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Evaluation of storage conditions and efficiency of a novel microencapsulated *Salmonella* phage cocktail for controlling *S.* enteritidis and *S.* typhimurium *in-vitro* and in fresh foods

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ABSTRACT

S. Enteritidis and *S.* Typhimurium are typically linked to foodborne outbreaks. Phages have continued to expand in various food applications. In this study, microencapsulation is applied for enhancing the stability and efficacy of phages as bio-control agent. Microencapsulated phage cocktail kept in aluminium laminated foil bag (LF) at 4 °C showed the highest survivability with a titer loss of 0.5 log PFU/g after 12 weeks of storage. Titer loss of phage cocktail lysate > 4 log PFU/mL was observed after 12 weeks, at 4 °C. Color change of microencapsulated phage cocktail kept in LF at 4 °C did not show any significant difference during storage, and water activity (free water content) at 0.13 was found in these conditions. *In-vitro* study, *S.* Enteritidis and *S.* Typhimurium were decreased 1.79 and 3.63 log CFU/mL, respectively at 37 °C. Whereas, 0.43 and 0.76 log CFU/mL, respectively were observed at 10 °C. In foods, *S.* Enteritidis and *S.* Typhimurium were decreased 0.57 and 1.78 log CFU/cm^2, respectively in meat. Whereas, 0.86 and 1.2 log CFU/g, respectively were observed in sprout. Foods with/without microencapsulated phage cocktail showed non-significant differences in liking scores after 2 days of storage. Overall, microencapsulated phage cocktail suggests another alternative for phage-based biocontrol with improved stability and efficacy for food application.

1. Introduction

Salmonellosis, an infection caused by non-typhoidal *Salmonella* (NTS), has been reported as the major cause leading to high cases of hospitalization and death each year (Eng et al., 2015). In 2016, over 94,530 confirmed salmonellosis cases were reported in the European countries (European Food Safety Authority, 2017). From over 2,600 known 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 (Centers of Diseases Control and Prevention, 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). Recently the emergence of antibiotic-resistant *Salmonella* found in food has been emphasized, control of *Salmonella* in foods is particularly needed to reduce the spread of *Salmonella* with this characteristics (Choe et al., 2014; Hong et al., 2016)

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 that are commonly used in meat products which have some negative effects on human health (Kazi and Annapure, 2016). Generally, phages can be added to certain foods without affecting the quality and sensory of foods (Fister et al., 2016). Phages have been previously 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, the lysate form of phages could lead to a decrease in the efficacy as indicated by several limitations, for example, rapid loss of the phage titer and undesirable form for some food applications. In this study, microencapsulation technique is of interest for use to protect the phages and improve the phage efficiency and applications. Microencapsulated phage can be defined as another form of phages upon microencapsulation process which allows phage particles to be surrounded by other substances which are normally called wall material. Mixture of the phage particles and wall materials are then transformed by various techniques (e.g., spray drying, extrusion, and freeze drying) to a very

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small size up to a nano-scale. While phage encapsulation technique has been previously studied, most studies reported the potential form of microencapsulated phage as alginate-based beads (Colom et al., 2017; Ma et al., 2008). There are also limited reports on applications of microencapsulated phages in foods, while most of its uses related to therapeutic medicine have been reported (Saboo et al., 2016; Soykut et al., 2019; Tang et al., 2015; Vinner et al., 2019). In addition, the microencapsulated form of phages as alginate-based beads is speculated to lead to some limitations for food applications due to its detectable size and physical properties when applied to foods. Microencapsulated form of phages as a dry powder is thus of interest for further investigation to improve phage 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 a long-term storage of 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). However, certain environmental factors such as temperature and UV light could affect the survivability of phage particles during storage (Jończyk et al., 2011). Suitable storage conditions including temperature packaging types are needed to investigate. This study evaluated the stability of the newly developed microencapsulated phage cocktail kept in two major packaging material types (aluminium laminated foil; LF and low density polyethylene; LDPE) at 4 °C and 25 °C. Aluminium laminated foil bag (LF) presented the wide application in food packaging. This is due to its desirable barrier function against the migration of moisture, oxygen or impact of light, providing shelf-life stability of food (Lamberti and Escher, 2007). Low density polyethylene (LDPE) is the general packaging material which provides the resistance properties towards acid/alkali and alcohol, moderate in oil, and greases (Emblem, 2012). Phage survivability, physical (color), and chemical properties (water activity) of microencapsulated phage cocktail were evaluated during a storage study of 12 weeks. The efficiency of microencapsulated phage cocktail in controlling *S.* Enteritidis and *S.* Typhimurium were investigated *in-vitro* and in food models, including raw chicken meat and sunflower sprout. The overall acceptability of food treated with microencapsulated phage was evaluated through sensory analysis.

