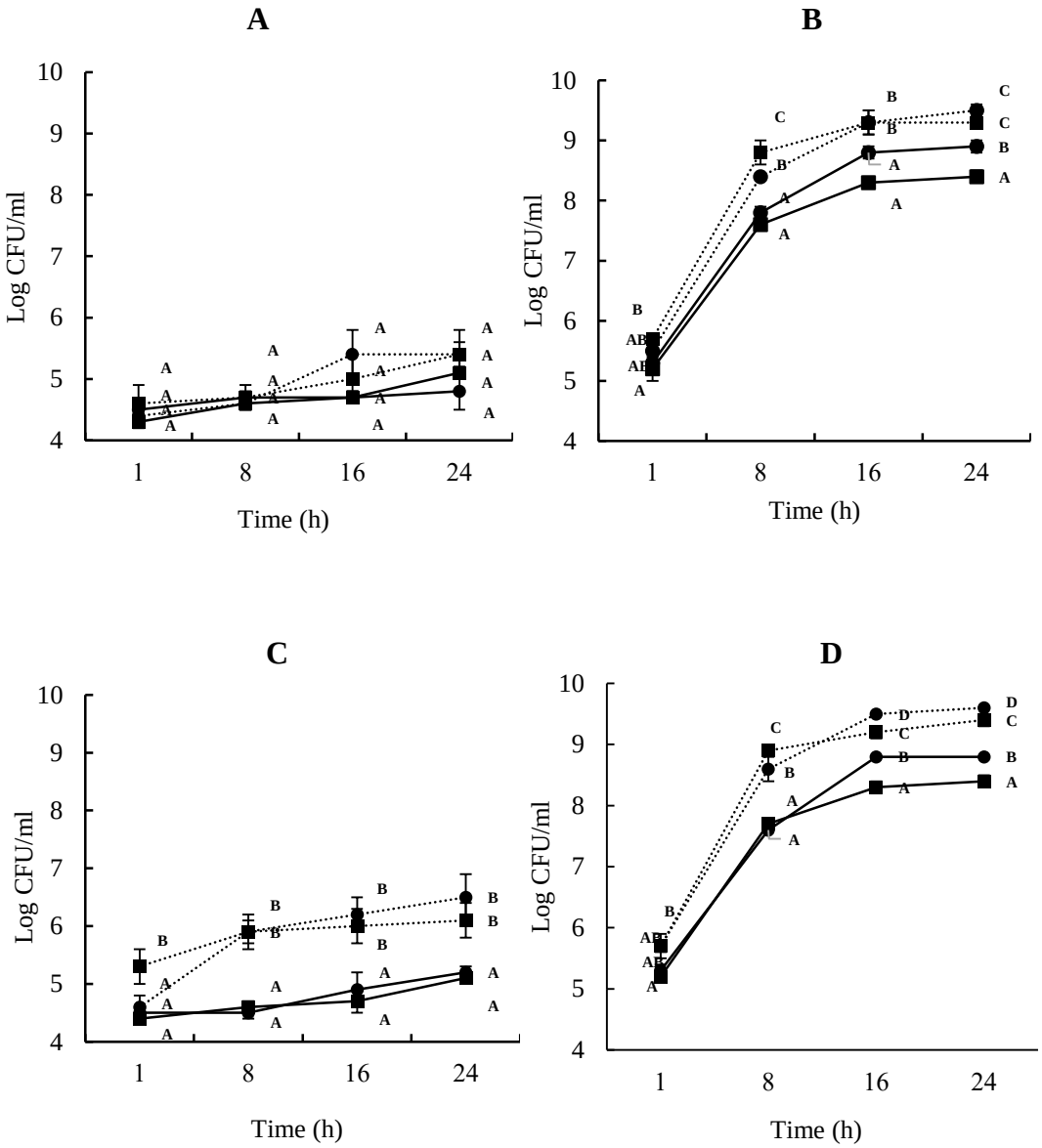


Figure 4.7. Efficiency of dry phage powder from the optimal formulation against *S.* Enteritidis at 10°C (A) and 30°C (B) and *S.* Typhimurium at 10°C (C) and 30°C (D). MOIs include: —●— (MOI 100) and —■— (MOI 10). Controls include: ···●··· (control of MOI 100) and ···■··· (control of MOI 10). Bars represent the mean standard deviation (n=3). Different uppercase letters on the time point within the same periods of time indicated significant differences ($p < 0.05$) between each MOI and control.

4.5 Conclusion

The combination of WPI/trehalose at 3:1 (w/w) showed the optimal formulation which provided the highest phage survivability in the powder form after freeze-drying in this study. Microstructure of the dry phage powder showed the complex particles of both wall materials, which showed the binding with H−bond as presented by FTIR analysis. The result from DSC analysis showed that the dry phage powder presented T_g at 63.43°C which is the indication of its physical stability over room temperature. Moreover, this newly developed phage powder formulation could protect phage particles from a wide range of pH conditions and showed the efficiency to reduce *S.* Enteritidis and *S.* Typhimurium by approximately 1 log unit. Finding suggests that dry phage powder can be further conveniently applied in various food products that represent various pH values in order to control *Salmonella* contamination. These results highlight the newly developed formulation of dry phage powder as a bio-control agent against *Salmonella* contamination in the food production chain.

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CHAPTER 5

EVALUATION OF STABILITY, STORAGE CONDITIIONS AND PHYSIO-CHEMICAL PROPERTIES OF ENCAPSULATED PHAGE COCKTAIL

5.1 Abstract

Application of phages has gained more interest in food industry in recent years. Although, phage as traditional form (liquid form) has the high potential to control target hosts, new form of phages can provide improved phage characteristics and applications. Microencapsulation is a technique which can improve the application of phage-based bio-control. In this study, our previously developed phage cocktail was transformed to be dry powder by using the optimal formulation which has been optimized. However, other aspects of the newly form of microencapsulated phage including packaging, conditions for storage and its stability still needed to be studied. In order to evaluate the suitable packaging, phage cocktail powder was kept in different materials of zip-locked bags (aluminium laminated foil; LF and low density polyethylene; LDPE). Survivability of phage cocktail as dry powder in different packaging and the traditional lysate form (kept in the conical tube) stored at 4°C and 25°C was evaluated for 12 weeks. The change of physical (color) and chemical (a_w) properties of phage cocktail powder were also evaluated. Survivability studies indicated that 4°C provided the higher survivability of phage cocktail in both dry powder and lysate forms as compared to 25°C. Phage cocktail powder kept in LF at 4°C showed the highest survivability with a minor loss in titer of 0.5 log PFU/g after 12 weeks of storage. Titer loss of phage cocktail lysate was as high as 4 log PFU/mL after 12 weeks kept at 4°C and a total phage loss of the lysate was observed after 5 weeks kept at 25°C. Change in color (ΔE value) of phage cocktail powder in LF at 4°C did not show any significant difference during storage, and a_w (0.13) was found to be lowest in these conditions. The data indicate that our developed phage cocktail powder provides the longer shelf-life as compare to lysate form. We suggest to storage our phage cocktail powder in LF material and store at low temperature (4°C). Under these conditions phage cocktail powder could maintain its desirable stability in term of food quality and food safety.

5.2 Introduction

The efficiency of bacteriophage as liquid form (lysate) combats *Salmonella* spp. has been reported by several previous studies (Spricigo *et al.,* 2013; Álvarez and Biosca, 2017; Ramirez *et al.,* 2018). However, using phages in this lysate form could decrease their efficacy as by several limitations, for example, short self-life, and weight associated with more space required for storage and transportation. The rapidly decrease of phage titer as lysate form has been reported (Malik *et al*., 2017; Merabishvili *et al*., 2013). Therefore, the new form of phage is needed to develop. Although, the efficiency of phage as bio-control has been approved (Petsong and Vongkamjan, 2015; FDA, 2015). The effect of food additive on the quality and safety of food should be concern. Color and water activity (a_w) are the major factors which influence to the sensory acceptability and microbial growth, respectively (Clydesdale, 1990; Langová *et al.,* 2012). Microencapsulated form of phages as a dry powder is more desirable for applications in various food products from farms to industries while maintaining quality and safety of the food products. Microencapsulated phages have been reported to confer long-term period of storage up to 21 years (Ackermann *et al.,* 2004) and showed high efficiency for controlling bacterial pathogens (Dini *et al*. 2012; Ma *et al.,* 2012; Saez *et al.,* 2011). In order to maintain the properties especially physical and chemical of product, the packaging is the important factor which is needed to be focused on. Low density polyethylene (LDPE) is the general packaging material which is common use with the low cost. LDPE has the properties as very good in acid/alkali and alcohol resistance, moderate in oil, and greases resistance, and quite poor as gases barrier (Emblem, 2012). Aluminium laminated foil bag presented the wide application in food packaging. With the desirable barrier function against the migration of moisture, oxygen and volatile aroma, impact of light, and provide shelf-life stability of food (Lamberti and Escher, 2007). This chapter evaluated stability of the developed microencapsulated phage cocktail as dry powder stored in two major packaging material types (aluminium laminated foil; LF and low density polyethylene; LDPE bag) stored at 4°C (refrigerator temperature) and 25°C (room temperature) for 12 weeks. Survivability of phage cocktail powder kept in different packaging and phage cocktail lysate at 4°C and 25°C was compared. Physical (color) and chemical properties (a_w) of phage cocktail powder during storage time were also evaluated.

5.3 Materials and methods

5.3.1 Development of encapsulated phage cocktail

In this study, a phage cocktail preparation consisted of approximately 10^8 PFU/mL of phages KP4, KP5 and KP50 was prepared using a ratio of 1:1:1 for each phage. Titer of the phage cocktail was confirmed as 10^9 PFU/mL on *S.* Enteritidis (FSL S5-371). The formula for phage encapsulation previously optimized in 4.3.3 was used and up-scale. Phage cocktail lysate was mixed with the coating materials consisting of whey protein isolate (WPI) and Trehalose at ratio of 3:1(w/w) to achieve a final concentration of 10% total solid (200 g). After homogenization for 1 h, a phage cocktail lysate (10^9 PFU/mL) was added to achieve 10% (v/v) of the final volume of mixture of coating agents. The mixture was solidified under -40°C for 24 h. Frozen mixture was tempered at -50°C using a laboratory scale freeze-dryer for 48 h. Size of freeze-dried samples was sorted through 100 mesh sieve, in order to achieve the dry powder with approximately 150 µm in size. Phages titer after freeze-drying were enumerated by the plaque assay following a procedure mentioned in 4.3.4.1.

5.3.2 Stability of phage cocktail powder during storage

Stability of phage cocktail powder was investigated during a storage for 12 weeks. Two packaging types, aluminium laminated foil bag (LF) and low density polyethylene (LDPE) bags were used to keep the phage cocktail powder. Phage cocktail lysate kept in a conical tube was used to compare with phage cocktail powder. Both forms were stored under refrigeration condition (4°C) and at room temperature (25°C). Samples were taken weekly for 12 weeks in order to evaluate the properties, including survivability, change in color and water activity (a_w). For survivability, 1 mL of the phage cocktail lysate or 1 g of phage cocktail powder was taken for enumeration by the plaque assay as mentioned above. For phage cocktail dry powder, 10 mL of PBS was added into 1 g of samples before phage enumeration.

For color evaluation, samples were evaluated by the CIE L*a*b* system. The instrument was standardized using a standard white plate. CIE L* (lightness), a* (redness/greenness), b* (yellowness/blueness) were determined using a Hunter Lab Miniscan colorimeter (HunterLab Reston, VA, USA). A numerical total color

difference (ΔE*) was calculated by: $\Delta E^* = [(L^* - L^*ref)^2 + (a^* - a^*ref)^2 + (b^* - b^*ref)^2]^{1/2}$. The control sample (sample at week 0) was used as reference value (Francis and Clydesdale, 1975). Water activity of phage cocktail powder was measured by Aqua-Lab Water Activity Meter (Series 3, Decagon Devices, Inc., Pullman, WA) at 25°C.

5.3.3 Statistical analysis

Statistical analysis was analyzed for the experiments, including color and a_w of phage cocktail powder during storage in different packaging materials (LF and LDPE) and different temperatures (4°C and 25°C). The differences between ΔE values of phage cocktail powder in different packaging types and temperatures were analyzed by Student's t-test. Analysis of variance (ANOVA) was used to compare results among each week of storage. Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p < 0.05$ using the statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Survivability of encapsulated phage cocktail and phage cocktail lysate during storage at low temperature (4°C) and room temperature (25°C)

By applying the optimal formulation according to 4.3.3 for preparation of phage powder, phage survivability after freeze-drying of a scaled-up production (200 g/batch) showed 99.9% of phage survival. Data suggest that this formulation provides high encapsulation efficiency even in larger scale of production.

Phage cocktail in a traditional lysate form and a novel form developed as a dry powder via freeze-drying were compared for the rate of phage survival during 12 weeks of storage at low temperature (4°C) and room temperature (25°C). Phage cocktail powder showed stable phage titer with a slight loss of phage titer (approximately 0.5 log PFU/mL) during 12 weeks at 4°C (Figure 5.1). At 25°C, total phage loss in a phage cocktail powder was observed after 7 weeks. For phage cocktail lysate, a decrease of approximately 1 log PFU/mL was observed after 5 weeks and 4 log PFU/mL after 12 weeks at 4°C. Whereas, this phage lysate form lost all titer after 4 weeks of storage at 25°C. Generally, the phage titers in the lysate form have been

shown to drop rapidly (Malik *et al.,* 2017; Merabishvili *et al.,* 2013). However, microencapsulation has been suggested to improve phage survival during processing and storage. Similar to our finding, microencapsulated *Staphylococcus* phage in a alginate bead form could maintain phage titers for over 3 months at 4°C (El Haddad *et al.,* 2018). Phage powder could extend the stability for a long-term storage of up to 21 years (Ackermann *et al.,* 2004). Storage at room temperature (25°C) could cause thermal instability which may disturb the embedded phages structure. Vandenheuvel *et al*., 2014 have previously reported high stability of encapsulated phage as a dry powder at low temperatures (4°C) and relative humidity of 0%. However, high humidity could cause crystallization of the amorphous matrix which could destroy the encapsulated phages (Vandenheuvel *et al.,* 2014). Overall, a novel form of encapsulated phage cocktail in this study can provide an improved form for a long-term storage for phages as compared to the traditional phage lysate form. In addition, storage at low temperature (4°C) indicates an optimal condition for storage the phage cocktail in both dry powder and lysate forms.

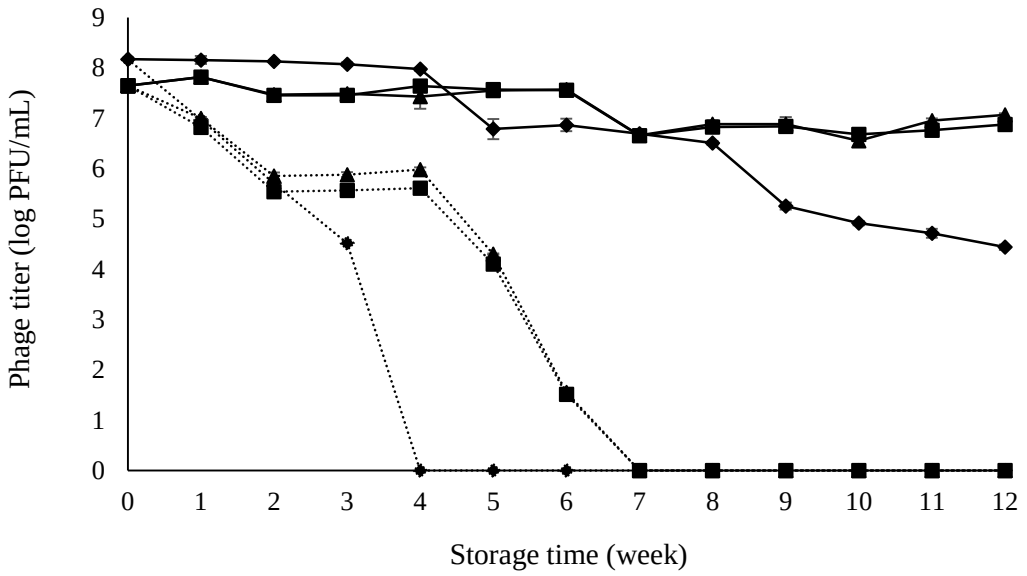

Figure 5.1. Phage cocktail survivability in the dry powder form and lysate form during storage. Phage cocktail powder form kept in aluminium laminated foil bag; LF at 4°C (—▲—), low density polyethylene; LDPE bag at 4°C (—■—), LF at 25°C (···▲···) and LDPE bag at 25°C (···■···). Phage cocktail lysate kept in conical tube at 4°C (—◆—), and 25°C (···✦···). Bars represents the mean standard deviation (n=3).

5.4.2 Evaluation of physical and chemical properties of encapsulated phage cocktail in various packaging types and temperatures

For storage at 4°C and 25°C, phage cocktail powder in both packaging types, LF and LDPE bags, showed similar phage titer during 12 weeks of storage (Figure 5.1). Evaluation of the stability of physical property of phage cocktail powder showed that color (ΔE values) of phage cocktail powder was not significantly different when kept in neither LF nor LDPE bag at 4°C or 25°C on a given week of sample evaluation (Table 5.1). When compared ΔE values of phage cocktail powder kept in LDPE bag, values with significant difference were observed between at 4°C and 25°C. However, ΔE values showed non-significant difference for phage cocktail powder kept in LF bag at both temperature conditions. A minor change in color provides a desirable quality of phage cocktail powder in storage condition. For evaluation of the chemical property, the initial a_w of phage cocktail powder was 0.3 (Table 5.2). After 4 weeks of storage, a_w of phage cocktail powder kept in both LDPE bag and LF bag stored at 4°C increased to 0.06 and 0.08, respectively. After 12 weeks at 4°C, a_w were 0.3 and 0.13 for phage cocktail powder kept in LDPE bag and LF bag, respectively. In this study, at 25°C, a_w of phage cocktail powder increased continuously. The a_w of phage cocktail powder contained in LF bag was 0.32 which was lower than that kept in LDPE bag with a_w of 0.43. Overall, phage cocktail powder stored in both packaging types at both 4°C and 25°C showed a_w less than 0.6 during a storage time of 3 months. This low a_w indicates the safety of this dry form of phage cocktail from microbial proliferation including bacteria, yeast, and mold during storage time (Fennema, 1996). Color is one of the most important characteristic which related to food quality and effects consumer judgment of other sensory characteristics (Clydesdale, 1991; Titova *et al.,* 2015). The a_w is now regarded as one of the most important indicators of food quality and safety. This parameter indicates water availability for physic-chemical stability of low moisture products and growth of microorganisms. The less a_w can inhibit the onset of undesirable reactions such as lipid oxidation and microbial growth (Langová *et al.*, 2012).

This study compared the physio-chemical properties of phage cocktail powder in 2 types of packaging material (LF and LDPE bag) kept at 4°C and 25°C. Our results indicate that LF bag could maintain the color of phage cocktail powder at

4°C and 25°C during 12 weeks of storage. However, our findings suggest that a_w of phage cocktail powder relies upon the storage temperature rather than the packaging type. At 4°C, a_w of phage cocktail powder showed a slight increase compared to that kept at 25°C for both LF and LDPE bags. In comparison, LF bag has higher ability to protect the contained material from light and moisture effect to the physico-chemical changes of contained material compared to LDPE. This study suggests LF bag as the most suitable packaging for storage phage cocktail powder at refrigeration temperature for maintaining the physio-chemical properties of this product.

Table 5.1. Color evaluation (ΔE) of phage cocktail powder kept in aluminium laminated foil bag (LF) and low density polyethylene bag (LDPE) and different temperature during storage.

Week	ΔE at 4°C		ΔE at 25°C	
	LF	LDPE	LF	LDPE
1	5.85 ± 0.10	6.24 ± 0.55^{bc}	6.31 ± 0.62	7.25 ± 0.11^{a}
2	6.97 ± 0.92	5.27 ± 0.17^{ab}	7.33 ± 0.68	6.79 ± 0.96^{ab}
3	6.20 ± 0.46	7.68 ± 0.68^{d}	6.74 ± 0.42	8.00 ± 0.85^{bc}
4	6.89 ± 0.24	5.86 ± 0.31^{b}	7.20 ± 0.12	6.80 ± 0.63^{ab}
5	6.11 ± 0.66	7.06 ± 0.92^{cd}	7.19 ± 0.03	8.37 ± 0.91^{c}
6	6.74 ± 0.52	7.19 ± 0.18^{cd}	7.04 ± 0.18	6.91 ± 0.31^{ab}
7	6.77 ± 2.59	9.76 ± 1.47^{e}	6.97 ± 0.35	8.64 ± 0.70^{c}
8	5.23 ± 0.28	5.08 ± 0.16^{ab}	ND	ND
9	5.64 ± 0.18	4.48 ± 0.79^{a}	ND	ND
10	6.41 ± 0.72	5.59 ± 0.33^{ab}	ND	ND
11	6.17 ± 0.44	5.75 ± 0.29^{b}	ND	ND
12	5.64 ± 0.18	4.48 ± 0.79^{a}	ND	ND

All values indicate mean ± standard deviation. Different letters indicated significant differences ($p < 0.05$) between ΔE of sample kept in LDPE at each temperature during storage. Non- differences ($p < 0.05$) between ΔE of sample kept in LF at each temperature during storage, sample kept in the same temperature (different packaging)

and kept in same packaging (different temperature). ND (not detected) due to the complete loss in phage titer at 25°C since week 8 (see figure 5.1)

Table 5.2. Water activity (a_w) of phage cocktail powder kept in aluminium laminated foil bag (LF) and low density polyethylene bag (LDPE) and different temperatures during storage.

Week	a_w at 4°C		a_w at 25°C	
	LF	LDPE	LF	LDPE
0	$0.03 \pm 0.00_{A}$	$0.03 \pm 0.00_{A}$	$0.03 \pm 0.00_{A}$	$0.03 \pm 0.00_{A}$
1	$0.05 \pm 0.00_{B}^{a}$	$0.08 \pm 0.00_{A}^{*a}$	$0.15 \pm 0.00_{B}^{b}$	$0.16 \pm 0.03_{B}^{*b}$
2	$0.06 \pm 0.00_{BC}^{a}$	$0.06 \pm 0.01_{A}$	$0.13 \pm 0.01_{B}^{b}$	$0.23 \pm 0.07_{B}^{*b}$
3	$0.07 \pm 0.00_{BC}^{*a}$	$0.06 \pm 0.01_{A}$	$0.13 \pm 0.02_{B}^{b}$	$0.35 \pm 0.06_{C}^{*b}$
4	$0.08 \pm 0.01_{BC}^{a}$	$0.06 \pm 0.01_{A}$	$0.15 \pm 0.00_{B}^{b}$	$0.34 \pm 0.00_{C}^{*b}$
5	$0.08 \pm 0.01_{BC}^{a}$	$0.15 \pm 0.01_{B}^{*a}$	$0.25 \pm 0.01_{C}^{b}$	$0.38 \pm 0.01_{C}^{*b}$
6	$0.08 \pm 0.00_{BC}^{a}$	$0.15 \pm 0.01_{B}^{*a}$	$0.38 \pm 0.03_{D}^{b}$	$0.40 \pm 0.06_{C}^{*b}$
7	$0.10 \pm 0.00_{C}^{a}$	$0.17 \pm 0.01_{B}^{*a}$	$0.32 \pm 0.01_{E}^{b}$	$0.43 \pm 0.05_{C}^{*b}$
8	$0.10 \pm 0.00_{CD}$	$0.18 \pm 0.04_{B}^{*}$	ND	ND
9	$0.11 \pm 0.02_{CD}$	$0.18 \pm 0.04_{B}^{*}$	ND	ND
10	$0.12 \pm 0.01_{CD}$	$0.21 \pm 0.05_{C}^{*}$	ND	ND
11	$0.12 \pm 0.01_{D}$	$0.30 \pm 0.04_{D}^{*}$	ND	ND
12	$0.13 \pm 0.01_{D}$	$0.33 \pm 0.00_{D}^{*}$	ND	ND

All value indicate mean ± standard deviation. Sign (*) on the standard deviation indicates significant differences ($p < 0.05$) between a_w of sample kept in the same temperature and different packaging. Different lowercase letters on the standard deviation indicate significant differences ($p < 0.05$) between a_w of sample kept in the same packaging type and different temperature. Different uppercase letters under standard deviation indicate significant differences ($p < 0.05$) between a_w of sample kept in the same condition during storage.

5.5 Conclusion

Our previously developed phage cocktail was used as a core and transformed to dry phage powder by encapsulation technique using the optimal formulation. A scaled-up production was successfully performed while high phage survivability was remained, indicating high encapsulation efficiency even in larger scale of production. Other aspects of the newly form of microencapsulated phage including packaging, conditions for storage and its stability were studied. Overall, our phage cocktail as dry powder form showed longer shelf-life as compared to the traditional lysate form. Low temperature (4°C) presented a better condition for storage our phage cocktail in both lysate and dry powder forms as compared to room temperature (25°C). Aluminium laminated foil bag provided the better properties to maintain the physio-chemical properties of our microencapsulated product compared to LDPE material. In summary, data here suggest suitable conditions to store our encapsulated phage cocktail powder while maintaining its stability and physio-chemical properties of for over 3 months. These aspects will be useful for further development of the encapsulated phage cocktail powder for other applications and commercialization.

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CHAPTER 6

EFFICIENCY AND APPLICATION OF ENCAPSULATED PHAGE COCKTAIL

6.1 Abstract

S. Enteritidis and *S.* Typhimurium are the most important serovars which often related to food contamination and foodborne illnesses. Application of phage as bio-control has gained wider of interest in the food industry. In this chapter, the efficiency of developed encapsulated phage cocktail against *S.* Enteritidis and *S.* Typhimurium *in-vitro* and in high risk foods, including chicken meat, sunflower sprout and animal feed was evaluated. *In-vitro* study, phage cocktail powder showed promising efficacy against *S* Enteritidis and *S.* Typhimurium *in-vitro* study as indicated by a reduction of 1.79 and 3.63 log CFU/mL, respectively at 37°C and 0.43 and 0.76 log CFU/mL, respectively at 10°C. In chicken meat, *S.* Enteritidis and *S.* Typhimurium were reduced by approximately 0.57 and 1.78 log CFU/cm^2, respectively. In sunflower sprout, *S.* Enteritidis and *S.* Typhimurium were reduced by approximately 0.86 and 1.2 CFU/g, respectively. In animal feed, *S.* Enteritidis and *S.* Typhimurium were reduced by approximately 1.92 and 1.74 log CFU/g, respectively. Sensory evaluation indicated that food models with/without phage cocktail powder showed non-significant differences in liking scores after 2 days of storage. Overall, the newly developed phage cocktail powder suggests another alternative for phage-based bio-control with improved the efficacy for food application.

6.2 Introduction

Salmonellosis, an infection caused by non-typhoidal *Salmonella* (NTS), has been reported as the major cause leading to high number of hospitalizations and deaths each year (Eng *et al.,* 2015). In 2016, over 94,530 confirmed salmonellosis cases were reported in the European countries (EFSA, 2017). Among over 2,600 serovars of *Salmonella*, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium are the top two most commonly reported serovars responsible for NTS infections worldwide (Eng *et al.,* 2015). *Salmonella* contamination in foods and outbreaks have been reported continuously each year (CDC, 2018).

Previous outbreaks in the United States, EU, Australia and Asia were often associated with *Salmonella* contamination in poultry meat and fresh produce such as sprout and fresh leafy greens (Ford *et al.*, 2016; Reddy *et al.*, 2016; Söderqvist, 2017; Vital *et al.*, 2014). *Salmonella* contamination in human mostly related to animal product through animal ingestion of *Salmonella* contaminated feed (Davies and Wales 2010). *Salmonella* contaminated in dry pet food caused 79 infected cases salmonellosis have been reported (FDA, 2013). The cases of animal feed contaminated *Salmonella* have been reported continuously (CDC, 2018). In addition, since the emergence of issue linked to antibiotic-resistant *Salmonella* found in food has been emphasized, control of *Salmonella* in foods is particularly needed to reduce to spread of *Salmonella* with this characteristics (Choe *et al.*, 2014; Hong *et al.*, 2016). Previously, phages have been evaluated for their efficiency as bio-control agents on foods in the form of the traditional phage suspension or lysate (liquid form) (Álvarez and Biosca, 2017; Ramirez *et al.,* 2018; Spricigo *et al.,* 2013).

However, using phages in this lysate form could decrease their efficacy as by several limitations. Our previous studies showed that a new form of phages as dry powder obtained from encapsulation technique could provide improved product characteristics for applications due to consistent physio-chemical properties. In addition, microencapsulated phages have been reported to confer long-term period of storage up to 21 years (Ackermann *et al.,* 2004) and showed high efficiency for controlling bacterial pathogens (Ma *et al.,* 2012). This study investigated the efficiency of phage cocktail powder in controlling *S.* Enteritidis and *S.* Typhimurium *in-vitro* and in food models, including raw chicken meat, sunflower sprout and animal feed. The overall acceptability of phage cocktail powder on foods and feed was evaluated by sensory analysis.

6.3 Materials and methods

6.3.1 *Salmonella* strains and phage cocktail used in this study

A total of four *Salmonella* strains (Table 6.1) and three phages (KP4, KP5 and KP50) were used in this chapter. *S.* Enteritidis and *S.* Typhimurium were used as the challenged hosts. *S.* Anatum was used as the propagate host of *Salmonella* phage KP4 and KP5. *S.* Agona H2-016 was used as the propagate host of *Salmonella* phage KP50. Phage cocktail preparation consisted of approximately 10^6 PFU/mL of each phage was prepared using a ratio of 1:1:1. Titer of the phage cocktail was confirmed as 10^7 PFU/mL on *S.* Enteritidis. For all experiments, overnight cultures were prepared following a procedure mentioned in 2.3.2.

Table 6.1. *Salmonella* strains used in this study.

Serovars	Strain ID	Source (Country)
Agona	H2-016	Pig slaughterhouse (Thailand)
Anatum	FSL A4-525	Bovine (US)
Enteritidis	FSL S5-371	Human (US)
Typhimurium	H2-001	Pig slaughterhouse (Thailand)

6.3.2. Efficiency of encapsulated phage cocktail against *S.* Enteritidis and *S.* Typhimurium *in-vitro*

The efficiency of phage cocktail powder against *S.* Enteritidis and *S.* Typhimurium was investigated at 10°C and 37°C. Overnight cultures of *S.* Enteritidis and *S.* Typhimurium were prepared following a procedure mentioned in 2.3.2, followed by washing step following a procedure mentioned in 4.2.4.7. Washed *Salmonella* pellets were suspended in TSB to achieve the level of 10^5 CFU/mL. Phage cocktail powder was mixed with TSB and diluted to achieve the phage concentration at 10^7 PFU/mL. The co-culture consisting of 5 mL of phage cocktail powder solution and 5 mL of each *Salmonella* culture was incubated at 10°C or 37°C in a shaking incubator at 220 rpm for 12 h. Controls with the only culture of *S.* Enteritidis or *S.* Typhimurium in TSB were included. The cell numbers of *S.* Enteritidis and *S.* Typhimurium from co-culture and controls at each temperature were enumerated every 4 h for 12 h by a spread plate technique on TSA. Plates were incubated for 24 h for surviving colony enumeration.

6.3.3 Efficiency of encapsulated phage cocktail against *S.* Enteritidis and *S.* Typhimurium in foods

Chicken meat, sunflower sprout and animal feed were prepared and inoculated *S.* Enteritidis and *S.* Typhimurium following a procedure mentioned in 3.3.5. Phage cocktail powder (prepared in chapter 5) 0.1 g of phage cocktail powder with the concentration of 10^8 PFU/g was evenly sprinkled to each piece of chicken and sunflower sprout in a separate sterile zip-lock bag. Powder of freeze-dried WPI/trehalose (without phage cocktail) was added to samples inoculated with *Salmonella* as controls. Treatments and controls were stored at 4°C. Cell numbers of *Salmonella* were enumerated at day 0, 1, 2, 3 and 4 on XLD ager. Cell numbers were also confirmed on TSA.

6.3.4 Evaluation of the overall acceptability of consumers towards the quality of food products applied with phage cocktail powder

A total of 30 panelists from the faculty of Agro-Industry, Prince of Songkla University were included for the evaluation of the overall acceptability of the food products treated with 0.1 g of microencapsulated phage cocktail. Food samples without microencapsulated phage cocktail were also prepared for evaluation. Liking scores were given for color, odor, appearance, and overall liking of samples (with and without microencapsulated phage cocktail) using a nine-point Hedonic scale (1 = dislike extremely, 5 = neither like nor dislike and 9 = like extremely).

6.3.5 Statistical analysis

Statistical analysis was analyzed for the experiments, including (i) the reduction of *S.* Enteritidis or *S.* Typhimurium on each food models during time period, (ii) sensory and (iii) color evaluation of food models with/without phage cocktail powder during storage. The differences between two treatments were compared within a given storage time, including liking scores of food models with/without phage cocktail powder for each sensory parameter, and ΔE values of food models with/without phage cocktail powder were analyzed by Student's t-test. The analysis of Analysis of variance (ANOVA) were used to compare the results of (i), (ii) and (iii) among storage time. Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p < 0.05$ using the statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and discussion

6.4.1 Efficiency of phage cocktail powder against *S.* Enteritidis and *S.* Typhimurium at 10°C and 37°C *in-vitro* and food models during storage time

The efficacy of phage cocktail powder for controlling *S.* Enteritidis and *S.* Typhimurium *in-vitro* was investigated at 10°C and 37°C. The initial inoculum level of 5 log CFU/mL of *S.* Enteritidis and *S.* Typhimurium was challenged with the phage cocktail powder. At 10°C, the numbers of both *S.* Enteritidis and *S.* Typhimurium in the control were slightly increased (< 0.5 log CFU/mL) after 12 h, while the numbers of *Salmonella* isolates increased continuously up to approximately 9 log CFU/mL after 8 h at 37°C. For phage treatment at 10°C, the highest *Salmonella* reduction was observed after 8 h of the treatment initiation presenting a reduction of 0.43 log CFU/mL and 0.76 log CFU/mL for *S.* Enteritidis and *S.* Typhimurium, respectively (Figure 6.1). After phage treatment initiation at 37°C, the highest *Salmonella* reduction was observed at 4 h presenting a reduction of 1.79 log CFU/mL and 3.63 log CFU/mL for *S.* Enteritidis and *S.* Typhimurium, respectively. Overall, phage cocktail powder could control *S.* Enteritidis and *S.* Typhimurium at both 10°C and 37°C. However, the optimal temperature for controlling.

Two selected foods (raw chicken meat and sunflower sprout) and animal feed that have shown high likelihood of having *Salmonella* contamination were used in this study. An initial inoculum of approximately 5 log CFU of *S.* Enteritidis and *S.* Typhimurium were spiked on each sample. Phage cocktail powder at the optimal multiplicity of infection (MOI) of 100 was applied on samples. For samples applied with the phage cocktail dry powder, the numbers of *S.* Enteritidis were decreased by 0.57 log CFU/cm^2, 0.86 log CFU/g and 1.92 log CFU/g in chicken meat, sunflower sprout and animal feed, respectively after 4 days of storage. The numbers of *S.* Typhimurium were decreased by 1.78 CFU/ cm^2, 1.2 log CFU/ g and 1.74 log CFU/g in chicken meat, sunflower sprout and animal feed, respectively (Figure 6.2). *In-vitro* study suggests the efficiency of phage cocktail powder to control *S.* Enteritidis and *S.* Typhimurium at 10°C and 37°C within 8 h and 4 h of phage treatment initiation, respectively. In our previous study, phages composed in the cocktail revealed the burst size of >90 PFU/cell and >100 PFU/cell on *S.* Enteritidis and *S.* Typhimurium, respectively. These phages showed the latent period from 5-40 min. The high burst size and short latent period are normally related

to the efficiency of phages to kill their bacterial hosts (Bao *et al*., 2011). Our study suggests a potential wide-range temperatures for our phages against *Salmonella* serovars.

The efficiency of the traditional phage cocktail lysate for controlling *Salmonella* in chicken meat, fresh produce and animal feed has been investigated by several previous studies (Spricigo *et al.,* 2013; Heyse *et al.,* 2015; Soffer *et al.,* 2016). A reduction of 0.9 and 2.2 log CFU/g of *S.* Enteritidis and *S.* Typhimurium, respectively on raw chicken meat was observed upon a treatment of a phage cocktail (3 phages) within 7 days at 4°C (Spricigo *et al.,* 2013). Controlling *Salmonella* in dry pet food was investigated by Heyse *et al*. (2015). Approximately >1 log reduction of *Salmonella* were observed after treated with phage cocktail comprising 6 *Salmonella* phages for 60 min (Heyse *et al.,* 2015). The commercial *Salmonella* phages (SalmoLyse®) were investigated their efficiency to reduce *Salmonella* in pet food and raw pet food ingredients. Approximately 3 log reduction was observed after tested (Soffer *et al.,* 2016). The efficiency of *Salmonella* phage as encapsulated form against *Salmonella* spp. has been reported. Colom *et al*., 2016 developed encapsulated *Salmonella* phages using alginate/$CaCO_3$. The result showed that encapsulated phage provided the higher efficiency to reduce the number of *Salmonella* infection in boiler chickens (Colom *et al*., 2016). Overall findings here suggest that our phage cocktail powder showed high efficiency in controlling *S.* Enteritidis and *S.* Typhimurium in representative fresh foods and animal feed. This suggests a potential alternative form of phage cocktail which provides comparable efficiency for controlling *Salmonella* on food matrices as the traditional phage lysate.

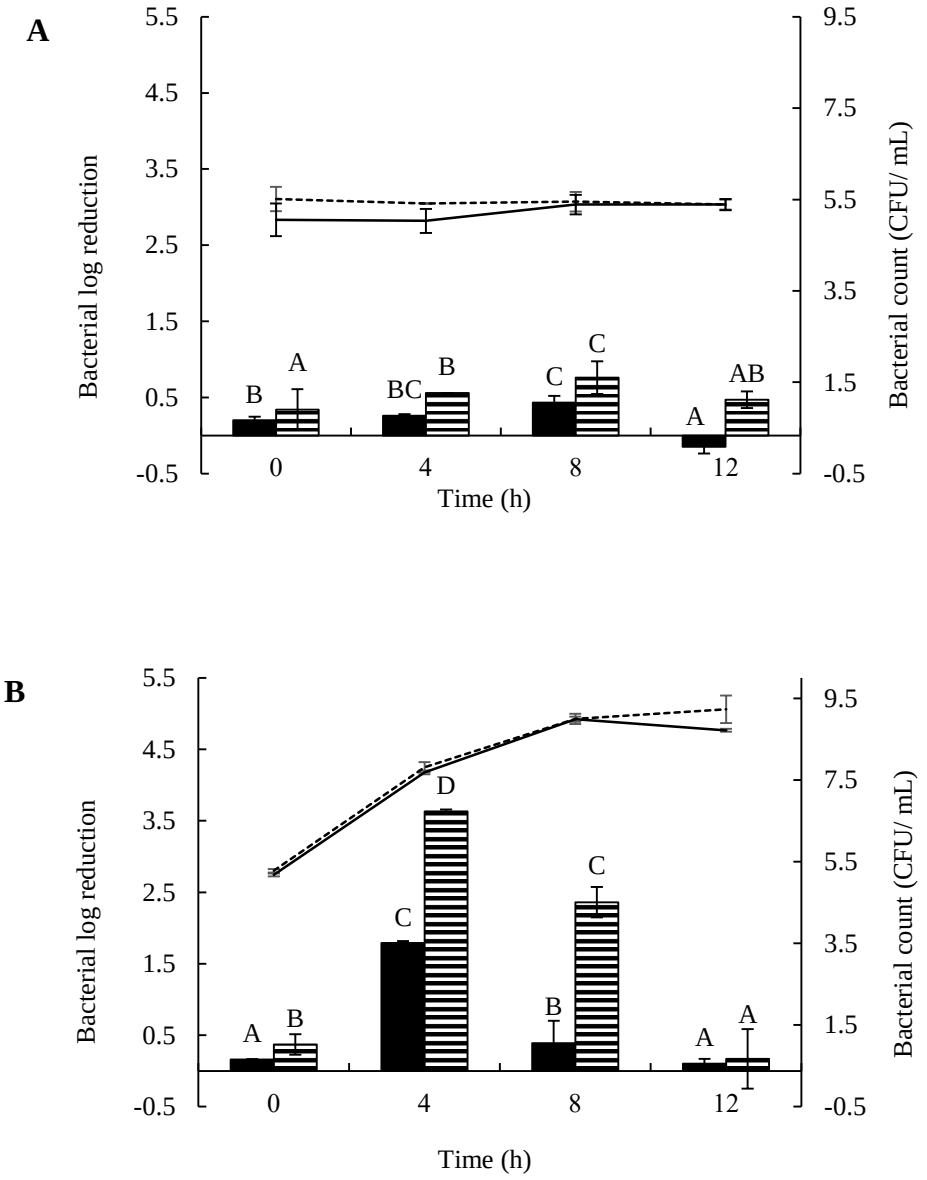

Figure 6.1. Log reduction of *S.* Enteritidis (■) and *S.* Typhimurium (▤) treated with microencapsulated phage cocktail compared to control (non-treated); *S.* Enteritidis (dash line) and *S.* Typhimurium (solid line) at 10°C (A) and 37°C (B) for 12 h. Bars represent the mean standard deviation (n = 3). Different uppercase letters indicated significant differences ($p < 0.05$) between log reductions of each *Salmonella* strains (*S.* Enteritidis or *S.* Typhimurium) during incubation time.