2. Materials and methods

2.1. Salmonella strains and Salmonella phages used in this study

A total of four strains of *Salmonella* were used in this study. Of these, two *Salmonella* strains were obtained from the Food Safety Lab, Cornell University, including *Salmonella enterica* serovar Anatum FSL A4-525 which was isolated from bovine in the United States and *Salmonella enterica* serovar Enteritidis FSL S5-371 which was isolated from human in the United States. Other two *Salmonella* isolates were obtained from the Faculty of Microbiology, Mahidol University, including *Salmonella enterica* serovar Agona H2-016, and *Salmonella enterica* serovar Typhimurium H2-001. Both were isolated from pig slaughterhouse in Thailand. *Salmonella* strains were stored in 15% glycerol at −80 °C as working stocks. For all experiments, a single colony of *Salmonella* grown on Tryptone Soya Agar (TSA; Oxoid, Hampshire, UK) was transferred into 5 mL of Tryptone Soya Broth (TSB; Oxoid, Hampshire, UK) and incubated at 37 °C for 16−18 h for preparation of overnight culture.

Three *Salmonella* bacteriophages were included in a phage cocktail in this study (SLP004, SLP005 and SLP050). These phages were isolated from environmental samples from poultry farm in Songkhla province, Thailand in 2014. The three phages have been previously characterized as broad host range, belonging to the *Siphoviridae* family. A phage cocktail preparation consisted of approximately 3×10^6 PFU/mL to 5×10^6 PFU/mL of each individual phage. In addition, high efficiency of plating (EOP) of the three phages has been confirmed on both *S.* Enteritidis and *S.* Typhimurium (data not shown).

2.2. Preparation of phage cocktail and phage titer determination

Lysate stock of each phage was used to prepare 10-fold serial dilutions in Phosphate Buffered Saline (PBS, pH 7.4). Appropriate dilutions were used to prepare the overlay (0.7% TSA) with a given host to yield semi-confluent lysis. Overlay agar was harvested with 10 mL of Salt-Magnesium buffer (SM buffer), followed by centrifugation at 3213 × *g* for 15 min at 4 °C. Supernatant was filtered through a 0.22-μm syringe filter and stocks of phage lysate were kept at 4 °C. Titers of each phage lysate was determined by serially diluted in PBS and 100 μl of each dilution was mixed with 300 μl of the *S.* Enteritidis host and 4 mL of overlay (Moreno Switt et al., 2013). The mixture was poured on bottom agar, followed incubation at room temperature for 16−24 h. Plaques present on each plate of a given dilution were enumerated (Vongkamjan et al., 2012).

2.3. Development of microencapsulated phage cocktail

In this study, the formula for phage encapsulation previously optimized was used. Phage cocktail lysate was mixed with the wall materials consisting of whey protein isolate (WPI) (Davisco Foods International, USA) and trehalose dihydrate (Wako Pure Chemical Industries, Ltd, Japan) at ratio of 3:1 (w/w) to achieve a final concentration of 10% total solid. 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 a coating mixture. The mixture was solidified at −40 °C for 24 h. Frozen mixture was tempered at −50 °C using a laboratory scale freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) for 48 h. Phages titer after freeze-drying were enumerated by the plaque assay as mentioned above.