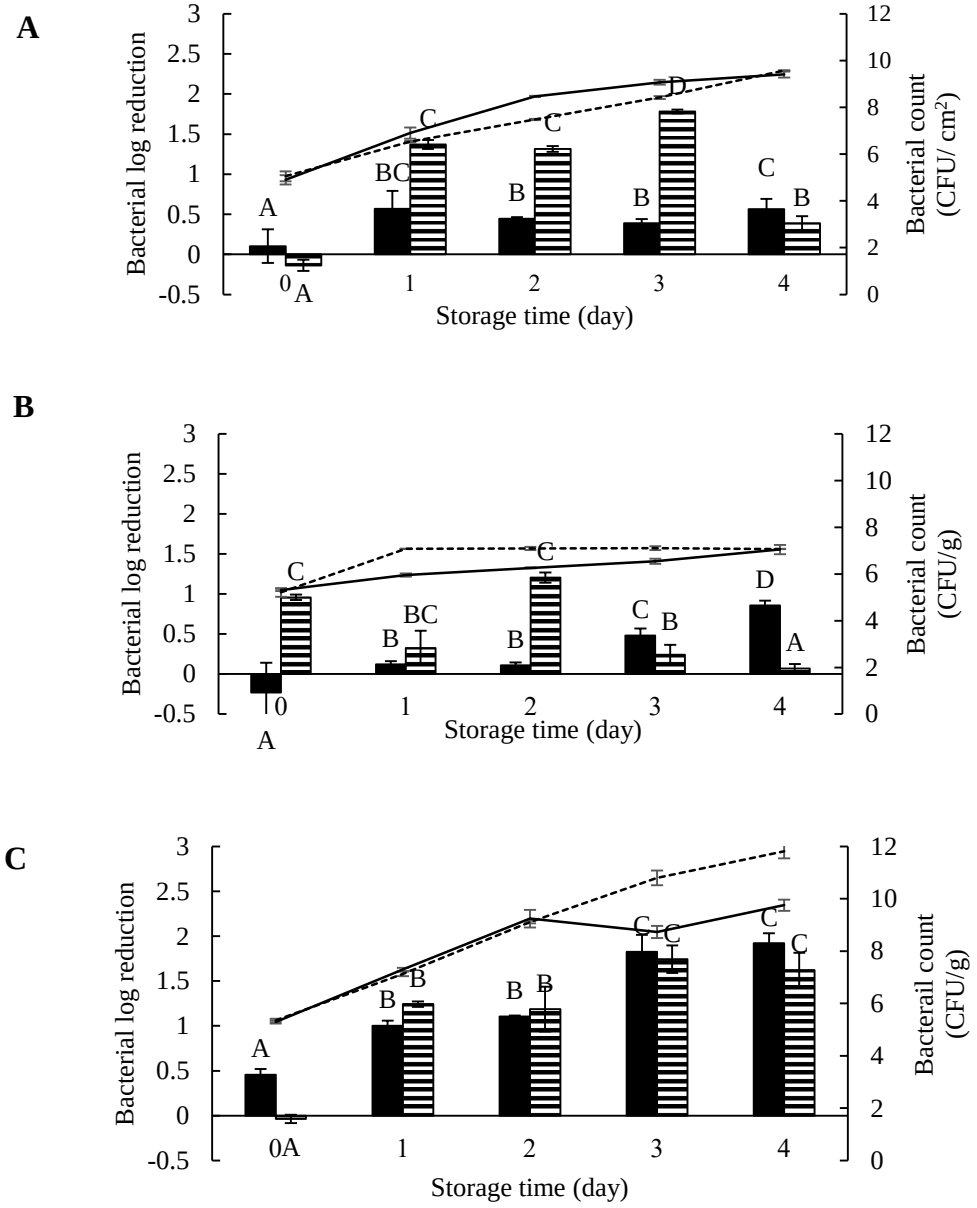

Figure 6.2. Log reduction of *S.* Enteritidis (■) and *S.* Typhimurium (▤) on chicken meat (A) sunflower sprout (B) and animal feed (C) treated with microencapsulated phage cocktail compared to control (non-treated); *S.* Enteritidis (dash line) and *S.* Typhimurium (solid line) at 4°C for 4 days. Bar represents the mean standard deviation (n = 3). Different uppercase letters indicated significant differences ($p < 0.05$) between log reductions of each *Salmonella* strains (*S.* Enteritidis or *S.* Typhimurium) during storage time.

6.4.2 Acceptability of fresh food applied with phage cocktail powder

This study evaluated the acceptability of raw chicken meat, sunflower sprout and animal feed with and without the phage cocktail powder and stored for 5 days

at 4°C. Scores on the appearance of color, odor, appearance, and overall liking of samples were observed (Table 6.2). For raw chicken meat, the score of samples without the phage cocktail powder was higher than that with the phage cocktail powder on all parameters tested for day 0 and day 1 of storage. From day 2, the scores of the meat samples with and without phage cocktail powder showed non-significant difference on odor, appearance, and the overall liking. Interestingly, the scores on color of raw chicken meat with the phage cocktail powder showed significant higher than control. For sunflower sprout, the trend of scores was similar to that observed in raw chicken meat for day 0, and 1. On day 2 and day 3, the scores on appearance and the overall liking of sprout with the phage cocktail powder increased and showed significantly higher than that observed in sprout without the phage cocktail powder. However, the score of both chicken meat and sunflower sprout with/without the phage cocktail powder showed scores under 6 (unacceptable quality) on day 4 for all parameters tested. For animal feed, on day 0 and 1, the score of samples without phage cocktail powder were higher than samples with phage cocktail powder significantly on all parameters evaluated. On day 2, the scores of samples with/without phage cocktail powder were not significantly different on all parameters evaluated. On day 3, the scores of odor, appearance and overall liking of samples without phage cocktail powder were higher. However, the score on color appearance was not significantly different. On day 4, the scores of color, appearance and overall liking of samples with/without phage cocktail powder were not significantly different. Interestingly, the score of odor of samples with phage cocktail powder was significantly higher that samples without phage cocktail powder. From table 6.3, color evaluation (ΔE) of raw chicken meat and animal feed with/without the phage cocktail powder showed significantly difference in the values since day 3, while the significant difference in ΔE values was detected in day 4 for sunflower sprout with/without the phage cocktail powder. Overall, data here indicate that raw chicken meat and sunflower sprout applied with phage cocktail powder was acceptable for over 3 days of storage at 4°C, whereas animal feed was acceptable for over 2 days for storage at 30°C.

Table 6.2. Liking score of chicken meat, sunflower sprout and animal feed applied with phage cocktail powder during storage.

Sample	Day	Color		Odor		Appearance		Overall liking	
		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Chicken meat	0	$7.9 \pm 1.0^{*c}$	6.4 ± 1.1^{ab}	$7.3 \pm 1.3^{*d}$	6.2 ± 1.5^{ab}	$7.7 \pm 1.0^{*c}$	6.5 ± 1.3^{ab}	$7.6 \pm 0.9^{*c}$	6.3 ± 1.2^{ab}
	1	$7.6 \pm 0.9^{*c}$	6.7 ± 1.3^{ab}	7.1 ± 1.3^{cd}	6.8 ± 1.4^{ab}	$7.5 \pm 0.9^{*c}$	6.8 ± 1.4^{b}	$7.5 \pm 0.8^{*c}$	6.7 ± 1.3^{b}
	2	6.4 ± 1.4^{b}	$7.0 \pm 1.0^{b*}$	6.5 ± 1.3^{bc}	6.6 ± 1.1^{b}	6.3 ± 1.1^{b}	6.3 ± 1.2^{ab}	6.4 ± 1.3^{b}	6.5 ± 0.9^{b}
	3	6.7 ± 1.3^{b}	6.6 ± 1.3^{ab}	6.2 ± 1.4^{b}	6.4 ± 1.3^{b}	6.1 ± 1.4^{b}	6.5 ± 1.3^{ab}	6.2 ± 1.3^{b}	6.6 ± 1.1^{b}
	4	5.6 ± 1.9^{a}	6.1 ± 1.6^{a}	5.4 ± 1.7^{a}	5.7 ± 1.3^{a}	5.1 ± 2.0^{a}	5.7 ± 1.6^{a}	5.2 ± 1.8^{a}	5.8 ± 1.5^{a}
Sunflower sprout	0	$7.9 \pm 0.6^{*d}$	6.8 ± 1.2^{b}	7.6 ± 0.8^{d}	7.2 ± 0.9^{c}	$7.8 \pm 0.8^{*c}$	6.8 ± 1.0^{c}	$7.9 \pm 0.6^{*c}$	6.9 ± 0.8^{b}
	1	$6.9 \pm 1.4^{*c}$	6.0 ± 1.5^{a}	6.8 ± 1.3^{c}	6.5 ± 1.4^{b}	$6.9 \pm 1.2^{*b}$	6.2 ± 1.6^{bc}	$6.9 \pm 1.1^{*b}$	6.2 ± 1.4^{b}
	2	6.1 ± 1.4^{b}	6.4 ± 1.2^{ab}	6.6 ± 1.2^{bc}	6.5 ± 1.2^{b}	5.3 ± 1.3^{a}	$5.9 \pm 1.2^{*b}$	6.0 ± 1.4^{a}	6.3 ± 1.3^{b}
	3	5.3 ± 1.5^{a}	$6.5 \pm 1.3^{*ab}$	6.1 ± 1.4^{ab}	6.4 ± 1.5^{ab}	4.9 ± 1.5^{a}	$6.4 \pm 1.2^{*bc}$	5.4 ± 1.4^{a}	$6.4 \pm 1.2^{b*}$
	4	6.2 ± 1.4^{b}	5.8 ± 1.3^{a}	5.9 ± 1.3^{a}	5.7 ± 1.5^{a}	5.5 ± 1.6^{a}	5.1 ± 1.7^{a}	5.6 ± 1.5^{a}	5.1 ± 1.4^{a}
Animal feed	0	7.2 ± 1.3^{cB}	6.1 ± 1.4^{abA}	7.1 ± 1.4^{cA}	6.6 ± 1.4^{cA}	7.4 ± 0.9^{cB}	6.4 ± 1.2^{cA}	7.1 ± 1.2^{cB}	6.2 ± 1.2^{bA}
	1	7.3 ± 1.1^{cB}	6.1 ± 1.5^{abA}	6.9 ± 1.0^{cB}	5.6 ± 2.1^{bA}	6.9 ± 0.9^{cB}	5.6 ± 1.5^{bcA}	7.0 ± 0.9^{cB}	5.7 ± 1.7^{bA}
	2	6.2 ± 0.9^{bA}	6.4 ± 1.4^{bA}	5.8 ± 1.4^{bA}	5.4 ± 1.5^{bA}	5.6 ± 1.2^{bA}	5.8 ± 1.5^{bcA}	5.9 ± 1.2^{bA}	5.9 ± 1.3^{bA}
	3	5.7 ± 1.5^{abA}	5.9 ± 1.4^{abA}	5.9 ± 1.6^{bB}	4.5 ± 1.4^{aA}	5.6 ± 1.5^{bB}	5.0 ± 1.4^{abA}	5.7 ± 1.5^{bB}	4.9 ± 1.3^{aA}
	4	5.4 ± 1.9^{aA}	5.3 ± 1.7^{aA}	3.5 ± 1.5^{aA}	4.7 ± 1.7^{abB}	4.2 ± 1.6^{aA}	4.6 ± 1.5^{aA}	4.2 ± 1.5^{aA}	4.7 ± 1.6^{aA}

All values indicate mean ± standard deviation from thirty panelists. Sign (*) on the standard deviation of each appearance at a given day indicates significant differences ($p < 0.05$) between control (non-treated) and treatment. Different lowercase letters on the standard deviation of control and treatment of each appearance indicate significant differences ($p < 0.05$) during storage.

Table 6.3. Color evaluation (ΔE) of fresh foods and animal feed treated with phage cocktail powder during storage.

Sample	Day	Control	Treatment
Chicken meat	1	3.22 ± 0.35^{a}	1.47 ± 0.77^{a}
	2	14.24 ± 1.51^{c}	12.44 ± 0.88^{c}
	3	12.66 ± 1.70^{bc}	10.48 ± 0.97^{b}
	4	$10.00 \pm 1.75^{b*}$	18.43 ± 0.87^{d}
Sunflower sprout	1	5.83 ± 3.69^{a}	3.19 ± 1.35^{a}
	2	21.11 ± 4.85^{c}	19.13 ± 3.49^{b}
	3	$11.32 \pm 0.15^{ab*}$	20.16 ± 2.84^{b}
	4	$15.62 \pm 5.04^{bc*}$	22.25 ± 3.18^{b}
Animal feed	1	$2.36 \pm 0.51_{A}$	$0.63 \pm 0.53_{A}$
	2	$6.23 \pm 1.66_{C}$	$2.48 \pm 0.13_{B}$
	3	$5.31 \pm 0.15_{BC}{}^{*}$	$2.57 \pm 0.71_{B}$
	4	$4.46 \pm 0.12_{B}{}^{*}$	$2.89 \pm 0.26_{B}$

All values indicate mean ± standard deviation. Sign (*) on the standard deviation of each fresh food at a given day indicates significant differences ($p < 0.05$) between control (non-treated) and treatment. Different letters on the standard deviation of control or treatment indicate significant differences ($p < 0.05$) during storage.

6.5 Conclusions

A newly developed form of phage cocktail as dry powder could provide improved product characteristics for applications due to consistent physio-chemical properties as reported in our previous studies. This study investigated the efficiency of phage cocktail powder in controlling *S.* Enteritidis and *S.* Typhimurium *in-vitro* and in food models, including raw chicken meat, sunflower sprout and animal feed. Phage cocktail as a dry powder developed here showed comparable efficiency for controlling *S.* Enteritidis and *S.* Typhimurium in both *in-vitro* and food models. *In-vitro* study showed that our dry phage cocktail provides the efficiency to reduce the number of *S.* Enteritidis and *S.* Typhimurium at both 10°C and 37°C. This dry phage cocktail powder also showed the efficiency to reduce the number *S.* Enteritidis and *S.* Typhimurium in fresh foods and animal feed during a storage period of 4 days at 4°C (for raw chicken meat and sunflower sprout) and 25°C (for animal feed). Sensory evaluation suggests the acceptability from consumers in raw chicken meat, sunflower sprouts and animal feed applied with phage cocktail powder during storage period of 3 days at 4°C (for raw chicken meat and sunflower sprout) and 2 days at 25°C (for animal feed). Data here suggest a potential use of phage cocktail powder as a bio-control agent for improving safety of fresh foods and animal feed that have been shown high risk from *Salmonella* contamination.

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CHAPTER 7

COMBINED EFFECT TO BACTERIOPHAGE AND ANTIBIOTIC ON THE INHIBITION OF THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN *SALMONELLA* TYPHIMURIUM

7.1 Abstract

This study was designed to evaluate the combined effects of bacteriophage and antibiotic on the reduction of the development of antibiotic-resistance in *Salmonella* Typhimurium LT2. The susceptibilities of *S.* Typhimurium to ciprofloxacin and erythromycin were increased when treated with bacteriophages, showing more than 10% increase in clear zone sizes and greater than twofold decrease in minimum inhibitory concentration values. The growth of *S.* Typhimurium was effectively inhibited by the combination of bacteriophage P22 and ciprofloxacin. The combination treatment effectively reduced the development of antibiotic resistance in *S.* Typhimurium. The relative expression levels of efflux pump-related genes (*acrA*, *acrB* and *tolC*) and outer membrane-related genes (*ompC*, *ompD* and *ompF*) were decreased at all treatments. This study provides useful information for designing new antibiotic therapy to control antibiotic-resistant bacteria.

7.2 Introduction

Non-typhoidal *Salmonella* Typhimurium and *S.* Enteritidis, are the most common pathogenic serovars that can cause gastroenteritis, enteric fever, infectious diarrheal, and septicemia (Acheson and Hohmann, 2001). Non-typhoidal *Salmonella* infections remain a serious public health problem worldwide, which resulted in approximately 94 million cases and 155,000 deaths annually (Ao *et al*., 2015). Furthermore, non-typhoidal *Salmonella* serovars represent a reservoir of antibiotic resistance determinants (Michael and Schwarz, 2016). This is responsible for the acquisition of multiple antibiotic resistance to β-lactams, fluoroquinolones, and tetracycline in non-typhoidal *Salmonella* serovars (Dahshan *et al*., 2010; Mather *et al*., 2013). The mechanisms of antibiotic resistance include enzymatic degradation of antibiotics, activation in efflux pump systems, and alteration in membrane permeability (Giedraitiené *et al*., 2011; Miró *et al*., 2015). The emergence and dissemination of antibiotic-resistant *Salmonella* have become a major problem in a clinical and hospital setting due to the frequent failure in antibiotic treatments (Prestinaci *et al*., 2015). Therefore, the novel approach to improving current antibiotic use is essential for control of antibiotic-resistant bacterial infections. Recently, bacteriophage has been renewed attention as an alternative approach for treatment of bacterial infections due to its effective specificity to target bacteria (Bardina *et al*., 2012). Bacteriophages can recognize the bacterial cell surface exposed receptors such as pili, flagella, capsule, membrane proteins, and lipopolysaccharides (Rakhuba *et al*., 2010; Stecher *et al*., 2004). Recent studies have reported the effectiveness of using bacteriophages in combating against bacterial pathogens, including bacteriophage cocktails and bacteriophage-antibiotic combinations (Jo *et al*., 2016; Perera *et al*., 2015; Ryan *et al*., 2012; Spricigo *et al*., 2013). Most researches highlights that the application of bacteriophages can be a promising strategy to overcoming antibiotic resistance. However, relatively few studies have evaluated the bacteriophage resistance mechanisms when bacteria are exposed to selective pressure. Therefore, the objective of this study was to investigate the efficiency of bacteriophage-antibiotic combination against *S.* Typhimurium and also evaluate bacterial resistance to bacteriophage.

7.3 Materials and methods

7.3.1 Bacterial strains and culture conditions

Strain of *Salmonella enterica* subsp. enterica serovar Typhimurium LT2 (ATCC 19585) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in trypticase soy broth (TSB) (BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37 °C for 20 h. The cultured cells were centrifuged at 30009×*g* for 20 min at 4°C. The collected cells diluted with phosphate- buffered saline (PBS, pH 7.2) to 10^8 CFU/mL.

7.3.2 Bacteriophage propagation and enumeration

Salmonella bacteriophage P22 (ATCC 97541) was propagated at 37 °C for 24 h in TSB containing *S.* Typhimurium as a suggested host. The propagated phages were harvested by centrifugation at 5000×*g* for 10 min. The harvested phages were filtered through a 0.2 μm filter to eliminate bacterial lysates. The phage titers were determined by using the double agar overlay plaque assay (Bielke *et al.,* 2007). In brief, phages were diluted with PBS. The diluted phages were mixed with TSB (0.5% agar) containing the host cells (10^7 CFU/mL) and then poured onto the agar lawn plates. The soft-agar overlaid plates were incubated at 37 °C for 24 h to count the phages which were expressed as plaque forming unit (PFU).

7.3.3 Agar diffusion test

The disk diffusion susceptibility was measured to deter- mine the synergistic effect of phage P22 and antibiotic. The Muller–Hinton agar plates containing *S.* Typhimurium (10^5 CFU/mL) were prepared without and with phage P22 (10^5 PFU/mL). The antibiotic disks, including cefotaxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), streptomycin (10 μg) and tetracycline (30 μg), were placed on the prepared Muller–Hinton agar plates and then incubated at 37 °C for 18 h. After incubation, the diameters of the zone of inhibition were measured in centimeters using a digital vernier caliper (The L.S. Starrett Co., Athol, MA, USA).

7.3.4 Preparation of antibiotic stock solutions

All antibiotics used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stock solutions of cefotaxime in water, chloramphenicol in absolute ethanol, ciprofloxacin in glacial acetic acid, erythromycin in absolute ethanol, streptomycin in water, and tetracycline in absolute ethanol were prepared at a concentration of 1024 mg/ml. The antibiotic working solutions were freshly prepared prior to use.

7.3.5 Antibiotic susceptibility assay

The susceptibility of *S.* Typhimurium to each antibiotic was evaluated in the absence and presence of phage P22. *S.* Typhimurium (10^5 CFU/mL) was inoculated in 96-well microtiter plates containing twofold serial dilution of each antibiotic (cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, streptomycin, or tetracycline) without and with phage P22. The inoculated 96-well plates were incubated for 18 h at 37°C. The growth of *S.* Typhimurium was observed at 600 nm (Lin *et al*., 2010) and fitted to exponential function using Microcal Origin® 8.0 (Microcal Software Inc., Northampton, MA, USA).

7.3.6 Time-kill curve analysis

The inhibitory effects of phage P22, ciprofloxacin, and combination against *S* Typhimurium was evaluated by the time-kill assay. Phage P22 (10^5 PFU/mL), ciprofloxacin (1 × MIC; 0.016 μg/mL) and combination were inoculated with *S.* Typhimurium (10^5 CFU/mL) in TSB. Each treatment was cultured for 20 h at 37 °C. The growth of *S.* Typhimurium was measured at every 4 h interval. After 20 h culture, samples were collected for the analyses of antibiotic resistance and gene expression.