2.4. Stability of microencapsulated form (dry powder) of phage cocktail during storage

Stability of phage cocktail as dry 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 the microencapsulated form. 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 food quality, a_w is generally used to indicate the appearance of food product. The change of a_w can be affected by the chemical and biochemical reactions of food product (Fontana, 2000). For food safety, a_w indicates water availability for growth of microorganisms and physicochemical stability of low-moisture products (Langová et al., 2012). For survivability, 1 mL of the phage cocktail lysate or 1 g of microencapsulated phage cocktail was taken for enumeration by the plaque assay as mentioned above. For microencapsulated form, 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 using a Hunter Lab Miniscan colorimeter (HunterLab Reston, VA, USA). A numerical total color difference (ΔE*) was calculated by the following equation: $\Delta E^* = [(L^* - L^*_{ref})^2 + (a^* - a^*_{ref})^2 + (b^* - b^*_{ref})^2]^{1/2}$, where L* is lightness, a* is redness/greenness and b* is yellowness/blueness. The control sample (sample at week 0) was used as reference value (Francis and Clydesdale, 1975). Water activity of microencapsulated phage cocktail was measured by Aqua-Lab Water Activity Meter (Series 3, Decagon Devices, Inc., Pullman, WA) at 25 °C. The experiment was performed in three independent studies.

2.5. Efficiency of microencapsulated phage cocktail against S. Enteritidis and S. Typhimurium in-vitro

The efficiency of microencapsulated phage cocktail against *S.* Enteritidis and *S.* Typhimurium was investigated at 10 °C and 37 °C. Overnight cultures of *S.* Enteritidis and *S.* Typhimurium were prepared as mentioned earlier, 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 in TSB to achieve the level of 10^5 CFU/mL. Microencapsulated phage cocktail was mixed with TSB and diluted to achieve the phage concentration at 10^7 PFU/mL. The co-culture consisting of 5 mL of microencapsulated phage cocktail solution and 5 mL of each *Salmonella* culture was incubated at 10 °C or 37 °C in a shaking incubator (ThermoStableTM IS-30 model, DAIHAN Scientific, Korea) at 220 rpm for 12 h. Controls with 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 survived colony enumeration. The experiment was performed in three independent studies.

2.6. Efficiency of microencapsulated phage cocktail against S. Enteritidis and S. Typhimurium in foods

Chicken meat and sunflower sprout were selected as representative high-risk foods of *Salmonella* contamination. Samples were purchased from the supermarkets on the day of study. To eliminate *Salmonella* that may be present, both samples were soaked in 50 ppm free chlorine concentration solution (Sigma-Aldrich, St. Louis, Mo, USA) for 5 min. Subsequently, samples were soaked and washed in sterile distilled water for 5 min three times. Chicken meat was aseptically cut into pieces of approximately 5 × 5 cm^2 and sunflower sprout was aseptically weight approximately 5 g. Cell suspension of *S.* Enteritidis or *S.* Typhimurium with the initial level of 10^5 CFU/mL was added to each food by evenly spread on the food surface to achieve inoculation level of approximately 10^5 CFU/piece or 10^5 CFU/5 g. Samples were left at 25 °C for 10 min to allow the cells to adapt to the conditions on food samples tested. Microencapsulated phage cocktail 0.1 g with the concentration of 10^8 PFU/g (newly prepared) 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 selective media for *Salmonella*, Xylose-Lysine-Desoxycholate agar (XLD; Oxoid, Hampshire, UK). Cell numbers were also confirmed on TSA. The experiment was performed in three independent studies.

2.7. Evaluation of the overall acceptability of consumers towards the quality of food products applied with microencapsulated phage cocktail

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 microencapsulated phage cocktail. Food samples without microencapsulated phage cocktail were also prepared for evaluation. Liking scores were given for color, odor, freshness, 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).

2.8. Statistical analysis

Statistical analysis was analyzed for the experiments, including color, and a_w of microencapsulated phage cocktail during storage in different packaging materials (LF and LDPE) and different temperatures (4 °C and 25 °C), log reduction of *S.* Enteritidis or *S.* Typhimurium *in-vitro* and in foods, sensory and color evaluation of food models with/without microencapsulated phage cocktail during storage. All experimental assays were performed in three independent studies. The differences between two treatments were compared within a given storage time, including (i) ΔE values of microencapsulated phage cocktail in different packaging types and temperatures, (ii) liking scores of food models with/without microencapsulated phage cocktail for each sensory parameter, and (iii) ΔE values of food models with/without microencapsulated phage cocktail were analyzed by Student's t-test. The analysis of (i), (ii), (iii) and also log reduction of *S.* Enteritidis or *S.* Typhimurium *invitro* and in foods study were performed using Analysis of variance (ANOVA) to compare results among each day and 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).

3. Results

3.1. Microencapsulated phage cocktail as a dry powder form remained high titers during a storage time at low temperature (4 °C)

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 refrigeration temperature (4 °C) and room temperature (25 °C). Microencapsulated phage cocktail as a dry powder showed stable phage titer with a slight loss of phage titer (approximately 0.5 log PFU/g) during 12 weeks at 4 °C (Fig. 1). At 25 °C, total phage loss in a dry powder form 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, the phage lysate lost all titer after 4 weeks of storage 25 °C.

3.2. Aluminium laminated foil bag (LF) and low temperature provided microencapsulated phage cocktail with more desirable physical and chemical properties

For storage at 4 °C and 25 °C, microencapsulated phage cocktail in both packaging types, LF and LDPE bags, showed similar phage titer during 12 weeks of storage (Fig. 1). Evaluation of the stability of physical property of microencapsulated phage cocktail showed that color (ΔE values) of a dry phage powder was not significantly different ($p < 0.05$) when kept in neither LF nor LDPE bag at 4 °C or 25 °C on a given week of sample evaluation (Table 1). When compared ΔE values of a dry phage powder kept in LDPE bag, significant difference ($p < 0.05$) was observed between storage at 4 °C and 25 °C. However, ΔE values showed non-significant difference ($p < 0.05$) for microencapsulated phage cocktail kept in LF bag at both temperature conditions.

For evaluation of the chemical property, the initial a_w of microencapsulated phage cocktail was 0.03 (Table 2). In this study, a_w of microencapsulated phage cocktail increased continuously at both 4 °C and 25 °C. After 4 weeks of storage, a_w of microencapsulated phage cocktail 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 0.33 and 0.13 for microencapsulated phage cocktail kept in LDPE bag and LF bag, respectively. At 25 °C, after 7 weeks of storage, a_w of microencapsulated phage cocktail contained in LF bag was 0.32 which was lower than that kept in LDPE bag with a_w of 0.43.

3.3. Microencapsulated phage cocktail showed high efficiency against S. Enteritidis and S. Typhimurium at 10 °C and 37 °C in-vitro as well as in raw chicken meat and sunflower sprout at refrigeration temperature

The efficacy of microencapsulated phage cocktail for controlling *S.* Enteritidis and *S.* Typhimurium *in vitro* was investigated at 10 °C and

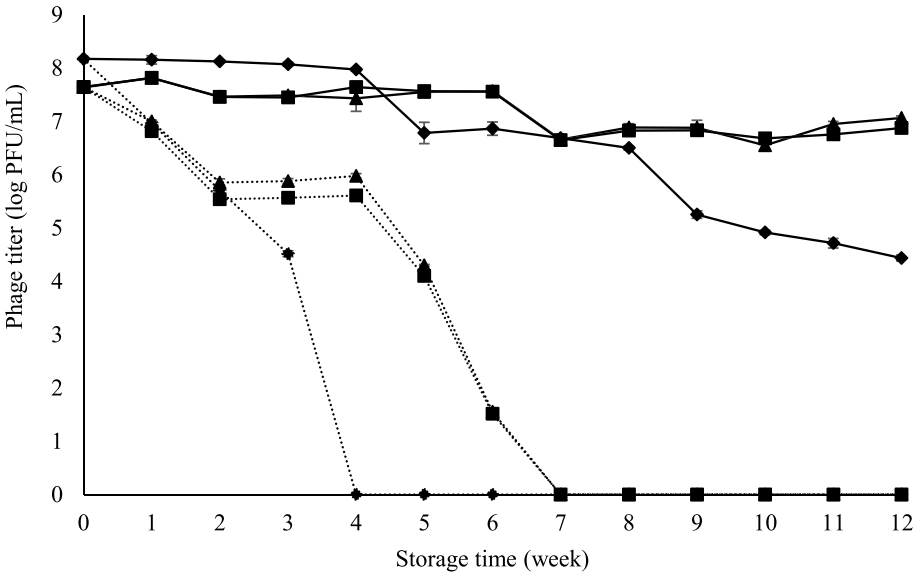

Fig. 1. Phage survivability in the microencapsulated form and phage lysate form during storage. Microencapsulated phage cocktail (powder form) kept in aluminium laminated foil bag; LF at 4 °C (—▲—), low density polyethylene; LDPE bag at 4 °C (—■—), LF at 25 °C (···▲···), and LDPE bag at 25 °C (···■···). Phage cocktail lysate kept in conical tube at 4 °C (—◆—), and 25 °C (···◆···). Bars represents the mean standard deviation (n = 3).