7.3.7 Antibiotic resistance profile

The changes in antibiotic resistance phenotype after the time-kill assay were estimated by using a disk diffusion assay. *S.* Typhimurium cells cultured in the control, phage P22 alone, ciprofloxacin alone, and combination were streaked onto Muller–Hinton agar plate. The antibiotic disks (cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, streptomycin, and tetracycline) were placed on the prepared Muller–Hinton agar plates and incubated at 37°C for 18 h. After incubation, the antibiotic susceptibility was compared by measuring the diameters of clear zone.

7.3.8 Quantitative PCR assay

Total RNA from *S.* Typhimurium cells cultured in the control, phage P22 alone, ciprofloxacin alone, and combination was extracted according to the protocol of RNeasy Protect Bacteria Mini kit (Qiagen, Hilden, Germany), and then cDNA was synthesized according to the QuantiTech reverse transcription procedure (Qiagen). For qPCR assay, the reaction mixture (10 µl of 2 × QuantiTect SYBR Green PCR Master, 2 µl of each primer (Table 7.1), 2 µl of cDNA, and 4 µl of RNase-free water) was amplified using an iCycler iQ^{TM} system (Bio-Rad Laboratories, Hemel Hempstead, UK). The mixture was denatured at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 15 s. The comparative method (Livak and Schmittgen, 2001) was used to evaluate the relative gene expression in *S.* Typhimurium cells cultured in phage P22 alone, ciprofloxacin alone, and combination.

Table 7.1. Primer sequences used in qPCR analysis for *S.* Typhimurium.

Gene	Molecular function	Primer sequence[a]
16S rRNA	Reference gene	F: AGGCCTTCGGGTTGTAAAGT
		R: GTTAGCCGGTGCTTCTTCTG
acrA	Multidrug efflux system	F: AAAACGGCAAAGCGAAGGT
		R: GTACCGGACTGCGGGAATT
acrB	Multidrug efflux system	F: TGAAAAAAATGGAACCGTTCTTC
		R: CGAACGGCGTGGTGTCA
ompC	Outer membrane protein C	F: TCGCAGCCTGCTGAACCAGAAC
		R: ACGGGTTGCGTTATAGGTCTGAG
ompD	Outer membrane protein D	F: GCAACCGTACTGAAAGCCAGGG
		R: GCCAAAGAAGTCAGTGTTACGGT
ompF	Outer membrane protein F	F: AGTGGGTTCAATCGATTATG
		R: GAATATATTTCGCCAGATCG
tolC	Multidrug efflux system	F: GCCCGTGCGCAATATGAT
		R: CCGCGTTATCCAGGTTGTTG

[a] F forward, R reverse

7.3.9 Statistical analysis

All experiments were conducted in duplicate for three replicates. Data were analyzed using the Statistical Analysis System software. The general linear model and Fisher's least significant difference procedures were used to determine significant mean differences at $p < 0.05$.

7.4 Results and discussion

7.4.1 Antibiotic susceptibility patterns of *S.* Typhimurium in the presence of phages

The antibiotic susceptibility of *S.* Typhimurium LT2 was evaluated in the absence and presence of phage P22 using disk diffusion assay (Table 7.2). The clear zone sizes of cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, streptomycin and tetracycline disks were increased to 13, 10, 22, 25, 9 and 12%, respectively, in the presence of phage P22. The growth of phages was stimulated by antibiotics (Comcau *et al*., 2007), leading to the enhanced lysis of host bacterium, *S.* Typhimurium LT2. This result is a good agreement with previous report that the increased plaque size and phage titer were observed after antibiotic treatment (Kamal and Dennis, 2015). The MIC values of antibiotics against *S.* Typhimurium LT2 were evaluated in the absence of presence of phage P22 (Figure 7.1). The antibiotic susceptibilities of LT2 were noticeably increased in the presence of phage P22 compared to the absence of phage P22, showing more than two-fold decrease in MIC values; cefotaxime (0.06–0.03 μg/mL), chloramphenicol (4–0.25 μg/mL), ciprofloxacin (0.016–0.008 μg/mL), erythromycin (64–8 μg/mL), streptomycin (64–16 μg/mL) and tetracycline (2–1 μg/mL). The decreased MIC values in *S.* Typhimurium LT2 treated with combination might be due to the changes in permeability and efflux pump activity (Moya-Torres *et al*., 2014). Antibiotics play an important role in the increased burst size and reduced latent period of phages, leading to an increase in the susceptibility of bacteria (Ryan *et al*., 2012). However, antibiotics can interfere with the phage replication within bacteria cells, resulting in the reduction in the phage- antibiotic combination effect (Chaudhry *et al*., 2017). Therefore, further studies are needed to understand the mechanisms of phage-host interplay and antibiotic resistance for phage-antibiotic based therapeutic approach.

Table 7.2. Antibiotic disk diffusion (cm) of *S.* Typhimurium in the absence and presence of phages.

Antibiotic	*S.* Typhimurium	
	No phage	Phage
Cefotaxime	$3.61 \pm 0.11^{1}{}_{aA}$	$4.08 \pm 0.14_{aA}$
Chloramphenicol	$2.85 \pm 0.07_{bA}$	$3.12 \pm 0.09_{bA}$
Ciprofloxacin	$3.54 \pm 0.08_{aA}$	$4.32 \pm 0.12_{aB}$
Erythromycin	$1.27 \pm 0.06_{eA}$	$1.58 \pm 0.06_{dB}$
Streptomycin	$1.59 \pm 0.06_{dA}$	$1.73 \pm 0.08_{dA}$
Tetracycline	$2.14 \pm 0.07_{cA}$	$2.39 \pm 0.11_{cA}$

[1]Means with different letters (a–e) within a column are significantly different at $p < 0.05$ and means with different letters (A–B) within a row are significantly different at $p < 0.05$.

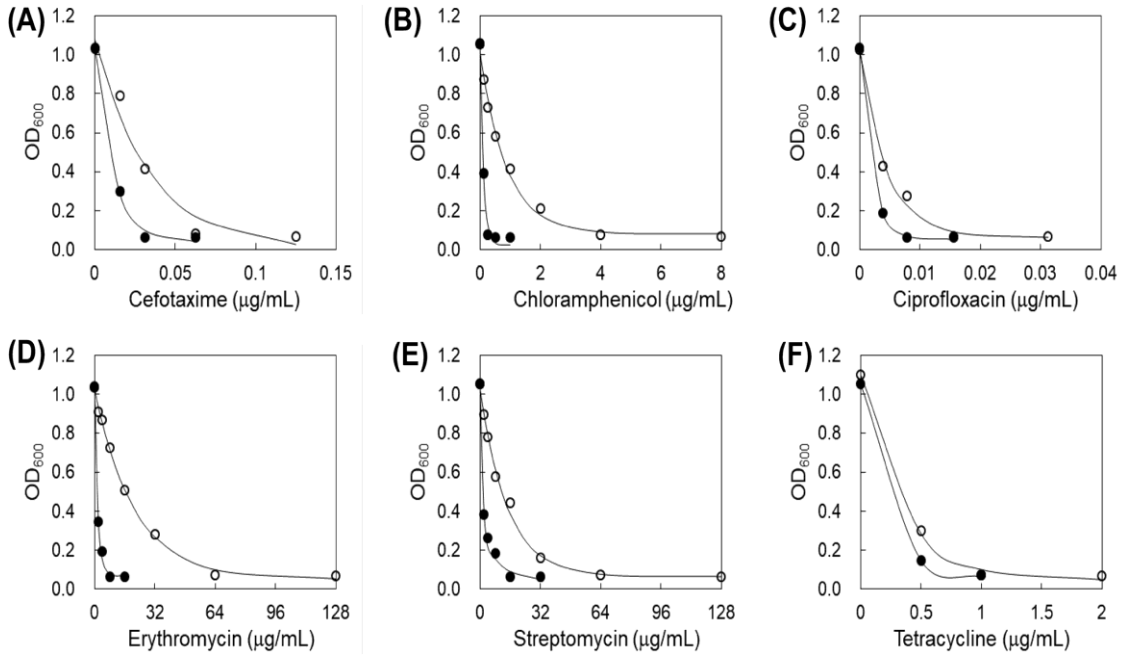

Figure 7.1. Antibiotic susceptibility of *S.* Typhimurium in the absence (opened circle) and presence (filled circle) of bacteriophages.

7.4.2 Combined inhibitory effect of phage and ciprofloxacin on the growth of *S.* Typhimurium

The antimicrobial effect of phage P22 alone, ciprofloxacin alone, and combination against *S.* Typhimurium LT2 was evaluated at 37°C for 20 h (Figure 7.2). Ciprofloxacin was used to evaluate the induction of antibiotic resistance, representing the second generation of fluoroquinolone. Compared to the control, the growth of *S.* Typhimurium LT2 was significantly inhibited by all treatments throughout the incubation. The most considerable reduction was observed for the combination treatment, resulting in more than 5 log reduction up to 12 h of incubation. However, the number of *S.* Typhimurium LT2 treated with phage P22 was increased after 12 h incubation. This might be due to the presence of bacterial cells resistant to bacteriophage P22 (Labrie *et al*., 2010). The ability of develop resistance to antibiotics was evaluated in *S.* Typhimurium LT2 treated with phage P22, ciprofloxacin, and combination after 20 h incubation (Figure 7.3). The antibiotic susceptibilities varied in the treatments. Compared to the control, the enhanced susceptibilities of *S.* Typhimurium LT2 to erythromycin, streptomycin, and tetracycline were observed at the combination treatment, while *S.* Typhimurium LT2 treated with phage P22 alone and antibiotic alone was less sensitive to erythromycin and streptomycin. The results suggest that appropriate antibiotic use can improve the antimicrobial activity when combined with phages. The phage-antibiotic combination can be possibly used to reduce the resistance to phages and antibiotics (Muniesa *et al*., 2013; Oechslin *et al*., 2017).

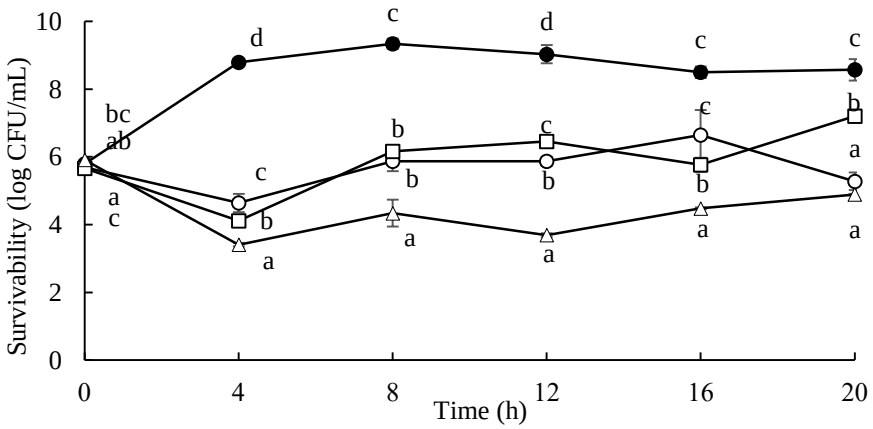

Figure 7.2. Survival of *S.* Typhimurium incubated with none (control; opened circle), bacteriophage alone (filled square), ciprofloxacin alone (filled triangle) and combination of bacteriophage and ciprofloxacin (filled circle) at 37°C for 20 h.

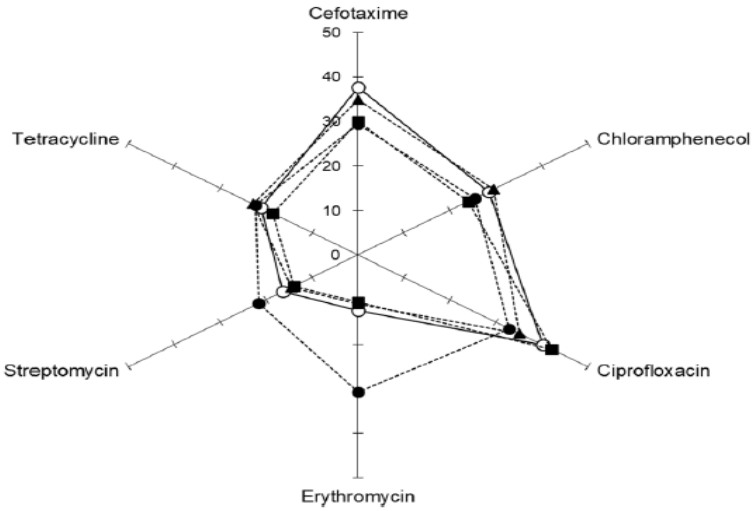

Figure 7.3. Radar plot of antibiotic resistance profiles (disk diffusion in cm) of *S.* Typhimurium incubated with none (control; opened circle), phage P22 alone (filled square), ciprofloxacin alone (filled triangle) and combination of phage P22 and ciprofloxacin (filled circle) at 37 °C for 20 h. Antibiotic disks include cefotaxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), streptomycin (10 μg) and tetracycline (30 μg).

7.4.3 Differential gene expression of *S.* Typhimurium treated with phage, ciprofloxacin, and combination

The relative expression of efflux pump-related genes (*acrA*, *acrB* and *tolC*) and outer membrane-related genes (*ompC*, *ompD* and *ompF*) was observed in *S.* Typhimurium LT2 treated with phage P22 alone, antibiotic alone, and combination, compared to the control (Figure 7.4). The relative expression levels of genes used in this study were decreased at all treatments. The antibiotic resistance in bacteria is associated with the efflux pump systems, including ATP-binding cassette (ABC), major facilitator (MF), small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE), and resistance- nodulation-cell division (RND) (Nikaido *et al*., 2008). The tripartite efflux transporter complex, AcrAB-TolC, is mainly responsible for the antibiotic resistance in *S.* Typhimurium, which has a broad specificity to various substrates such as β-lactams, fluoroquinolones, tetracycline and erythromycin (Kobayashi *et al*., 2014; Ricci and Piddock, 2009). In structure, *acrA*, *acrB* and *tolC* are located in the periplasmic protein, the inner membrane, and the outer membrane, respectively (Nikaido *et al*., 1998). The

downregulation of efflux pump-related genes (*acrA*, *acrB* and *tolC*) and outer membrane-related genes (*ompC*, *ompD* and *ompF*) are responsible for the reduced permeability and efflux of antibiotics, leading to the increase in the antibiotic susceptibility of *S.* Typhimurium LT2 (Moya-Torres *et al*., 2014; Piddock, 2014; Rushdy *et al*., 2013). Moreover, the decrease in *acrAB–tolC* leads to reduce the virulence of *Salmonella* because *acrA*, *acrB* and *tolC* are required for efficient adhesion invasion of epithelial cells and macrophages (Webber and Piddock, 2003). Phage combined with antibiotic, known as phage-antibiotic synergy (PAS), can be an alternative way to control bacterial infections. PAS can also be a possible strategy for reducing the emergence of phage-resistant mutants (Oechslin *et al.*, 2017). In conclusion, the significant findings are that the antibiotic susceptibility of *S.* Typhimurium LT2 was significantly increased when combined with phages and the growth of *S.* Typhimurium LT2 was synergistically inhibited by the combination of phage P22 and antibiotic. The combined treatment of phage P22 and ciprofloxacin could downregulate the expression of efflux pump-related genes (*acrA*, *acrB*, and *tolC*) and outer membrane-related genes (*ompC*, *ompD*, and *ompF*), resulting in the increased antibiotic susceptibility of *S.* Typhimurium LT2. These results highlight the advantages of using phage-antibiotic combination to prevent the emergence of antibiotic resistant strains.

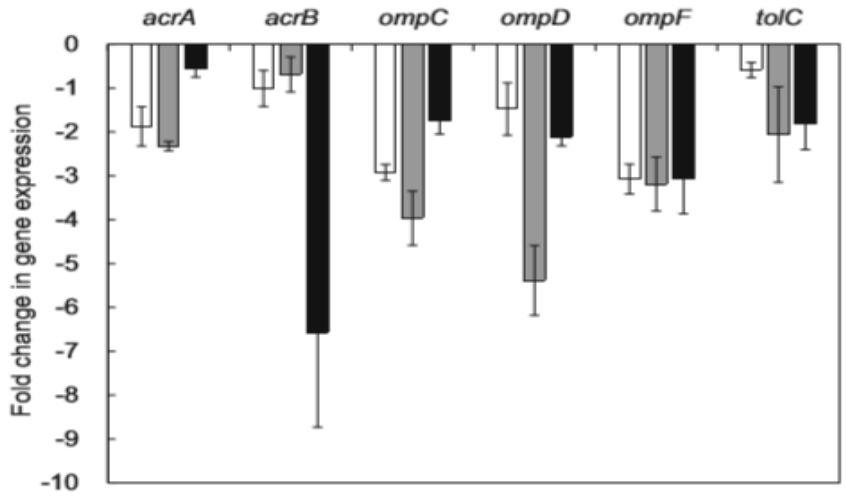

Figure 7.4. Relative gene expression *S.* Typhimurium LT2 treated with phage P22 alone (opened square), ciprofloxacin alone (filled square) and combination of phage P22 and ciprofloxacin (filled square) at 37 °C for 20 h.

7.5 Conclusion

In this study, we investigated the effect of antibiotic-phage combination in controlling *Salmonella* and the occurrence of antibiotic-resistant *Salmonella*. Results showed that the combination provided the improvement of antibiotic efficiency to control *Salmonella* and also decreased the development of antibiotic resistance mechanisms compared to using of antibiotic alone. Overall, the synergistic effect of antibiotic and phage provides an effective control against *Salmonella* while reducing the chance of development of resistant mechanisms of bacteria.

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CHAPTER 8

SUMMARY AND SUGGESTIONS

According to the concern of *Salmonella* outbreaks related food-borne illness salmonellosis and the limitations of phage as traditional lysate form, this study aimed to develop encapsulated *Salmonella* phages as dry powder by microencapsulation to control *Salmonella* spp. in food matrices. We have developed a collection of *Salmonella* phages in this study. These phages were isolated from various animal farms with the link to various *Salmonella* serovars. Phenotypic and genotypic characterizations of our isolated phages revealed that phages provide the broad spectrum to kill *Salmonella* serovars from various sources and regions. The wide range of estimated genome sizes indicated the diversity of phages isolated here. To improve the antibacterial efficiency, we thus developed the highest effective phages from the collection as phage cocktail to control the two major *Salmonella* serovars, *S.* Enteritidis and *S.* Typhimurium. Of 36 phages, three phages (KP4, KP5 and KP50) were selected for further development of phage cocktail. Electron microscopy revealed the evidences which support that these phages should be classified into the order *Caudovirales* and family *Siphoviridae*. All three phages showed the desirable biology as the large burst size and short latent period on both target *Salmonella* serovars, indicating the high potential to disrupt bacterial host cells. Genome sequencing analysis and annotations revealed that all three phages presented as putative virulent phages. All three phages showed the absence of genes associated lysogeny module, virulence/toxin and antibiotic resistance. Our developed phage cocktail showed the efficiency to control target *Salmonella* serovars in both *in-vitro* study and in food (raw chicken meat and sunflower sprout) and feed models. Data suggest the potential to control *Salmonella* contamination along food production chain by phage cocktail.

A new form of phage as dry powder was developed in order to extend the applications and improve the limitation of the traditional phage lysate form. We thus optimized the optimal formulation of coating materials (WPI/trehalose) which provide the effective protection of phages in the process of freeze-drying. Among the formulations tested, WPI/trehalose at the ratio of 3:1 (w/w) presented as the optimal formulation. In order to understand the characteristics and stability of our microencapsulated phage (dry phage powder) in the harsh conditions (temperatures and pH), we thus evaluated the

specific functional properties of dry phage powder. The external morphology of phage powder presented by SEM images indicated the complex of coating materials (WPI and trehalose) of phage powder. FTIR analysis revealed the occurrence of H−bonding between coating materials. While, DSC analysis revealed T_g of phage powder at 63.43°C. Dry phage powder also showed the stability in various temperatures (4°C, 25°C and 50°C) and wide range of pH (pH 1.5 to pH 9.5). Data here indicate the physical stability of phage power over room temperature and potential of encapsulation to protect phages from the harsh conditions.

Developed phage cocktail was also transformed to be dry powder using the optimized formulation. Dry phage cocktail powder was investigated the optimal storage conditions including packaging material and temperature which provide the longest shelf-life and the stability of physio-chemical properties of this product over 12 weeks of storage. The results indicate that dry phage cocktail powder kept in aluminium laminated foil bag at 4° provided the highest phage survivability and non-significant changing of physical (color) and chemical (a_w) of dry phage cocktail powder during storage. Dry phage cocktail powder also showed the efficiency to decrease the number of target *Salmonella* serovars in both *in-vitro* study and in foods (raw chicken meat and sunflower sprout) and feed models. Foods and feed applied with dry phage cocktail powder were also evaluated the acceptability of the consumers. Results indicate that the foods applied with dry phage cocktail powder were accepted for 3 days of storage, while the applied feed was accepted for 2 days of storage. Overall, this developed form of phage cocktail can be applied on foods and food products related high risk of *Salmonella* contamination with the overall acceptance by the sensory analysis.