Table 1
Color evaluation (ΔE) of microencapsulated phage cocktail 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 lowercase letters indicate significant difference ($p < 0.05$) between ΔE of sample kept in LDPE at each temperature during a 12-week storage. No significant difference ($p < 0.05$) was observed between (i) ΔE of samples kept in LF at each temperature during a 12-week storage, (ii) ΔE of samples kept in LF and LDPE at the same temperature and (iii) ΔE of samples kept at 4 °C and 25 °C for each type of packaging. ND (not detected) due to the complete loss in phage titer at 25 °C since week 8 (see Fig. 1).

Table 2
Water activity (a_w) of microencapsulated phage cocktail 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 ± 0.00$_{A}$	0.03 ± 0.00$_{A}$	0.03 ± 0.00$_{A}$	0.03 ± 0.00$_{A}$
1	0.05 ± 0.00$_{B}^{a}$	0.08 ± 0.00$_{A}^{*a}$	0.15 ± 0.00$_{B}^{b}$	0.16 ± 0.03$_{B}^{*b}$
2	0.06 ± 0.00$_{BC}^{a}$	0.06 ± 0.01$_{A}$	0.13 ± 0.01$_{B}^{b}$	0.23 ± 0.07$_{B}^{*b}$
3	0.07 ± 0.00$_{BC}^{*a}$	0.06 ± 0.01$_{A}$	0.13 ± 0.02$_{B}^{b}$	0.35 ± 0.06$_{C}^{*b}$
4	0.08 ± 0.01$_{BC}^{a}$	0.06 ± 0.01$_{A}$	0.15 ± 0.00$_{B}^{b}$	0.34 ± 0.00$_{C}^{*b}$
5	0.08 ± 0.01$_{BC}^{a}$	0.15 ± 0.01$_{B}^{*a}$	0.25 ± 0.01$_{C}^{b}$	0.38 ± 0.01$_{C}^{*b}$
6	0.08 ± 0.00$_{BC}^{a}$	0.15 ± 0.01$_{B}^{*a}$	0.38 ± 0.03$_{D}^{b}$	0.40 ± 0.06$_{C}^{*b}$
7	0.10 ± 0.00$_{C}^{a}$	0.17 ± 0.01$_{B}^{*a}$	0.32 ± 0.01$_{E}^{b}$	0.43 ± 0.05$_{C}^{*b}$
8	0.10 ± 0.00$_{CD}$	0.18 ± 0.04$_{B}^{*}$	ND	ND
9	0.11 ± 0.02$_{CD}$	0.18 ± 0.04$_{B}^{*}$	ND	ND
10	0.12 ± 0.01$_{CD}$	0.21 ± 0.05$_{C}^{*}$	ND	ND
11	0.12 ± 0.01$_{D}$	0.30 ± 0.04$_{D}^{*}$	ND	ND
12	0.13 ± 0.01$_{D}$	0.33 ± 0.00$_{D}^{*}$	ND	ND

All value indicate mean ± standard deviation. Sign (*) on the standard deviation indicates significant difference ($p < 0.05$) between a_w of samples kept in LF and LDPE at the same temperature. Different lowercase letters on the standard deviation indicate significant difference ($p < 0.05$) between a_w of samples kept at 4 °C and 25 °C for each type of packaging. Different uppercase letters under standard deviation indicate significant difference ($p < 0.05$) between a_w of samples kept in the same packaging type at each temperature during a 12-week storage.

37 °C. The initial inoculum level of 5 log CFU/mL of *S.* Enteritidis and *S.* Typhimurium was challenged with the newly prepared phage cocktail powder. The numbers of both *S.* Enteritidis and *S.* Typhimurium in the no phage-treated control increased after 12 h at 10 °C, and reached 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 (Fig. 2). 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, microencapsulated phage cocktail could control *S.* Enteritidis and *S.* Typhimurium at both 10 °C and 37 °C. However, the optimal temperature for controlling *in-vitro* was 37 °C.