In addition, our study indicates that the combination of bacteriophage and antibiotic showed the potential to improve the efficiency against *Salmonella* as compared to using of antibiotic alone. Moreover, this synergistic strategy could reduce the chance of development of resistance mechanisms in bacteria, suggesting a potential use to control *Salmonella* in various fields.

Overall, this study suggests that animal farm environments provided the abundant and diversity of *Salmonella* phages. Phages showed more lysis ability on hosts that were related to the environments of isolation. Formulation of phage as dry powder developed here provided the desirable properties for improving the stability of phages in the harsh conditions, long-term of storage and also maintain the efficiency of phages to

control the number of *Salmonella* serovars. Acceptability from consumers indicated that the characteristics of this product did not disrupt the quality of foods and feed. For food safety application, phage cocktail can be used as bio-control agent and phages need to be ensured the absence of the genes associated lysogeny module, virulence/toxin and antibiotic resistance.

This study provides a new effective model of encapsulated phages as dry powder form to control *Salmonella* serovars in foods and feed at the lab scale. For future work, scaling up the production together with studying the parameters needed for scale-up production is of our interest. The developed encapsulated phages can be used for controlling *Salmonella* contamination along the food production chain including farm environments (i.e. wastewater treatment, crop and manure). In addition, the application of dry phage powder developed in this study can also be applied in the solution in order to use as liquid form while phages are being protected in unfavorable conditions for the improved effectiveness (i.e. animal drinking water and animal carcasses). However, whole genome sequencing is recommended to confirm the absence of concerned genes or gene clusters in the phage genome before use in full-scale production. In addition, further related studies including use of phages with other non-thermal treatments such as high-pressure, pulsed electrical fields, pulsed light and ultrasound are also of our study in order to provide synergistic effects for controlling *Salmonella* contamination.

APPENDIX

Supplemental table 1. *Salmonella* phages isolated from various animal farms in this study

No.	ID	Sample (animal farm)	Isolated host
1	KP1	Chicken feed (poultry farm 3)	Anatum (A4-525)
2	KP2	Drinking water (poultry farm 3)	Anatum (A4-525)
3	KP3	Goat feces	Anatum (A4-525)
4	KP4	Soil (chicken farm 2)	Anatum (A4-525)
5	KP5	Chicken feces (poultry farm 3)	Anatum (A4-525)
6	KP6	Chicken feed (farm 2)	Anatum (A4-525)
7	KP7	Soil (poultry farm 2)	Anatum (A4-525)
8	KP8	Chicken feed (poultry farm 2)	Anatum (A4-525)
9	KP9	Soil (poultry farm 2)	Anatum (A4-525)
10	KP10	Chicken feed (poultry farm 2)	Anatum (A4-525)
11	KP11	Soil (poultry farm 2)	Anatum (A4-525)
12	KP12	Soil (poultry farm 2)	Anatum (A4-525)
13	KP13	Soil (poultry farm 2)	Anatum (A4-525)
14	KP20	Soil (poultry farm 2)	Give (H2-016)
15	KP21	Soil (poultry farm 2)	Give (H2-016)
16	KP22	Chicken feed (poultry farm 2)	Give (H2-016)
17	KP23	Chicken feed (poultry farm 2)	Kentucky (W1-010)

Supplemental table 1. (Cont.)

No.	ID	Sample (animal farm)	Isolated host
18	KP24	Chicken feed (poultry farm 2)	Kentucky (W1-010)
19	KP25	Chicken feed (poultry farm 2)	Kentucky (W1-010)
20	KP34	Chicken feces (poultry farm 1)	Virchow (H2-117)
21	KP36	Bovine feces	Virchow (H2-117)
22	KP38	Bovine feces	Stanley (H2-002)
23	KP39	Bovine feces	Stanley (H2-002)
24	KP40	Soil (poultry farm 3)	Typhimurium (H2-001)
25	KP41	Drinking water (poultry farm 3)	Typhimurium (H2-001)
26	KP43	Chicken feces (poultry farm 3)	Typhimurium (H2-001)
27	KP44	Chicken feed (poultry farm 3)	Typhimurium (H2-001)
28	KP45	Drinking water (poultry farm 3)	Typhimurium (H2-001)
29	KP46	Drinking water (poultry farm 3)	Agona (H2-016)
30	KP47	Chicken feces (poultry farm 3)	Agona (H2-016)
31	KP48	Chicken feed (poultry farm 3)	Agona (H2-016)
32	KP49	Soil (poultry farm 3)	Agona (H2-016)
33	KP50	Chicken feces (poultry farm 3)	Agona (H2-016)
34	KP53	Chicken feces (poultry farm 3)	Typhimurium (H2-001)
35	KP54	Soil (poultry farm 3)	Typhimurium (H2-001)
36	KP55	Chicken feed (poultry farm 3)	Typhimurium (H2-001)

Supplemental table 2. Host range classification based on lysis profiles of *Salmonella* phages on *Salmonella* hosts from Thailand and the US.

Phage ID	% host strains being lysed by each phage (level of host range[a])		
	Salmonella hosts from Thailand	*Salmonella* hosts from US	All *Salmonella* hosts tested
KP1	44 (broad)	13 (intermediate)	29 (intermediate)
KP2	44 (broad)	13 (intermediate)	29 (intermediate)
KP3	4 (narrow)	4 (narrow)	4 (narrow)
KP4	44 (broad)	9 (narrow)	27 (intermediate)
KP5	52 (broad)	9 (narrow)	31 (broad)
KP6	8 (narrow)	9 (narrow)	8 (narrow)
KP7	12 (intermediate)	4 (narrow)	8 (narrow)
KP8	12 (intermediate)	4 (narrow)	8 (narrow)
KP9	40 (broad)	9 (narrow)	25 (intermediate)
KP10	20 (intermediate)	4 (narrow)	13 (intermediate)
KP11	12 (intermediate)	13 (intermediate)	13 (intermediate)
KP12	12 (intermediate)	9 (narrow)	10 (narrow)
KP13	4 (broad)	9 (narrow)	6 (narrow)
KP20	24 (intermediate)	4 (narrow)	15 (intermediate)
KP21	12 (intermediate)	0 (narrow)	6 (narrow)
KP22	12 (intermediate)	0 (narrow)	6 (narrow)
KP23	28 (intermediate)	4 (narrow)	17 (intermediate)
KP24	24 (intermediate)	9 (narrow)	17 (intermediate)
KP25	28 (intermediate)	9 (narrow)	19 (intermediate)
KP34	60 (broad)	48 (broad)	54 (broad)
KP36	52 (broad)	9 (narrow)	31 (broad)
KP38	16 (intermediate)	9 (narrow)	13 (intermediate)
KP39	8 (narrow)	4 (narrow)	6 (narrow)
KP40	8 (narrow)	0 (narrow)	4 (narrow)
KP41	8 (narrow)	0 (narrow)	4 (narrow)
KP43	8 (narrow)	4 (narrow)	6 (narrow)

Supplemental table 2. (Cont.)

Phage ID	% host strains being lysed by each phage (level of host range[a])		
	Salmonella hosts from Thailand	*Salmonella* hosts from US	All *Salmonella* hosts tested
KP44	8 (narrow)	0 (narrow)	4 (narrow)
KP45	12 (intermediate)	4 (narrow)	8 (narrow)
KP46	44 (broad)	17 (intermediate)	31 (broad)
KP47	32 (broad)	9 (narrow)	21 (intermediate)
KP48	40 (broad)	13(intermediate)	27 (intermediate)
KP49	52 (broad)	4 (narrow)	29 (intermediate)
KP50	48 (broad)	13 (intermediate)	31 (broad)
KP53	8 (narrow)	0 (narrow)	4 (narrow)
KP54	8 (narrow)	0 (narrow)	4 (narrow)
KP55	8 (narrow)	0 (narrow)	4 (narrow)

[a]Level of the phage host range was classified when presenting lysis on *Salmonella* hosts: l-10% (narrow); 11-30% (intermediate); >31% (board).

Supplemental table 3. Blast annotation function of phage KP4.

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
1	49	1722	1674	Capsid and scaffold protein	*Salmonella* phage SE131	0.0	87%
2	1784	2068	285	Hypothetical protein	*Salmonella* phage SE131	1e-61	99%
3	2082	2273	192	Hypothetical protein SaPh711_gp011	*Salmonella* phage 7-11	2e-37	100%
4	2321	3010	690	Hypothetical protein SaPh711_gp012	*Salmonella* phage 7-11	2e-169	100%
5	3020	3796	777	Hypothetical protein SaPh711_gp013	*Salmonella* phage 7-11	3e-156	98%
6	3789	6737	2949	Phage tail fibers	*Salmonella* phage SE131	0.0	99%
7	6741	7181	441	Hypothetical protein	*Salmonella* phage SE131	6e-98	97%
8	7247	7498	252	Hypothetical protein SaPh711_gp016	*Salmonella* phage 7-11	0.0	99%
9	7495	8385	891	Putative minor structural protein	*Salmonella* phage 7-11	0.0	96%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
10	8399	10114	1716	Hypothetical protein SaPh711_gp018	*Salmonella* phage 7-11	6e-163	99%
11	10114	10338	225	Hypothetical protein SaPh711_gp019	*Salmonella* phage 7-11	2e-46	100%
12	10350	11588	1239	Putative DNA injection protein	*Salmonella* phage 7-11	6e-163	99%
13	11619	12515	897	Hypothetical protein	*Salmonella* phage SE131	1e-105	00%
14	12526	13071	546	Hypothetical protein SaPh711_gp022	*Salmonella* phage 7-11	3e-105	100%
15	13071	14708	1638	Hypothetical protein SaPh711_gp023	*Salmonella* phage 7-11	0.0	100%
16	14720	19717	4998	Hypothetical protein SaPh711_gp027	*Salmonella* phage 7-11	0.0	100%
17	19791	19922	132	Hypothetical protein SaPh711_gp028	*Salmonella* phage 7-11	6e-10	100%
18	19971	20399	429	Putative endonuclease	*Salmonella* phage 7-11	3e-100	100%
19	20396	20815	420	Hypothetical protein SaPh711_gp030	*Salmonella* phage 7-11	2e-69	100%
20	20769	20930	162	Hypothetical protein CR3_265	*Cronobacter* phage CR3	1.1	35%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
21	21316	21026	291	Hypothetical protein SaPh711_gp031	*Salmonella* phage 7-11	0.0	99%
22	22052	21297	756	Hypothetical protein	*Salmonella* phage SE131	5e-60	99%
23	22249	22049	201	Hypothetical protein	*Salmonella* phage SE131	2e-30	100%
24	22511	22242	270	Hypothetical protein	*Salmonella* phage SE131	5e-60	99%
25	22675	22508	168	Hypothetical protein	*Salmonella* phage SE131	2e-30	100%
26	22820	22680	141	Hypothetical protein SaPh711_gp035	*Salmonella* phage 7-11	5e-25	100%
27	23133	22813	321	Hypothetical protein SaPh711_gp036	*Salmonella* phage 7-11	2e-70	99%
28	23467	23123	345	Putative GTP-binding protein	*Salmonella* phage 7-11	1e-79	100%
29	23699	23553	147	Hypothetical protein SaPh711_gp038	*Salmonella* phage 7-11	3e-59	98%
30	23883	23677	207	Hypothetical protein SaPh711_gp039	*Salmonella* phage 7-11	6e-27	100%
31	24313	23873	441	Hypothetical protein SaPh711_gp040	*Salmonella* phage 7-11	1e-106	100%
32	24567	24328	240	Hypothetical protein	*Salmonella* phage SE131	0.0	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
33	24928	24560	369	Hypothetical protein SaPh711_gp042	*Salmonella* phage 7-11	3e-59	98%
34	25223	24918	306	Hypothetical protein PAK_P100040	*Pseudomonas* phage PAK_P1	1e-28	54%
35	26304	25216	1089	DNA ligase	*Salmonella* phage SE131	0.0	99%
36	26768	26352	417	Hypothetical protein SaPh711_gp044	*Salmonella* phage 7-11	1e-94	100%
37	27663	26752	912	Phage exonuclease	*Salmonella* phage SE131	0.0	100%
38	27845	27660	186	Hypothetical protein SaPh711_gp046	*Salmonella* phage 7-11	4e-36	100%
39	28412	27855	558	RNA polymerase ECF sigma factor	*Salmonella* phage 7-11	4e-135	100%
40	29275	28502	774	Hypothetical protein SaPh711_gp048	*Salmonella* phage 7-11	0.0	100%
41	30124	29810	315	Phage endolysin	*Salmonella* phage 7-11	5e-35	62%
42	30686	30138	549	Putative serine/threonine protein phosphatase	*Salmonella* phage SE131	2e-129	97%
43	30911	30765	147	Hypothetical protein	*Salmonella* phage SE131	9e-26	98%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
44	31327	30911	417	Hypothetical protein	*Salmonella* phage SE131	1e-95	98%
45	31539	31327	213	Hypothetical protein SaPh711_gp053	*Salmonella* phage 7-11	4e-43	100%
46	31749	31549	201	Hypothetical protein SaPh711_gp054	*Salmonella* phage 7-11	1e-41	100%
47	31973	31752	222	Hypothetical protein	*Salmonella* phage SE131	9e-47	99%
48	32133	31966	168	Hypothetical protein	*Salmonella* phage SE131	3e-32	98%
49	32878	32126	753	Putative Sir2-like protein	*Salmonella* phage 7-11	0.0	99%
50	33575	32871	705	Hypothetical protein SaPh711_gp057	*Salmonella* phage 7-11	2e-175	100%
51	33732	33550	183	Hypothetical protein SaPh711_gp058	*Salmonella* phage 7-11	5e-35	98%
52	33940	33722	219	Hypothetical protein SaPh711_gp059	*Salmonella* phage 7-11	6e-47	100%
53	34140	33937	204	Hypothetical protein SaPh711_gp060	*Salmonella* phage 7-11	7e-44	100%
54	34335	34219	117	Hypothetical protein SaPh711_gp061	*Salmonella* phage 7-11	3e-20	100%
55	36290	34455	1836	DNA polymerase	*Salmonella* phage 7-11	0.0	99%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
56	36591	36307	285	Hypothetical protein SaPh711_gp064	*Salmonella* phage 7-11	3e-59	100%
57	36860	36591	270	Hypothetical protein SaPh711_gp065	*Salmonella* phage 7-11	3e-49	100%
58	37585	37127	459	Hypothetical protein SaPh711_gp067	*Salmonella* phage 7-11	1e-106	99%
59	37971	37630	342	Hypothetical protein SaPh711_gp068	*Salmonella* phage 7-11	2e-57	100%
60	38153	37986	168	Hypothetical protein SaPh711_gp069	*Salmonella* phage 7-11	6e-32	100%
61	38328	38158	171	Hypothetical protein SaPh711_gp070	*Salmonella* phage 7-11	1e-33	100%
62	38828	38328	501	Hypothetical protein SaPh711_gp071	*Salmonella* phage 7-11	3e-121	99%
63	39190	38891	300	Hypothetical protein SaPh711_gp072	*Salmonella* phage 7-11	1e-64	99%
64	39639	39199	441	DNA-binding protein	*Salmonella* phage 7-11	3e-105	100%
65	40304	39639	666	Thymidylate synthase	*Salmonella* phage SE131	3e-164	100%
66	40532	40323	210	Hypothetical protein SaPh711_gp075	*Salmonella* phage 7-11	1e-43	100%
67	40699	40529	171	Hypothetical protein SaPh711_gp076	*Salmonella* phage 7-11	2e-33	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
68	40936	40703	234	Hypothetical protein SaPh711_gp077	*Salmonella* phage 7-11	1e-33	100%
69	41204	40938	267	Hypothetical protein SaPh711_gp078	*Salmonella* phage 7-11	7e-57	100%
70	41490	41194	297	Thiol-disulphide isomerase and thioredoxin	*Salmonella* phage 7-11	2e-44	100%
71	42233	41490	744	Phosphate starvation-inducible protein PhoH, predicted ATPase	*Salmonella* phage 7-11	2e-65	100%
72	42833	42246	588	Deoxycytidine triphosphate deaminase	*Salmonella* phage 7-11	2e-143	100%
73	42957	42844	114	Hypothetical protein	*Salmonella* phage SE131	1e-16	100%
74	43525	43019	507	DNA Polymerase	*Salmonella* phage 7-11	2e-122	100%
75	45337	43535	1803	Primase/helicase	*Salmonella* phage SE131	0.0	100%
76	45888	45385	504	Hypothetical protein SaPh711_gp085	*Salmonella* phage 7-11	2e-120	100%
77	46571	45909	663	Phage protein	*Salmonella* phage SE131	6e-163	99%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
78	46746	46558	189	Hypothetical protein SaPh711_gp087	*Salmonella* phage 7-11	2e-22	98%
79	47101	46733	369	Hypothetical protein SaPh711_gp088	*Salmonella* phage 7-11	4e-72	100%
80	47499	47236	264	Hypothetical protein SaPh711_gp089	*Salmonella* phage 7-11	2e-54	97%
81	47806	47486	321	Hypothetical protein SaPh711_gp090	*Salmonella* phage 7-11	6e-73	100%
82	48010	47807	204	Hypothetical protein SaPh711_gp091	*Salmonella* phage 7-11	5e-42	100%
83	48220	48011	210	Hypothetical protein SaPh711_gp092	*Salmonella* phage 7-11	2e-44	97%
84	48410	48228	183	Hypothetical protein SaPh711_gp093	*Salmonella* phage 7-11	6e-32	100%
85	49224	48646	579	Hypothetical protein SaPh711_gp094	*Salmonella* phage 7-11	5e-138	100%
86	49791	49282	510	Hypothetical protein SaPh711_gp095	*Salmonella* phage 7-11	9e-74	66%
87	49997	49791	207	Hypothetical protein SaPh711_gp096	*Salmonella* phage 7-11	2e-42	100%
88	50185	50000	186	Hypothetical protein	*Salmonella* phage SE131	5e-36	100%
89	50414	50187	228	Hypothetical protein SaPh711_gp097	*Salmonella* phage 7-11	2e-49	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
90	50671	50414	258	Hypothetical protein SaPh711_gp098	*Salmonella* phage 7-11	9e-54	98%
91	51920	50661	1260	ATP-grasp enzyme	*Salmonella* phage 7-11	0.0	100%
92	52249	51998	252	Hypothetical protein SaPh711_gp100	*Salmonella* phage 7-11	4e-52	100%
93	52473	52249	225	Hypothetical protein SaPh711_gp101	*Salmonella* phage 7-11	2e-46	100%
94	54438	52489	1950	Glutamine amidotransferase	Salmonella phage 7-11	0.0	99%
95	56212	54506	1707	Hypothetical protein SaPh711_gp103	*Salmonella* phage 7-11	0.0	99%
96	56790	56185	606	Hypothetical protein SaPh711_gp104	*Salmonella* phage 7-11	5e-149	100%
97	57604	56801	804	Hypothetical protein	*Salmonella* phage SE131	1e-172	99%
98	58692	57619	1074	Hypothetical protein SaPh711_gp106	*Salmonella* phage 7-11	0.0	99%
99	59605	58694	912	Hypothetical protein SaPh711_gp107	*Salmonella* phage 7-11	0.0	100%
100	60077	59682	396	Hypothetical protein	*Salmonella* phage SE131	0.0	98%
101	60349	60152	198	Hypothetical protein SaPh711_gp109	*Salmonella* phage 7-11	7e-38	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
102	60619	60362	258	Hypothetical protein SaPh711_gp110	*Salmonella* phage 7-11	2e-44	85%
103	60974	60624	351	Putative endolysin	*Salmonella* phage 7-11	2e-81	100%
104	61540	61289	252	Glycosyltransferase family 2 protein	*Paenibacillus contaminans*	3.8	38%
105	61992	61540	453	Hypothetical protein	*Salmonella* phage SE131	9e-64	86%
106	62190	61996	195	Hypothetical protein SaPh711_gp114	*Salmonella* phage 7-11	3e-39	98%
107	62391	62218	174	Hypothetical protein SaPh711_gp115	*Salmonella* phage 7-11	2e-19	65%
108	62702	62388	315	Hypothetical protein SaPh711_gp116	*Salmonella* phage 7-11	4e-67	97%
109	63060	62839	222	Hypothetical protein SaPh711_gp118	*Salmonella* phage 7-11	7e-45	99%
110	64727	63057	1671	Nicotinamide phosphoribosyltransferase	*Salmonella* phage 7-11	0.0	100%
111	65620	64736	885	Ribose-phosphate pyrophosphokinase	*Salmonella* phage 7-11	0.0	97%
112	66601	65960	642	Hypothetical protein SaPh711_gp121	*Salmonella* phage 7-11	4e-150	100%
113	67306	66602	705	Hypothetical protein SaPh711_gp122	*Salmonella* phage 7-11	9e-177	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
114	67520	67398	123	Hypothetical protein	*Salmonella* phage SE131	6e-05	100%
115	68460	67540	921	Hypothetical protein	*Salmonella* phage SE131	0.0	99%
116	69754	68546	1209	Putative ATPase	*Salmonella* phage 7-11	0.0	100%
117	70011	69823	189	Hypothetical protein SaPh711_gp126	*Salmonella* phage 7-11	1e-52	100%
118	70233	70015	219	Hypothetical protein SaPh711_gp127	*Salmonella* phage 7-11	1e-36	98%
119	70473	70315	159	Hypothetical protein SaPh711_gp128	*Salmonella* phage 7-11	6e-28	100%
120	70588	70433	156	Hypothetical protein SaPh711_gp128	*Salmonella* phage 7-11	3e-05	100%
121	71075	70560	516	Putative anti-sigma factor Srd	*Salmonella* phage 7-11	9e-124	100%
122	71361	71053	309	Hypothetical protein SaPh711_gp130	*Salmonella* phage 7-11	2e-67	100%
123	71846	71604	243	Hypothetical protein	*Salmonella* phage SE131	6e-48	98%
124	72019	71867	153	Hypothetical protein SaPh711_gp132	*Salmonella* phage 7-11	8e-27	88%
125	72346	72116	231	Hypothetical protein SaPh711_gp133	*Salmonella* phage 7-11	4e-48	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
126	72557	72363	195	Hypothetical protein SaPh711_gp134	*Salmonella* phage 7-11	4e-37	100%
127	73448	73269	180	Hypothetical protein SaPh711_gp135	*Salmonella* phage 7-11	1e-33	100%
128	73599	73435	165	Hypothetical protein SaPh711_gp136	*Salmonella* phage 7-11	4e-32	100%
129	73885	73568	318	Hypothetical protein SaPh711_gp137	*Salmonella* phage 7-11	3e-72	99%
130	74168	73887	282	Hypothetical protein SaPh711_gp138	*Salmonella* phage 7-11	6e-58	96%
131	74536	74174	363	Hypothetical protein SaPh711_gp139	*Salmonella* phage 7-11	7e-81	98%
132	75028	74861	168	Hypothetical protein	*Salmonella* phage SE131	8e-33	98%
133	75203	75021	183	Hypothetical protein SaPh711_gp141	*Salmonella* phage 7-11	2e-36	100%
134	75372	75205	168	Hypothetical protein	*Salmonella* phage SE131	4e-20	98%
135	75746	75369	378	Hypothetical protein SaPh711_gp142	*Salmonella* phage 7-11	1e-90	100%
136	76113	75712	402	Hypothetical protein SaPh711_gp143	*Salmonella* phage 7-11	2e-93	100%
137	76352	76110	243	Hypothetical protein SaPh711_gp144	*Salmonella* phage 7-11	3e-51	100%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
138	76469	76356	114	Hypothetical protein	*Salmonella* phage SE131	3e-06	100%
139	76839	76456	384	Hypothetical protein	*Salmonella* phage SE131	1e-73	97%
140	77030	76842	189	Hypothetical protein	*Salmonella* phage SE131	8e-20	100%
141	77674	77033	642	Hypothetical protein SaPh711_gp147	*Salmonella* phage 7-11	5e-89	98%
142	78246	77878	369	Hypothetical protein	*Salmonella* phage SE131	3e-83	99%
143	78550	78425	126	Hypothetical protein	*Salmonella* phage SE131	9e-21	100%
144	79237	78620	618	Hypothetical protein	*Salmonella* phage SE131	3e-146	99%
145	82157	82552	396	Hypothetical protein	*Salmonella* phage SE131	3e-86	96%
146	82564	82797	234	Hypothetical protein SaPh711_gp002	*Salmonella* phage 7-11	4e-47	97%
147	83367	83525	159	Hypothetical protein SaPh711_gp003	*Salmonella* phage 7-11	5e-28	100%
148	83676	85160	1485	Phage terminase, large subunit	*Salmonella* phage 7-11	0.0	100%
149	85180	87531	2352	Phage portal protein	*Salmonella* phage 7-11	0.0	99%

Supplemental table 3. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
150	87570	87761	192	Hypothetical protein SaPh711_gp006	*Salmonella* phage 7-11	3e-36	100%
151	87761	88813	1053	Phage capsid and scaffold	*Salmonella* phage 7-11	0.0	99%
152	88940	89878	939	Phage major head protein	*Salmonella* phage 7-11	0.0	99%

Supplemental table 4. Blast annotation function of phage KP5.

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
1	560	886	327	Helix-turn-helix XRE-family of transcriptional regulators	*Salmonella* phage FSL SP-099	2e-67	94%
2	3516	934	2583	DNA primase/helicase, phage-associated	*Salmonella* phage FSL SP-019	0.0	99%
3	3794	3513	282	Endolysin	*Salmonella* phage 37	1e-24	100%
4	4034	4438	405	RecT protein	*Salmonella* phage 37	1e-14	100%
5	4501	4830	330	Hypothetical protein SP35_2	*Salmonella* phage 35	1e-62	100%
6	4823	6160	1338	Cas4-like protein	Escherichia phage Utah	0.0	97%
7	6215	6811	597	DUF2815 family protein	*Salmonella* enterica	1e-122	99%
8	6878	8917	2040	DNA polymerase I	*Salmonella* phage FSL SP-124	0.0	99%
9	8919	9206	288	VRR-NUC domain-containing protein	*Salmonella* virus SPN19	2e-64	99%
10	9253	10728	1476	ATP-dependent helicase	*Salmonella* phage Siskin	0.0	99%
11	10715	11284	570	DUF1441 family protein	*Salmonella* enterica	2e-138	100%

Supplemental table 4. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
12	11271	13349	2079	Terminase large subunit	*Salmonella* phage Siskin	0.0	100%
13	13360	13614	255	Putative head-to-tail joining protein W	*Salmonella* enterica	2e-56	100%
14	13611	15290	1680	Phage portal protein	*Staphylococcus* phage SA1	0.0	100%
15	15332	16603	1272	Prohead protease ClpP	*Staphylococcus* phage SA1	0.0	99%
16	16618	17037	420	Putative decorator protein D	*Salmonella* virus iEPS5	3e-96	100%
17	17050	18114	1065	Capsid protein E	*Salmonella* virus FSLSP030	0.0	99%
18	18176	18469	294	Hypothetical protein CHI_18	*Salmonella* virus Chi	2e-32	100%
19	18472	18837	366	Hypothetical protein SP37_28	*Salmonella* phage 37	1e-81	100%
20	18837	19463	627	Hypothetical protein SP030_00100	*Salmonella* virus FSLSP030	4e-136	100%
21	19460	19963	504	Hypothetical protein SP030_00105	*Salmonella* virus FSLSP030	5e-118	100%
22	19977	21116	1140	Major tail protein	*Salmonella* phage Siskin	0.0	99%
23	21213	21674	462	Tail assembly chaperone	Escherichia phage Utah	6e-75	100%

Supplemental table 4. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
24	21719	21916	198	Tail assembly chaperone	*Salmonella* virus Chi	2e-26	100%
25	21909	26204	4296	Tail tape measure protein	*Salmonella* phage 35	0.0	99%
26	26210	27898	1689	Distal tail protein	*Salmonella* virus Chi	0.0	97%
27	27908	28726	819	DUF2163 domain-containing protein	*Salmonella* enterica	0.0	100%
28	28738	28968	231	Tail assembly protein	*Enterobacter* phage Enc34	6e-25	85%
29	28968	29207	240	Tail tape measure protein	*Salmonella* phage 37	2e-26	98%
30	29197	33087	3891	Phage tail protein	*Salmonella* virus FSLSP088	0.0	97%
31	33087	33827	741	Tail fiber protein	*Salmonella* phage FSL SP-099	6e-167	100%
32	33837	34844	1008	Hypothetical protein SP37_51	*Salmonella* phage 37	0.0	99%
33	34855	35817	963	ATP-binding cassette sub-family C member 8 isoform 2	*Salmonella* phage 35	5e-41	100%

Supplemental table 4. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
34	35831	36850	1020	Putative short transient receptor potential channel 6 isoform X1	*Salmonella* phage 35	0.0	99%
35	36865	38094	1230	Tail protein P	*Salmonella* phage 37	2e-59	98%
36	38104	40233	2130	Putative capsid protein	*Salmonella* phage 37	0.0	99%
37	40296	40634	339	Putative endolysin 2	*Salmonella* virus SPN19	8e-74	100%
38	40638	41351	714	Putative endolysin 1	*Salmonella* virus SPN19	2e-172	100%
39	41527	41357	171	Endolysin like protein	*Salmonella* virus iEPS5	8e-17	95%
40	41529	41732	204	Hypothetical protein SP030_00195	*Salmonella* virus FSLSP030	8e-37	100%
41	42194	41733	462	Deoxyribosyl transferase	*Salmonella* phage Siskin	2e-108	98%
42	43105	42191	915	NinC protein	*Salmonella* virus FSLSP088	0.0	98%
43	43520	43125	396	Hypothetical protein SP37_65	*Salmonella* phage 37	2e-92	98%
44	44221	43517	705	DNA methylase	*Salmonella* phage 37	1e-167	97%

Supplemental table 4. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
45	44675	44223	453	Hypothetical protein	*Salmonella* enterica	5e-98	93%
46	44983	44672	312	Putative DNA-directed DNA polymerase protein family A	*Salmonella* phage 37	1e-55	82%
47	45261	44980	282	Hypothetical protein	*Salmonella* enterica	1e-60	98%
48	45854	45342	513	Putative thymidylate synthase complementing protein ThyX	*Salmonella* phage 37	1e-118	98%
49	46089	45844	246	Hypothetical protein SP019_00110, partial	*Salmonella* phage FSL SP-019	3e-17	100%
50	46256	46062	195	Hypothetical protein	*Salmonella* enterica	3e-39	100%
51	47241	46267	975	Hypothetical protein	*Salmonella* phage 37	3e-103	99%
52	48008	47322	687	DNA adenine methyltransferase	*Salmonella* phage 37	1e-163	97%
53	49108	48008	1101	Hypothetical protein BSPM4_0055	*Salmonella* phage BSPM4	0.0	97%
54	49710	49105	606	Hypothetical protein SP35_70	*Salmonella* phage 35	8e-149	100%

Supplemental table 4. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
55	50447	49707	741	Hypothetical protein CPT_Siskin_055	*Salmonella* phage Siskin	0.0	99%
56	50918	50643	276	Endolysin	*Salmonella* phage 37	1e-62	100%
57	51156	50923	234	Tail fiber protein	*Salmonella* phage 35	6e-34	100%
58	52232	51159	1074	Exonuclease	*Salmonella* phage Siskin	0.0	99%
59	52554	52213	342	Coat protein	*Salmonella* phage 35	3e-48	97%
60	52876	52541	336	Hypothetical protein SPN19_014	*Salmonella* virus SPN19	6e-78	100%
61	53303	52863	441	Hypothetical protein SPN19_013	*Salmonella* virus SPN19	3e-101	100%
62	53746	53375	372	RecT family protein	*Salmonella* phage 37	2e-72	100%
63	54507	54767	261	Hypothetical protein SP124_00050	*Salmonella* phage FSL SP-124	1e-56	100%
64	54781	55320	540	Hypothetical protein CHI_65	*Salmonella* virus Chi	5e-129	98%
65	55538	56161	624	Hypothetical protein BP12C_09	*Salmonella* phage BP12C	1e-136	99%
66	56173	56376	204	Hypothetical protein	*Salmonella* enterica	4e-42	100%

Supplemental table 4. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
67	56387	56935	549	Holin family protein	*Salmonella* phage 35	1e-14	97%
68	56932	57153	222	Putative membrane protein	*Salmonella* phage Siskin	8e-33	96%
69	57279	58427	1149	Putative tail component K-like protein	*Salmonella* phage 35	3e-61	90%
70	58439	58708	270	Hypothetical protein CPT_Siskin_071	*Salmonella* phage Siskin	5e-59	99%
71	58708	58920	213	Hypothetical protein SP35_89	*Salmonella* phage 35	1e-41	100%
72	58913	59164	252	Terminase large subunit	*Salmonella* phage 37	2e-49	95%
73	59157	59408	252	Head outer capsid protein	*Salmonella* phage 35	4e-30	87%
74	59843	59956	114	Hypothetical protein	*Salmonella* enterica	3e-12	78%

Supplemental table 5. Blast annotation function of phage KP50**.**

No.	Start	Stop	Length (bp)	Product	Related organism	E-value	Identity
1	1153	1296	144	Hypothetical protein, conserved	Plasmodium ovale wallikeri	5.6	45%
2	1757	2152	396	Hypothetical protein	*Salmonella* phage SE131	3e-86	96%
3	2164	2397	234	Hypothetical protein SaPh711_gp002	*Salmonella* phage 7-11	6e-47	97%
4	3037	3195	159	Hypothetical protein SaPh711_gp003	*Salmonella* phage 7-11	5e-28	100%
5	3346	4830	1485	Terminase large subunit	*Salmonella* phage 7-11	0.0	100%
6	4849	7200	2352	Portal protein	*Salmonella* phage 7-11	0.0	99%
7	7203	7430	228	Hypothetical protein SaPh711_gp006	*Salmonella* phage 7-11	3e-44	99%
8	7430	8482	1053	Scaffolding protein	*Salmonella* phage 7-11	0.0	99%
9	8609	9547	939	Capsid and scaffold protein	*Salmonella* phage SE131	0.0	99%
10	9626	11392	1767	Capsid and scaffold protein	*Salmonella* phage SE131	0.0	92%
11	11435	11758	324	Haloacid dehalogenase-like hydrolase	Bosea sp. CF476	4.2	40%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E-value	Identity
12	11811	12095	285	Hypothetical protein SaPh711_gp010	*Salmonella* phage 7-11	1e-62	100%
13	12109	12300	192	Hypothetical protein SaPh711_gp011	*Salmonella* phage 7-11	2e-37	100%
14	12348	13037	690	Hypothetical protein SaPh711_gp012	*Salmonella* phage 7-11	2e-169	100%
15	13047	13823	777	Hypothetical protein SaPh711_gp013	*Salmonella* phage 7-11	2e-150	98%
16	13816	16767	2952	Tail protein I	*Salmonella* phage SE131	0.0	96%
17	16771	17211	441	Hypothetical protein SaPh711_gp015	*Salmonella* phage 7-11	2e-99	99%
18	17277	17528	252	Hypothetical protein SaPh711_gp016	*Salmonella* phage 7-11	4e-50	100%
19	17525	18415	891	Putative structural protein	*Salmonella* phage 7-11	0.0	98%
20	18429	20144	1716	Hypothetical protein	*Salmonella* phage SE131	0.0	99%
21	20144	20368	225	Hypothetical protein SaPh711_gp019	*Salmonella* phage 7-11	2e-46	100%
22	20380	21618	1239	Putative DNA injection protein	*Salmonella* phage 7-11	1e-162	98%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E-value	Identity
23	21649	22545	897	Hypothetical protein	*Salmonella* phage SE131	1e-105	99%
24	22556	23101	546	Hypothetical protein SaPh711_gp022	*Salmonella* phage 7-11	1e-104	99%
25	23101	24738	1638	Hypothetical protein SaPh711_gp023	*Salmonella* phage 7-11	0.0	100%
26	24750	29747	4998	Hypothetical protein SaPh711_gp027	*Salmonella* phage 7-11	0.0	100%
27	29821	29952	132	Hypothetical protein SaPh711_gp028	*Salmonella* phage 7-11	5e-10	100%
28	30001	30429	429	Putative endonuclease	*Salmonella* phage 7-11	3e-100	100%
29	30426	30845	420	Hypothetical protein SaPh711_gp030	*Salmonella* phage 7-11	2e-69	100%
30	30799	30960	162	Hypothetical protein CR3_265	*Cronobacter* phage CR3	1.0	35%
31	31345	31055	291	Hypothetical protein	*Salmonella* phage SE131	1e-58	94%
32	32081	31326	756	Hypothetical protein	*Salmonella* phage SE131	0.0	99%
33	32278	32078	201	Hypothetical protein	*Salmonella* phage SE131	2e-38	92%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E-value	Identity
34	32540	32271	270	Hypothetical protein	*Salmonella* phage SE131	5e-60	99%
35	32704	32537	168	Hypothetical protein SaPh711_gp034	*Salmonella* phage 7-11	2e-30	99%
36	32849	32709	141	Hypothetical protein SaPh711_gp035	*Salmonella* phage 7-11	5e-25	100%
37	33162	32842	321	Hypothetical protein SaPh711_gp036	*Salmonella* phage 7-11	1e-70	100%
38	33496	33152	345	Putative GTP-binding protein	*Salmonella* phage 7-11	1e-79	100%
39	33728	33582	147	Hypothetical protein SaPh711_gp038	*Salmonella* phage 7-11	3e-26	98%
40	33912	33706	207	Hypothetical protein SaPh711_gp039	*Salmonella* phage 7-11	3e-43	100%
41	34342	33902	441	Hypothetical protein SaPh711_gp040	*Salmonella* phage 7-11	1e-106	100%
42	34566	34357	210	Hypothetical protein	*Salmonella* phage SE131	1e-22	98%
43	34711	34559	153	Hypothetical protein SaPh711_gp042	*Salmonella* phage 7-11	1e-27	98%
44	34866	34678	189	Hypothetical protein SaPh711_gp042	*Salmonella* phage 7-11	2e-27	98%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
45	35153	34917	237	Hypothetical protein CNR37_00158	Pseudomonas phage ventosus	1e-21	51%
46	36303	35215	1089	DNA ligase	*Salmonella* phage SE131	0.0	99%
47	36767	36351	417	Hypothetical protein SaPh711_gp044	*Salmonella* phage 7-11	1e-94	100%
48	37662	36751	912	Exonuclease	*Salmonella* phage SE131	0.0	100%
49	37844	37659	186	Hypothetical protein SaPh711_gp046	*Salmonella* phage 7-11	4e-36	100%
50	38411	37854	558	RNA polymerase ECF sigma factor	*Salmonella* phage 7-11	4e-135	100%
51	39274	38501	774	Hypothetical protein SaPh711_gp048	*Salmonella* phage 7-11	0.0	100%
52	40111	39812	300	Putative endolysin	*Salmonella* phage 7-11	1e-67	100%
53	40655	40128	528	Putative serine/threonine phosphatase	*Salmonella* phage 7-11	2e-129	100%
53	40898	40752	147	Hypothetical protein	*Salmonella* phage SE131	9e-26	98%
55	41314	40898	417	Hypothetical protein	*Salmonella* phage SE131	2e-95	98%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
56	41526	41314	213	Hypothetical protein SaPh711_gp053	*Salmonella* phage 7-11	4e-42	99%
57	41736	41536	201	Hypothetical protein SaPh711_gp054	*Salmonella* phage 7-11	1e-41	100%
58	41960	41739	222	Hypothetical protein	*Salmonella* phage SE131	6e-47	100%
59	42120	41953	168	Hypothetical protein	*Salmonella* phage SE131	3e-32	98%
60	42826	42113	714	Putative Sir2-like protein	*Salmonella* phage 7-11	1e-176	99%
61	43562	42858	705	Hypothetical protein SaPh711_gp057	*Salmonella* phage 7-11	2e-175	100%
62	43719	43537	183	Hypothetical protein SaPh711_gp058	*Salmonella* phage 7-11	5e-35	98%
63	43927	43709	219	Hypothetical protein SaPh711_gp059	*Salmonella* phage 7-11	6e-47	100%
64	44127	43924	204	Hypothetical protein SaPh711_gp060	*Salmonella* phage 7-11	7e-44	100%
65	44636	44319	318	Hypothetical protein	*Salmonella* phage SE131	4e-71	99%
66	46510	44675	1836	DNA polymerase	*Salmonella* phage 7-11	0.0	99%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
67	46811	46527	285	Hypothetical protein SaPh711_gp064	*Salmonella* phage 7-11	3e-59	100%
68	47080	46811	270	Hypothetical protein SaPh711_gp065	*Salmonella* phage 7-11	3e-49	100%
69	47366	47085	282	Hypothetical protein SaPh711_gp066	*Salmonella* phage 7-11	2e-60	92%
70	47752	47411	342	Hypothetical protein SaPh711_gp068	*Salmonella* phage 7-11	1e-54	100%
71	47934	47767	168	Hypothetical protein SaPh711_gp069	*Salmonella* phage 7-11	6e-32	100%
72	48109	47939	171	Hypothetical protein SaPh711_gp070	*Salmonella* phage 7-11	1e-33	100%
73	48609	48109	501	Hypothetical protein SaPh711_gp071	*Salmonella* phage 7-11	3e-121	99%
74	48971	48672	300	Hypothetical protein SaPh711_gp072	*Salmonella* phage 7-11	9e-65	99%
75	49420	48980	441	DNA-binding protein	*Salmonella* phage 7-11	3e-105	100%
76	50085	49420	666	Thymidylate synthase	*Salmonella* phage 7-11	1e-163	100%
77	50313	50104	210	Hypothetical protein SaPh711_gp075	*Salmonella* phage 7-11	1e-43	100%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
78	50450	50310	141	Hypothetical protein SaPh711_gp076	*Salmonella* phage 7-11	4e-25	100%
79	50717	50484	234	Hypothetical protein SaPh711_gp077	*Salmonella* phage 7-11	1e-39	100%
80	50985	50719	267	Hypothetical protein SaPh711_gp078	*Salmonella* phage 7-11	6e-57	100%
81	51271	50975	297	Thiol-disulphide isomerase and thioredoxin	*Salmonella* phage 7-11	2e-65	100%
82	52014	51271	744	PhoH-like protein	*Salmonella* phage 7-11	0.0	99%
83	52614	52027	588	dCTP deaminase	*Salmonella* phage 7-11	2e-143	100%
84	52738	52625	114	Hypothetical protein	*Salmonella* phage SE131	1e-16	100%
85	53306	52800	507	DNA polymerase	*Salmonella* phage 7-11	2e-122	100%
86	55118	53316	1803	Primase/helicase	*Salmonella* phage 7-11	0.0	100%
87	55669	55166	504	Hypothetical protein SaPh711_gp085	*Salmonella* phage 7-11	2e-120	100%
88	56352	55690	663	Hypothetical protein SaPh711_gp086	*Salmonella* phage 7-11	8e-164	100%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
89	56527	56339	189	Hypothetical protein SaPh711_gp087	*Salmonella* phage 7-11	2e-22	98%
90	56882	56514	369	Hypothetical protein SaPh711_gp088	*Salmonella* phage 7-11	4e-72	100%
91	57280	57017	264	Hypothetical protein SaPh711_gp089	*Salmonella* phage 7-11	1e-45	98%
92	57587	57267	321	Hypothetical protein SaPh711_gp090	*Salmonella* phage 7-11	6e-73	100%
93	57791	57588	204	Hypothetical protein SaPh711_gp091	*Salmonella* phage 7-11	2e-40	98%
94	58007	57792	216	Hypothetical protein SaPh711_gp092	*Salmonella* phage 7-11	1e-44	99%
95	58940	58362	579	Hypothetical protein SaPh711_gp094	*Salmonella* phage 7-11	4e-138	100%
96	59519	58998	522	Hypothetical protein SaPh711_gp095	*Salmonella* phage 7-11	9e-124	99%
97	59725	59519	207	Hypothetical protein SaPh711_gp096	*Salmonella* phage 7-11	2e-42	100%
98	59913	59728	186	Hypothetical protein	*Salmonella* phage SE131	5e-36	100%
99	60142	59915	228	Hypothetical protein SaPh711_gp097	*Salmonella* phage 7-11	2e-49	100%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
100	60399	60142	258	Hypothetical protein SaPh711_gp098	*Salmonella* phage 7-11	3e-54	99%
101	61648	60389	1260	ATP-grasp enzyme	*Salmonella* phage 7-11	0.0	99%
102	61977	61726	252	Hypothetical protein SaPh711_gp100	*Salmonella* phage 7-11	4e-52	100%
103	62207	61977	231	Hypothetical protein SaPh711_gp101	*Salmonella* phage 7-11	2e-46	100%
104	64166	62217	1950	Glutamine amidotransferase	*Salmonella* phage 7-11	0.0	100%
105	65940	64234	1707	Hypothetical protein SaPh711_gp103	*Salmonella* phage 7-11	0.0	99%
106	66518	65913	606	Hypothetical protein SaPh711_gp104	*Salmonella* phage 7-11	5e-149	100%
107	67332	66529	804	Hypothetical protein SaPh711_gp105	*Salmonella* phage 7-11	1e-172	100%
108	68420	67347	1074	Hypothetical protein SaPh711_gp106	*Salmonella* phage 7-11	0.0	99%
109	69333	68422	912	Hypothetical protein SaPh711_gp107	*Salmonella* phage 7-11	0.0	100%
110	69805	69410	396	Hypothetical protein SaPh711_gp108	*Salmonella* phage 7-11	4e-66	100%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
111	70076	69879	198	Hypothetical protein SaPh711_gp109	*Salmonella* phage 7-11	7e-38	100%
112	70346	70089	258	Hypothetical protein SaPh711_gp110	*Salmonella* phage 7-11	2e-55	100%
113	70701	70351	351	Putative endolysin	*Salmonella* phage 7-11	2e-81	100%
114	71267	71016	252	Glycosyltransferase family 2 protein	Prevotella shahii	1.9	45%
115	71719	71267	453	Hypothetical protein	*Salmonella* phage SE131	2e-81	95%
116	71945	71712	234	Hypothetical protein	*Salmonella* phage SE131	2e-48	95%
117	72142	71969	174	Hypothetical protein SaPh711_gp115	*Salmonella* phage 7-11	2e-19	65%
118	72453	72139	315	Hypothetical protein SaPh711_gp116	*Salmonella* phage 7-11	1e-67	98%
119	72811	72590	222	Hypothetical protein SaPh711_gp118	*Salmonella* phage 7-11	1e-44	97%
120	74478	72808	1671	Nicotinamide phosphoribosyl transferase	*Salmonella* phage 7-11	0.0	99%
121	75371	74487	885	Putative ribose-phosphate pyrophosphokinase	*Salmonella* phage 7-11	0.0	98%