Two selected foods (raw chicken meat and sunflower sprout) 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 food sample. Phage cocktail powder that was newly prepared was applied on food samples at the optimal multiplicity of infection of 100. For control, the numbers of both *S.* Enteritidis and *S.* Typhimurium of > 9 log CFU/cm^2 and 7 log CFU/g were observed during 4 days of storage at 4 °C in raw chicken meat and sunflower sprout, respectively. For foods applied with the phage cocktail powder, the numbers of *S.* Enteritidis were decreased by 0.57 log CFU/cm^2 and 0.86 log CFU/g in chicken meat and sunflower sprout, respectively after 4 days of storage. The numbers of

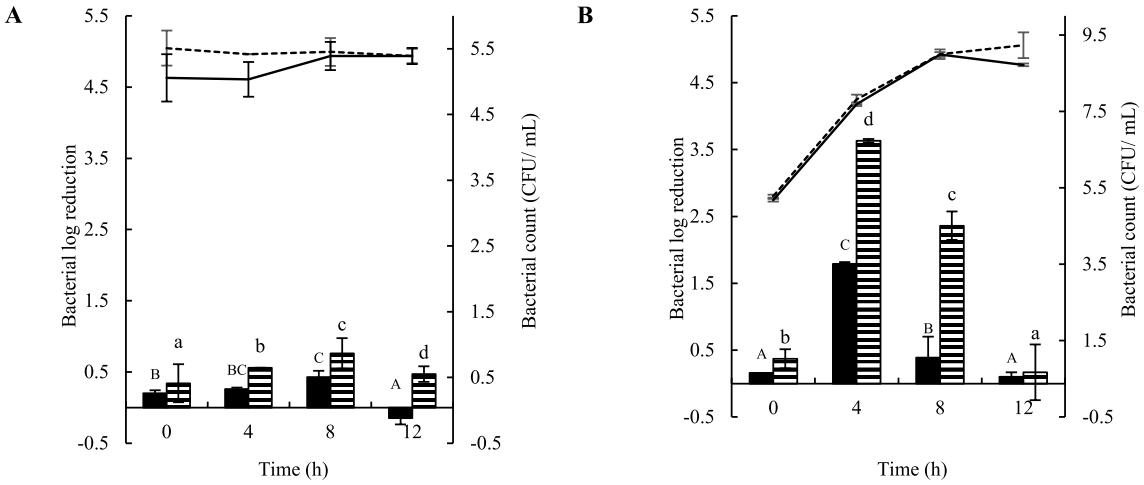

Fig. 2. Log reduction of *S.* Enteritidis (■) and *S.* Typhimurium (▨) treated with microencapsulated phage cocktail compared to control (non-treated); *S.* Enteritidis (dash line) and *S.* Typhimurium (solid line) at 10 °C (A) and 37 °C (B) for 12 h. Bars represent the mean standard deviation (n = 3). Different uppercase and lowecase letters indicated significant differences ($p < 0.05$) among log reduction of *S.* Enteritidis and *S.* Typhimurium, respectively during incubation time.

S. Typhimurium were decreased by 1.78 CFU/cm^2 and 1.2 log CFU/g in chicken meat and sunflower sprout, respectively (Fig. 3).

3.4. Fresh food applied with microencapsulated phage cocktail maintained the overall consumer acceptability

This study evaluated the acceptability of raw chicken meat and sunflower sprout with and without the phage cocktail powder and stored for 4 days at 4 °C. Scores on the appearance of color, odor, freshness, and overall liking of samples were observed. For raw chicken meat, the score of samples without the phage cocktail powder was significantly higher ($p < 0.05$) than that with the phage cocktail powder on all parameters tested for day 0 and day 1 of storage (Table 3). From day 2, the scores of the meat samples with and without microencapsulated phage cocktail showed non-significant difference ($p < 0.05$) on odor, freshness, and the overall liking. Interestingly, the scores on color of raw chicken meat with the phage cocktail powder showed significantly higher ($p < 0.05$) 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 3, the scores on freshness and the overall liking of sprout with the phage cocktail powder increased and showed significantly higher ($p < 0.05$) 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. From Table 4, color evaluation (ΔE) of raw chicken meat with/without the phage cocktail powder showed significantly difference ($p < 0.05$) in the values since day 3, while the significant difference ($p < 0.05$) in ΔE values was detected in day 4 for sunflower sprout with/without the phage cocktail powder.