Supplemental table 5. (Cont.)

No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
122	76352	75711	642	Hypothetical protein SaPh711_gp121	*Salmonella* phage 7-11	4e-150	100%
123	77057	76353	705	Hypothetical protein SaPh711_gp122	*Salmonella* phage 7-11	3e-176	99%
124	77271	77149	123	Hypothetical protein	*Salmonella* phage SE131	6e-05	100%
125	78211	77291	921	Hypothetical protein	*Salmonella* phage SE131	0.0	98%
126	79505	78297	1209	Putative ATPase	*Salmonella* phage 7-11	0.0	100%
127	79762	79574	189	Hypothetical protein SaPh711_gp126	*Salmonella* phage 7-11	1e-36	98%
128	79930	79766	165	Hypothetical protein SaPh711_gp127	*Salmonella* phage 7-11	4e-32	100%
129	80224	80066	159	Hypothetical protein SaPh711_gp128	*Salmonella* phage 7-11	6e-28	100%
130	80339	80184	156	Hypothetical protein SaPh711_gp128	*Salmonella* phage 7-11	2e-0	95%
131	80829	80311	519	Hypothetical protein	*Salmonella* phage SE131	1e-122	99%
132	81112	80804	309	Hypothetical protein SaPh711_gp130	*Salmonella* phage 7-11	1e-66	99%

Supplemental table 5. (Cont.)

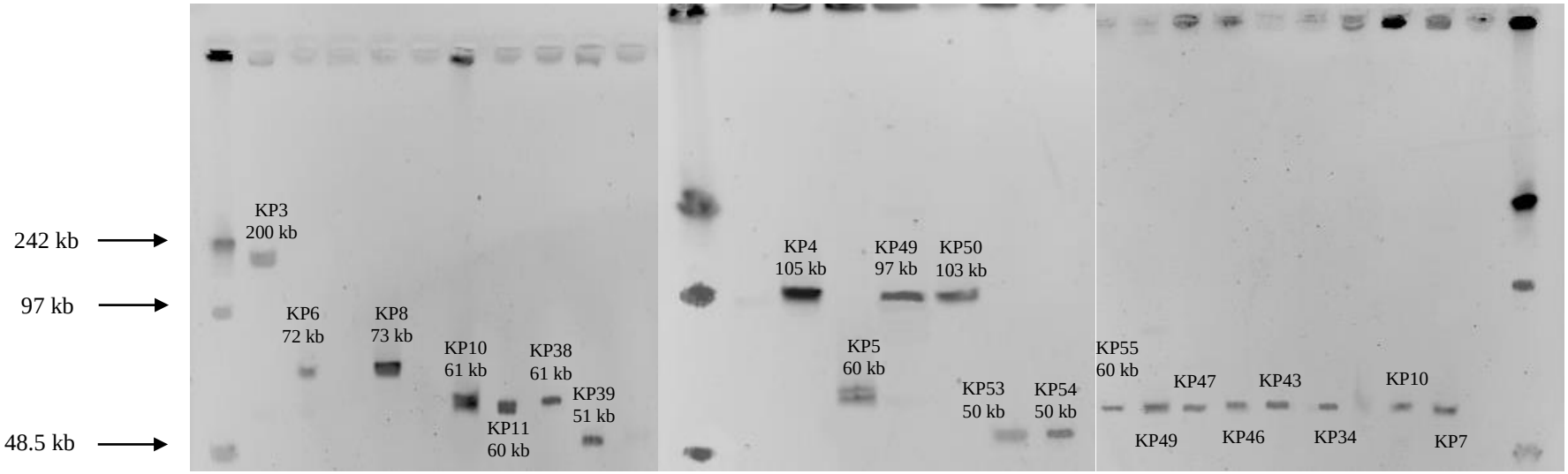
No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
133	81598	81356	243	Hypothetical protein SaPh711_gp131	*Salmonella* phage 7-11	2e-49	96%
134	81771	81619	153	Hypothetical protein SaPh711_gp132	*Salmonella* phage 7-11	5e-29	94%
135	82098	81868	231	Hypothetical protein SaPh711_gp133	*Salmonella* phage 7-11	3e-45	95%
136	82309	82115	195	Hypothetical protein SaPh711_gp134	*Salmonella* phage 7-11	4e-37	100%
137	83201	83022	180	Hypothetical protein SaPh711_gp135	*Salmonella* phage 7-11	1e-33	100%
138	83352	83188	165	Hypothetical protein SaPh711_gp136	*Salmonella* phage 7-11	1e-31	98%
139	83638	83321	318	Hypothetical protein	*Salmonella* phage SE131	4e-71	99%
140	83921	83640	282	Hypothetical protein SaPh711_gp138	*Salmonella* phage 7-11	7e-59	98%
141	84289	83927	363	Hypothetical protein	*Salmonella* phage SE131	1e-80	97%
142	84781	84614	168	Hypothetical protein SaPh711_gp140]	*Salmonella* phage 7-11	4e-31	98%
143	84956	84774	183	Hypothetical protein SaPh711_gp141	*Salmonella* phage 7-11	2e-36	100%

Supplemental table 5. (Cont.)

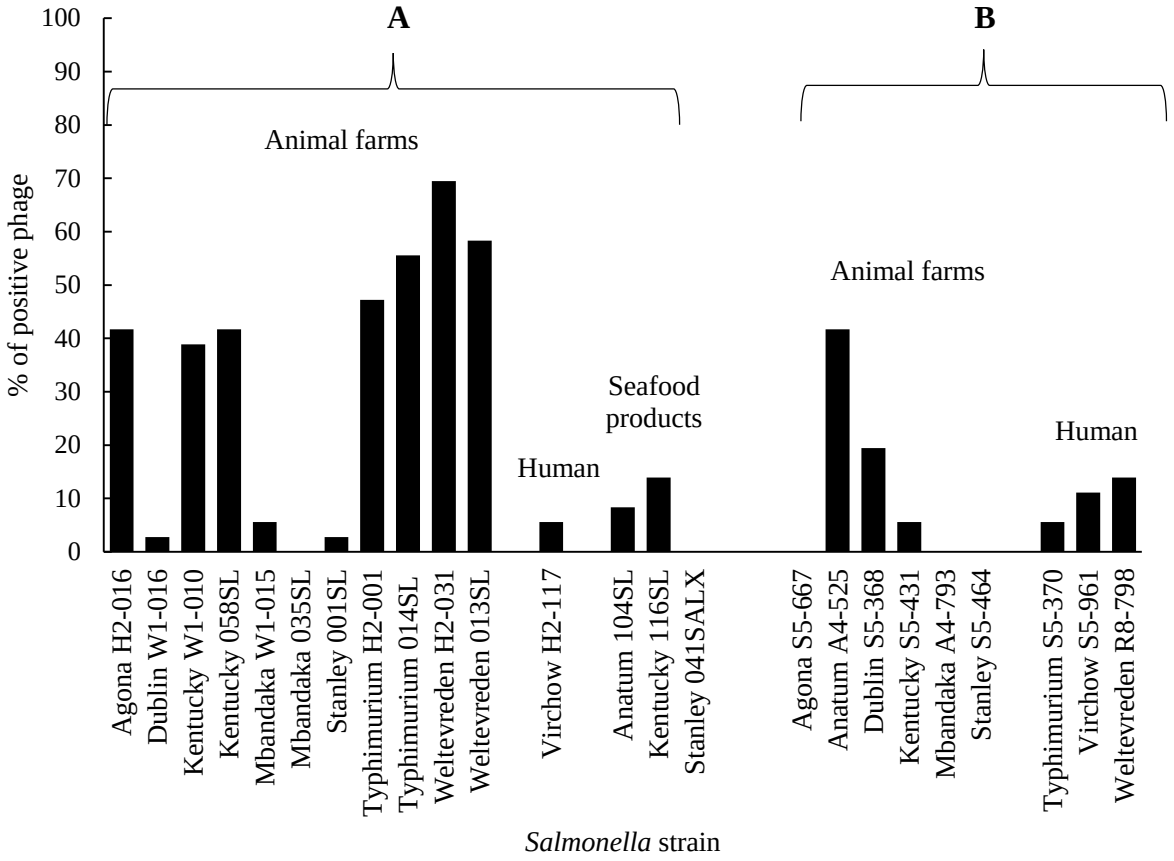
No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
144	85125	84958	168	Hypothetical protein	*Salmonella* phage SE131	1e-20	100%
145	85499	85122	378	Hypothetical protein SaPh711_gp142	*Salmonella* phage 7-11	3e-87	98%
146	85869	85465	405	Hypothetical protein SaPh711_gp143	*Salmonella* phage 7-11	1e-62	72%
147	86108	85866	243	Hypothetical protein SaPh711_gp144	*Salmonella* phage 7-11	3e-51	100%
148	86225	86112	114	Hypothetical protein	*Salmonella* phage SE131	3e-06	100%
149	86595	86212	384	Hypothetical protein	*Salmonella* phage SE131	2e-86	97%
150	86786	86598	189	Hypothetical protein SaPh711_gp146	*Salmonella* phage 7-11	2e-21	100%
151	87358	86789	570	Hypothetical protein	*Salmonella* phage SE131	2e-120	97%
152	87569	87360	210	Hypothetical protein SaPh711_gp148	*Salmonella* phage 7-11	2e-44	100%
153	87930	87562	369	Hypothetical protein	*Salmonella* phage SE131	3e-83	99%
154	88234	88109	126	Hypothetical protein	*Salmonella* phage SE131	9e-21	100%

Supplemental table 5. (Cont.)

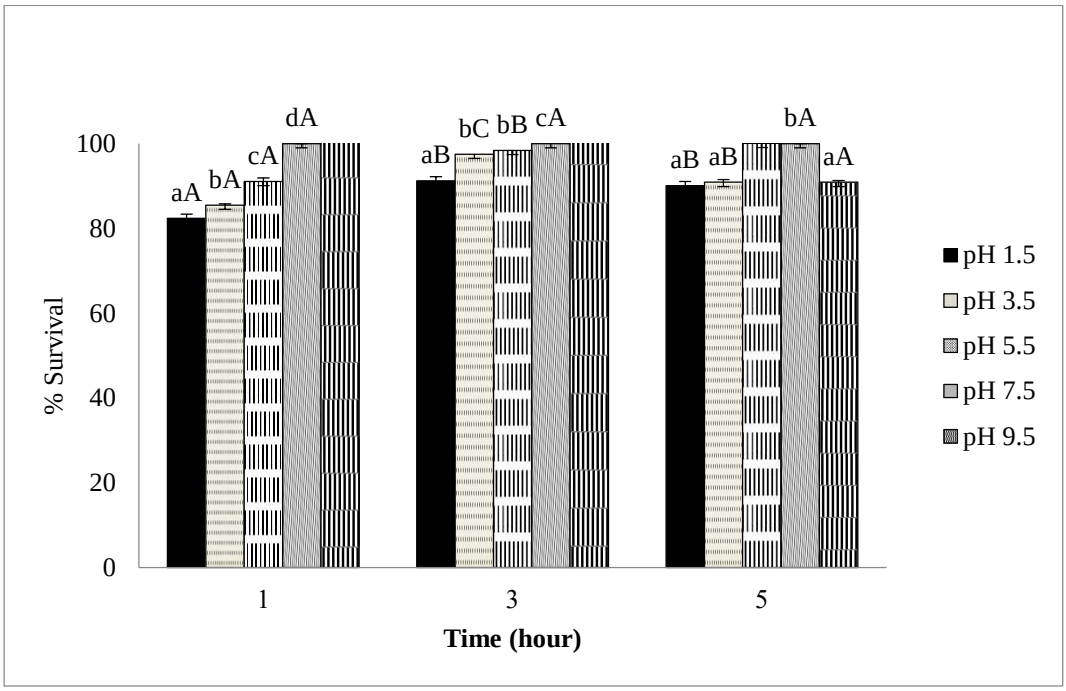
No.	Start	Stop	Length (bp)	Product	Related organism	E value	Identity
155	88921	88304	618	Hypothetical protein SaPh711_gp151	*Salmonella* phage 7-11	1e-141	96%



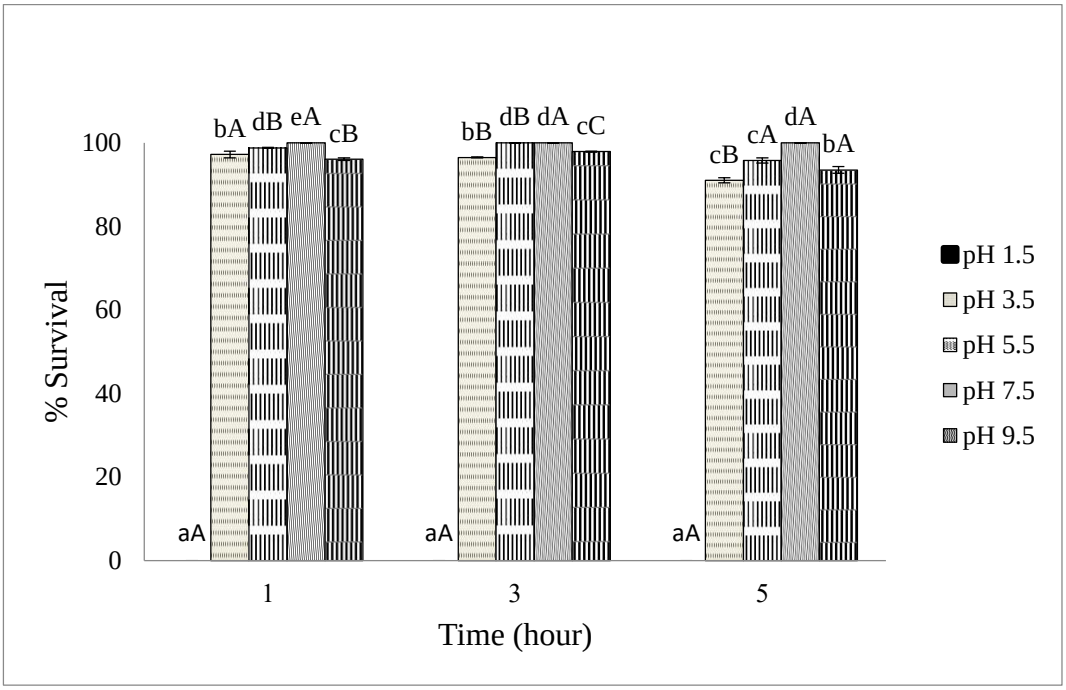
Supplemental figure 1. Estimated genome size (kb) of *Salmonella* phages isolated in this study.



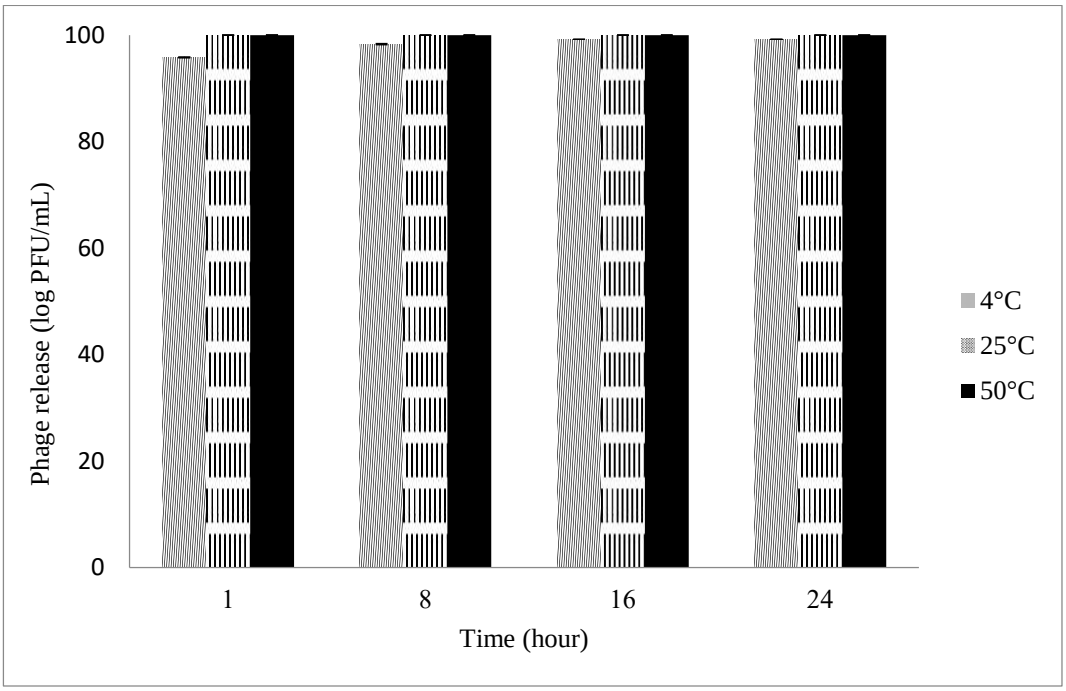
Supplemental figure 2. Percentage of positive phage on *Salmonella* strains (serovars) isolated from different sources in Thailand (A) and US (B).



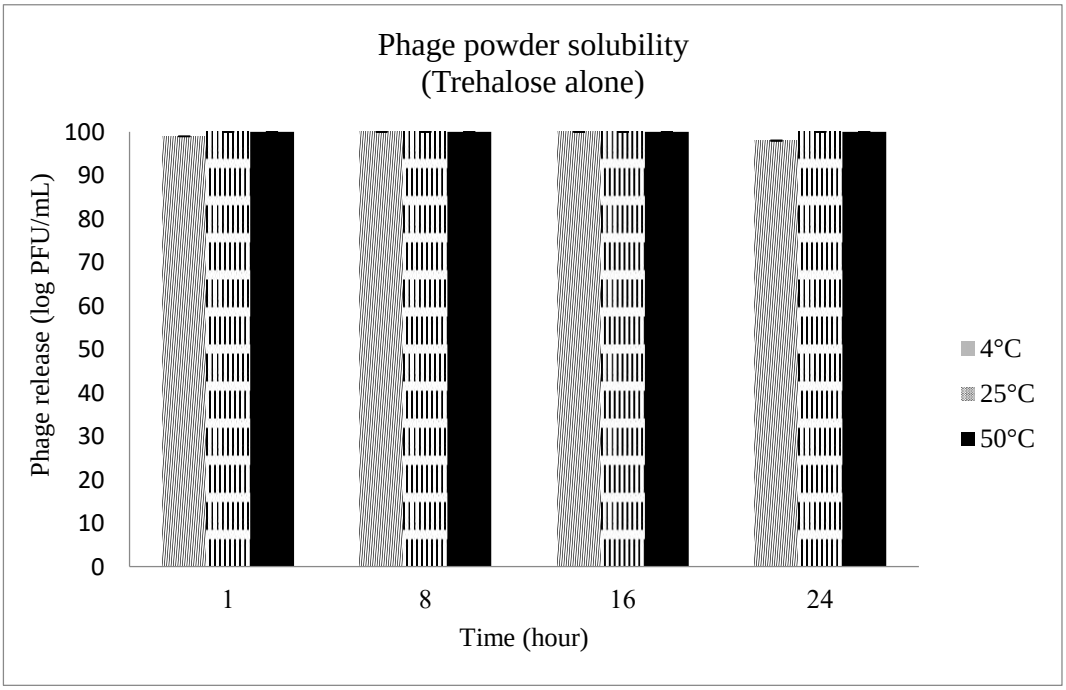
Supplemental figure 3 Stability of phage powder at various pH (WPI alone).



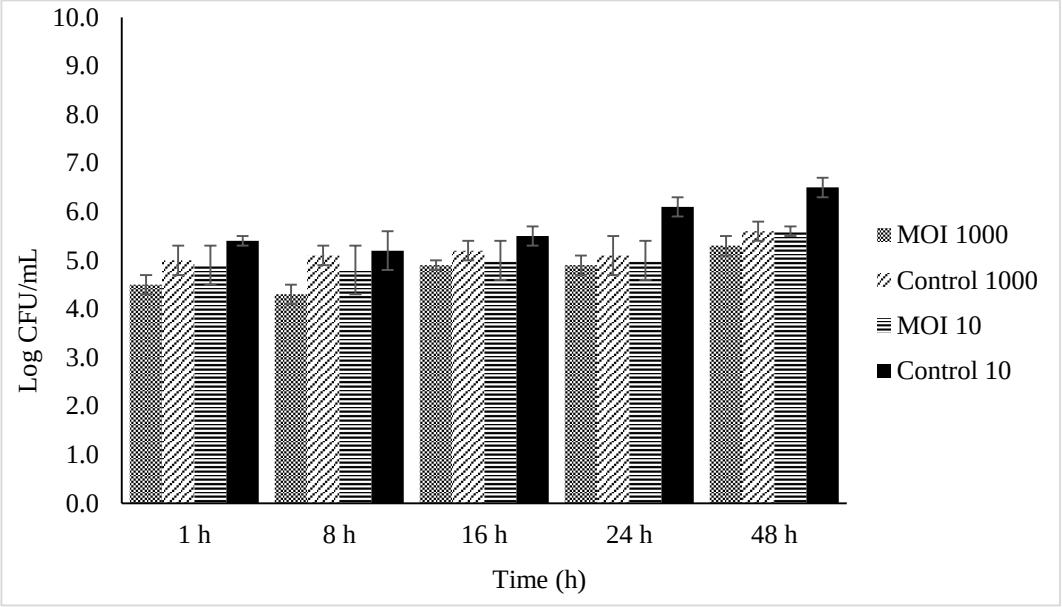
Supplemental figure 4 Stability of phage powder at various pH (trehalose alone).



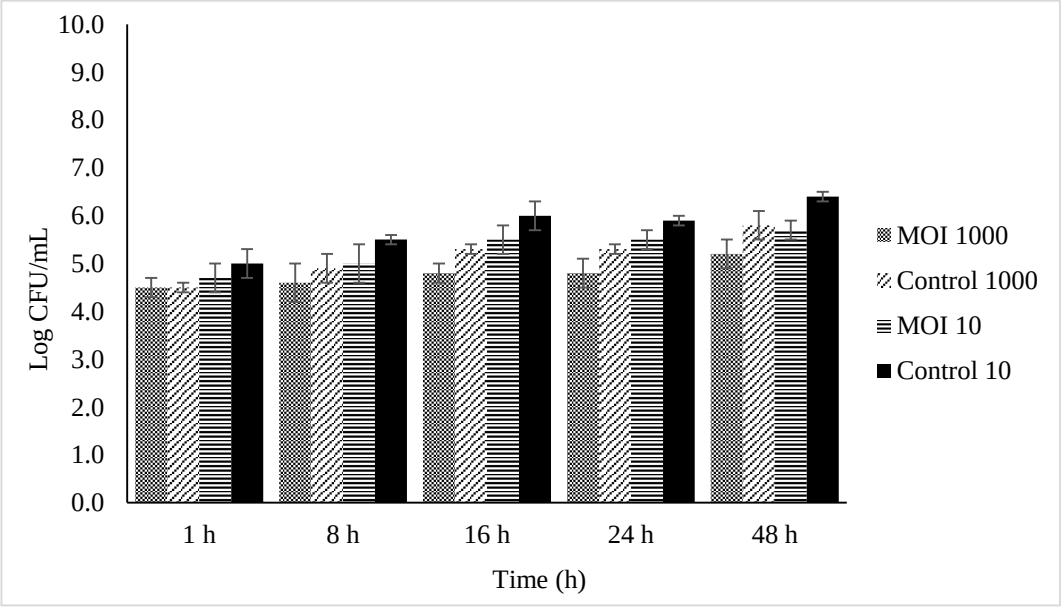
Supplemental figure 5 Stability of phage powder at various temperature (WPI alone).



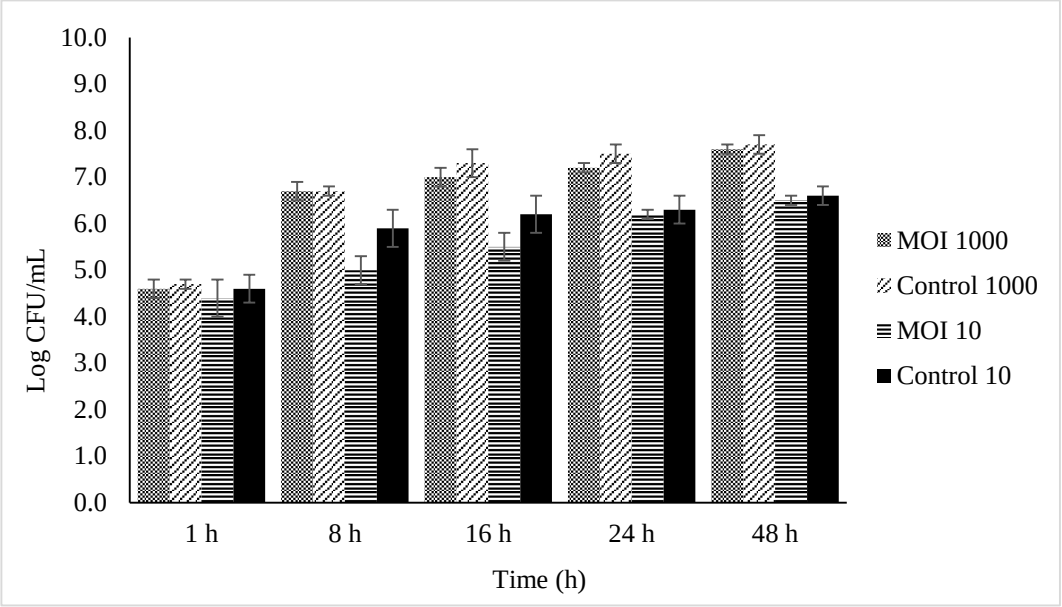
Supplemental figure 6 Stability of phage powder at various temperature (trehalose alone).



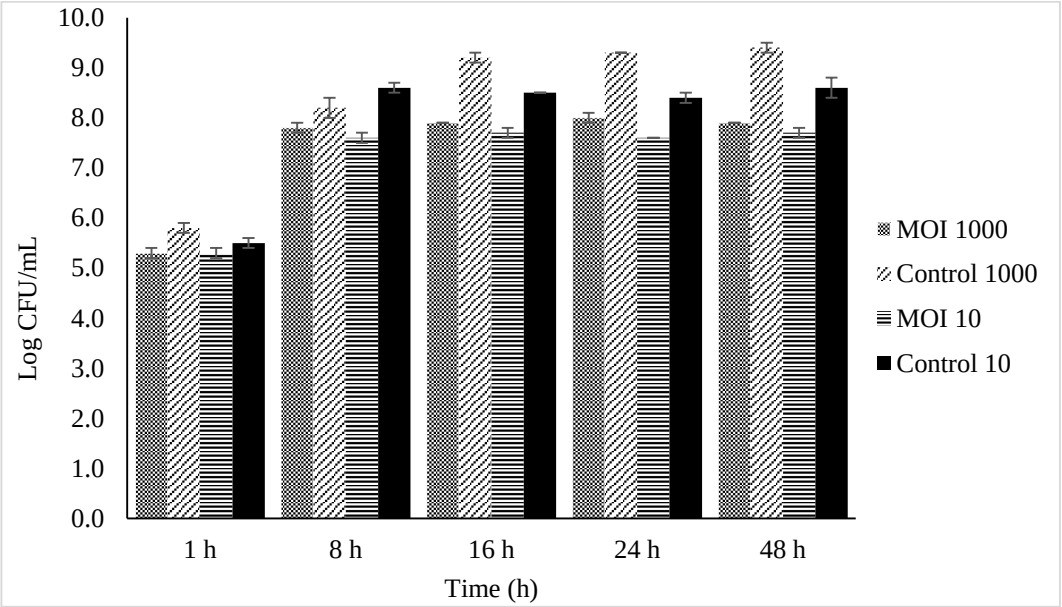
Supplemental figure 7 Efficiency of phage powder (WPI alone) to reduce *S.* Typhimurium at10°C.



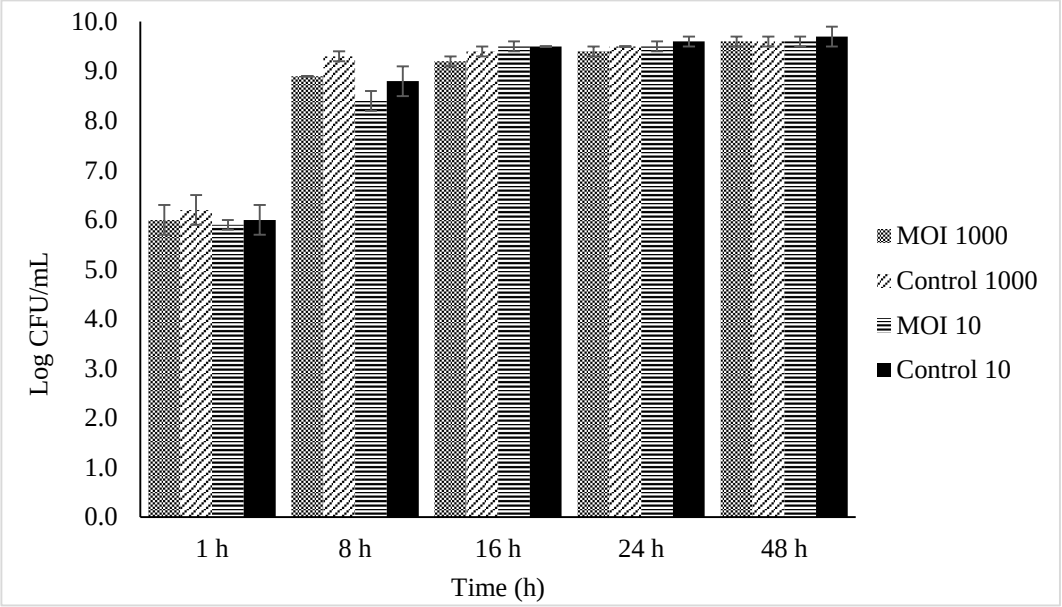
Supplemental figure 8 Efficiency of phage powder (trehalose alone) to reduce *S.* Typhimurium at10°C.



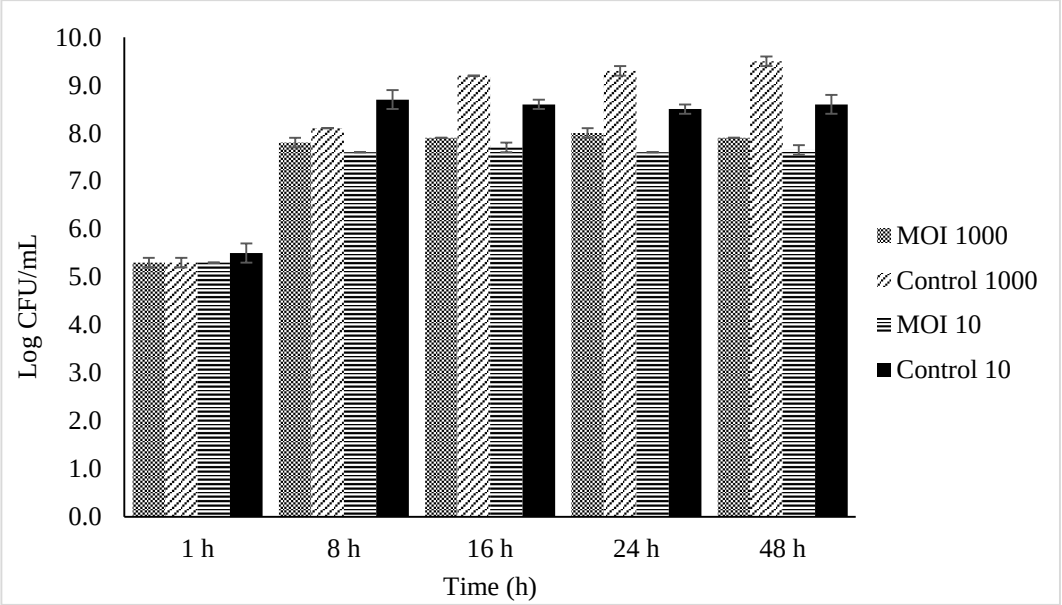
Supplemental figure 9 Efficiency of phage powder (trehalose alone) to reduce *S.* Enteritidis at 10˚C.



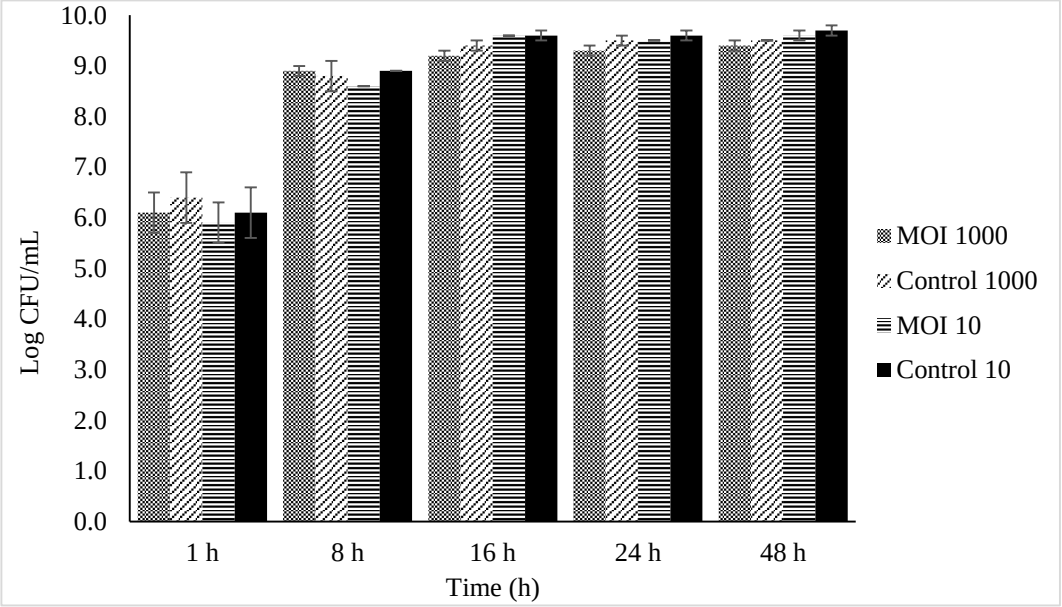
Supplemental figure 10 Efficiency of phage powder (WPI alone) to reduce *S.* Typhimurium at 30˚C.



Supplemental figure 11 Efficiency of phage powder (trehalose alone) to reduce *S.* Typhimurium at 30˚C.



Supplemental figure 12 Efficiency of phage powder (WPI alone) to reduce *S.* Enteritidis at 30˚C.



Supplemental figure 13 Efficiency of phage powder (trehalose alone) to reduce *S.* Enteritidis at 30˚C

VITAE

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Educational Attainment

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- Graduate School Dissertation Funding for Thesis from Prince of Songkla University.

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Awards:

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List of Publication:

Petsong, K., Uddin, M. J., Vongkamjan, K. and Ahn, J. 2018. Combined effect of bacteriophage and antibiotic on the inhibition of the development of antibiotic resistance in *Salmonella* Typhimurium. Food Sci Biotechnol. 27(4) : 1239-1244.

Petsong, K., Benjakul, S., Chaturonggakul, S., Moreno Switt, A. I. and Vongkamjan, K. 2019. Lysis profiles of *Salmonella* phages on *Salmonella* isolates from various sources and efficiency of a phage cocktail against *S*. Enteritidis and *S*. Typhimurium. Microorganisms. 7(4) : 100. https://doi.org/10.3390/microorganisms7040100.

List of book chapter:

Petsong, K., Vongkamjan, K. Applications of *Salmonella* bacteriophages in the food production chain. The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Program. Formatex 2015, 275–283.

List of Patent/Petty Patent:

Formulation of microencapsulated *Salmonella* bacteriophage as dry powder and the process. Petty patent, No. 1703001885.

List of Presentations:

K. Petsong, S. Benjakul, B. Sripaurya, S. Chaturongkakul, A. I. Moreno Switt, V. Vuddhakul, and K. Vongkamjan. Isolation and characterization of *Salmonella* phages isolated from various food-associated environments. 10th IMT-GT UNINET Conference 2016 (Bioscience: The Element of Life). December 1–2, 2016. Prince of Songkla University, Hat Yai, Thailand (Poster).

K. Petsong, S. Benjakul, B. Sripaurya, S. Chaturongkakul, A. I. Moreno Switt, V. Vuddhakul, and K. Vongkamjan. Isolation and characterization of *Salmonella* phages isolated from various food-associated environments. IV International Conference on Antimicrobial Research (ICAR2016). June 29-July 1, 2016. Terremolinos, Malaga, Spain (Poster).

K. Petsong, S. Benjakul, and K. Vongkamjan. Prevalence and host range diversity of *Salmonella* phages isolated from livestock farms in Thailand. International Union of Microbiological Societies (IUMS 2017 Singapore). July 17-21, 2017. Sands Expo & Convention Centre, Singapore (Poster).