4. Discussion

4.1. Stability of microencapsulated phage cocktail during a storage time

A novel phage powder form in this study suggests a better form for providing a longer shelf-life of phages as compared to the traditional phage lysate form. Generally, the phage titers in the lysate form have been shown to drop rapidly (Malik et al., 2017). However, microencapsulation has been suggested to improve phage survival during processing and storage. Microencapsulated form of phages could be well preserved and stable by the charge-charge interaction between

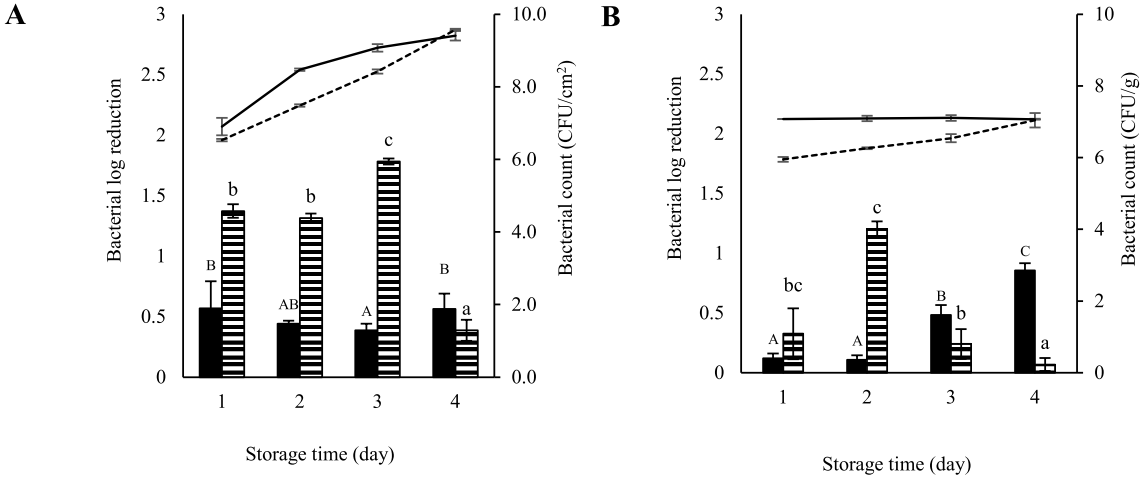

Fig. 3. Log reduction of *S.* Enteritidis (■) and *S.* Typhimurium (▤) on chicken meat (A) and sunflower sprout (B) treated with microencapsulated phage cocktail compared to control (non-treated); *S.* Enteritidis (dash line) and *S.* Typhimurium (solid line) at 4 °C for 4 days. Bar represents the mean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

Table 3
Liking score of chicken meat and sunflower sprout applied with microencapsulated phage cocktail during storage.

Liking score									
Sample	Day	Color		Odor		Freshness		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}

All values indicate mean ± standard deviation from 30 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 each appearance indicate significant differences ($p < 0.05$) during storage.

Table 4
Color evaluation (ΔE) of fresh foods treated with microencapsulated phage cocktail during storage at 4 °C.

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}

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.

phage particles and whey protein, and H-bonding between trehalose and whey protein. Similar to our findings, microencapsulated *Salmonella* phage as dry powder formulated by Eudragit S100 (polymer) or trehalose (sugar) could maintain the phage titers for 3 months during a storage at 4 °C with no statistically difference of phage titers during storage (Vinner et al., 2019). Moreover, phage as a dry powder has been reported its stability during a long-term storage of up to 21 years (Ackermann et al., 2004). Overall, our result suggests that low temperature (4 °C) provided the optimal condition for phage storage. Storage at room temperature (25 °C) could cause thermal instability which may disturb the embedded phages structure. Vandenheuvel et al. (2014) has previously reported high stability of encapsulated phage as a dry powder at low temperatures (4 °C) and relative humidity of 0%. While high humidity could cause crystallization of the amorphous matrix which could destroy the encapsulated phages (Vandenheuvel et al., 2014), our study has evaluated the phage stability at 65% relative humidity (for 4 °C) and 78% relative humidity (for 25 °C) with no crystallization observed during storage.

4.2. Aluminium laminated foil bag as an appropriate package for desirable physical and chemical properties of microencapsulated phage cocktail

This study compared the physiochemical properties of microencapsulated phage cocktail 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 the dry powder form of phage cocktail at 4 °C and 25 °C during 12 weeks of storage. Color is one of the most important characteristic which relates to food quality and it also effects consumer judgment of other sensory characteristics (Clydesdale, 1991; Titova et al., 2015). A minor change in color provides a desirable quality of the dry powder form in storage condition. However, our findings suggest that a_w of microencapsulated phage cocktail relies upon the storage temperature rather than the packaging type. At 4 °C, a_w of microencapsulated phage cocktail showed slight increase compared to that kept at 25 °C for both LF and LDPE bags. Overall, microencapsulated phage cocktail 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 storing time (Fennema, 1996). The a_w is now regarded as one of the most important indicators of food quality. The less a_w can inhibit the onset of undesirable reactions such as lipid oxidation and microbial growth.

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 the microencapsulated phage cocktail at refrigeration temperature for maintaining the physiochemical properties.

4.3. Control of S. Enteritidis and S. Typhimurium in-vitro and in fresh foods by microencapsulated phage cocktail

S. Enteritidis and *S.* Typhimurium are normally reported as the most important *Salmonella* serovars contaminated in foods worldwide (Acheson and Hohmann, 2001). *In-vitro* study suggests the efficiency of microencapsulated phage cocktail 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 to 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 the application of our phages against *Salmonella* serovars.

In this study, raw chicken meat and sunflower sprout were selected as the representative high-risk foods for *Salmonella* contamination (Antunes et al., 2016; Hanning et al., 2009). The efficiency of the traditional phage cocktail lysate for controlling *Salmonella* in chicken meat and fresh produce has been investigated by several previous studies (Kocharunchitt et al., 2009; Spricigo et al., 2013). A phage cocktail

comprising of 2 *Salmonella* phages showed the efficacy in reducing *S.* Oranienburg by 1 log CFU/g (Kocharunchitt et al., 2009). 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). In this study, a reduction of 0.57 log CFU/cm^2 of *S.* Enteritidis and 1.78 log CFU/cm^2 of *S.* Typhimurium in raw chicken meat was observed from the application of a phage cocktail powder. Application of a phage cocktail powder on sunflower sprout showed a reduction of 0.86 log CFU/g of *S.* Enteritidis and 1.2 log CFU/g of *S.* Typhimurium during storage at 4 °C for 4 days. Overall findings here suggest that the alternative novel form of phage cocktail still provide comparable efficiency for controlling *Salmonella* on food matrices as the traditional phage lysate. This is the first study to introduce a novel form of *Salmonella* phages for applying on several fresh food products. However, study on the effectiveness of a phage cocktail powder after storage may be needed to compare results with the newly prepared phage powder. Moreover, this form provides the advantages i.e. extended shelf-life, less weight of particles, thus easing the transportation, storage, and use. This form can be applied to various food products without affecting intrinsic properties of food.

4.4. The overall consumer acceptability on fresh foods treated with microencapsulated phage cocktail

The microencapsulated phage cocktail as a powder form has no effect on the appearance of raw chicken meat and sunflower sprout. Overall, food samples applied with the dry phage powder showed the desirable acceptability scores for the first 3 days of storage. On day 4, the score of all appearances of both chicken meat and sunflower sprout with/without microencapsulated phage cocktail showed under desirable acceptability, suggesting the poor quality of foods occurred after 3 days of storage. In this study, food samples were kept in the normal LDPE bag without quality control, which may cause rapid deterioration of food. Finding here suggest potential use of phage powder form on fresh foods with high moisture content or a_w (approximately 0.9).

5. Conclusion

This study has introduced a new form for phage cocktail against *Salmonella* serovars in fresh foods. Phages as a dry powder developed by encapsulation technique showed stable titers over 3 months. Laminated foil packaging was suggested as the most suitable packaging for phage cocktail powder storage at refrigerated condition while maintain physiochemical properties during storage. Microencapsulated phage cocktail showed comparable efficiency for controlling *S.* Enteritidis and *S.* Typhimurium in both *in-vitro* and food models (raw chicken meat and sunflower sprout) compared to the traditional lysate forms. However, this form provides the advantages over the lysate form i.e. long shelf-life, less weight of particles, thus easing the transportation, storage, and use. This form can be applied to various food products without affecting intrinsic properties of food. Sensory evaluation suggests the acceptability from consumers in raw chicken meat and sunflower sprouts applied with microencapsulated phage cocktail during storage. In summary, findings here suggest a potential use of microencapsulated phage cocktail as a bio-control agent for improving safety and quality of fresh foods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.05.008.

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