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Full Paper

Phage-mediated Biocontrol of *Salmonella enterica* ser. Typhimurium in Bacon

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Foodborne illnesses caused by *Salmonella* have long been a global public health concern. Whether through contaminated meat or water, or undercooked food, this poses a problem to countries like the Philippines that consume pork and chicken frequently. This study isolated three *Salmonella enterica* ser. Typhimurium phages (*svBEATS-5, -6,* and *-9*) from sewage samples to assess their potential biocontrol use against *Salmonella* Typhimurium in commercially bought bacon. The phages were characterized based on pH and thermal stability, and host range. Ideal conditions for the phages were identified at pH 7 and 37°C, with the most stable phages, *svBEATS-5* and *svBEATS-6*, exhibiting similar lytic activity at all pH levels and temperatures. To evaluate their efficacy as biocontrol agents, a phage cocktail comprising the three phages was applied over bacon spiked with *S. enterica* Typhimurium at 4°C and 30°C. Results showed that at both temperatures, the concentration of *Salmonella* Typhimurium decreased in vitro and meat samples, with a more significant reduction under refrigerated conditions. In addition, the phages could maintain viable concentrations at temperatures, 4°C and 30°C. The results suggest that the phages can be employed to control *Salmonella* Typhimurium in bacon and can be a viable alternative to using antimicrobials in bacon and other meat products.

Keywords: biocontrol, bacteriophage, bacon, food industry, antimicrobial resistance, *Salmonella enterica* serovar Typhimurium

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Introduction

Bacon is a cured meat product primarily produced from pork belly but has since diversified to be manufactured from a wide variety of meats (Soladeya et al., 2015). Intrinsically, bacons are described to have low salt concentration, high moisture, high concentration of nutrients and minerals, and a neutral pH (Casaburi et al., 2015). During the manufacturing stage, the meat is exposed to various materials, environment, equipment, and personnel which can be a source of microbial contamination influencing the dynamics of its bacterial community (Li et al., 2021). *Salmonella* is a rod-shaped, gram-negative bacterium that causes foodborne illnesses in both humans and animals, following the consumption of contaminated food (Centers for Disease Control and Prevention, 2023). The two species recognized within the genus *Salmonella* are *Salmonella enterica* and *Salmonella bongori*, each of which contains multiple serotypes that differ in their ability to cause disease (Lamas et al., 2018). Among the more than 2500 serotypes of *Salmonella, S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the dominant serotypes in many developed and developing countries (Wang et al., 2017).

Salmonella contamination in meat and poultry products remains a persistent challenge for the food industry, despite active biocontrol and preventative measures. Balita (2024) forecasted pork as the most consumed meat in the Philippines by 2023 with approximately 15 kilograms per person. The anticipated rise in meat and poultry consumption, particularly in Metro Manila, raises concerns about increased risk of *Salmonella*-related infections due to contaminated products (Paclibare et al., 2017). According to Santos et al. (2020), it was found that 56 out of 90 (62.22%) bacon purchased from different stalls in wet markets in Metro Manila are contaminated with *Salmonella*. While proper handling, storage, and cooking of food products can reduce the risk of human exposure to the bacterium, chemical-based preventive measures are employed to mitigate bacterial contamination (Robinson, 2014). Antimicrobials are also used in eradicating bacterial populations in food products, however, they only result in a short-term reduction of bacterial populations (Lucera et al., 2012). This temporary benefit, however, comes at a significant cost. The indiscriminate use of antimicrobials and biocides in primary production can be driven by several factors, including routine preventative use, attempts to promote faster growth in animals, and a lack of awareness about its long-term consequences (CDC, 2023; Aidara-Kane et al, 2018). These factors lead to the buildup reservoirs of antimicrobial-resistant microorganisms, and transferable resistance genes (Capita & Alonso-Calleja, 2013; Heuer et al., 2009; Samtiya et al., 2022).

To combat the selection and dissemination of antimicrobial resistance in the food chain, phage therapy can be an effective biocontrol alternative in food production (Goodridge & Bisha, 2011; Principi et al., 2019). Bacteriophages, classified as virulent or temperate phages, are viruses that infect and replicate in bacterial cells without harming human or animal cells (Kasman & Porter, 2021; Principi et al., 2019). Phages have multiple applications in the food industry as powerful biocontrol, bio-sanitation, biopreservation, and as therapeutic agents (Kazi & Annapure, 2016; Kasman & Porter, 2021). This is evidenced by numerous studies that have demonstrated that *Salmonella*-specific bacteriophages can effectively decrease the presence of *Salmonella* in a range of food items, including processed and raw chicken, ground meat, and meat products (Hashem et al., 2016; Whichard et al., 2003; Yeh et al., 2017).

The study will (1) isolate bacteriophages capable of lysing *Salmonella enterica* ser. Typhimurium, (2) characterize the isolated bacteriophages using plaque and virion morphology, host range, the effect of pH and temperature, and (3) measure the efficacy of the isolated bacteriophages as monophage and cocktail preparations in suppressing *Salmonella* Typhimurium in bacon at 4°C and 30°C.

This study contributes to the application and administration of phages as natural agents against the various food-borne pathogens in the food industry. In addition to helping improve phage research in the Philippines, the study can contribute to the elimination of the antibiotic-resistant bacteria that are currently threatening the local public health. A phage product can also be developed with the

help of this research to potentially ensure that meat products are free of pathogens when consumed.

Methodology

Bacterial Strain

Two (2) serovars of *Salmonella enterica* were used for this study, specifically *S. enterica* ser. Typhimurium and ser. Choleraesuis (Chiu et al., 2010). The strains were acquired from the University of Santo Tomas Collection of Microbials Strains (UST-CMS) and the Bacteriology Laboratory, National Yang Ming Chiao Tung University (NYCU) in Taiwan, respectively. Both pathogens were classified as Biosafety Level (BSL) 2 organisms, and all related experiments were performed in a BSL-2 Laboratory of the University of Santo Tomas. *Salmonella* Typhimurium was used as the host strain for isolation of phages and biocontrol testing in meat samples. *Salmonella* Choleraesuis was used for host range testing of the isolated phages. All bacterial strains were incubated at 37°C for 5-hr for working cultures and maintained at 10^8 colony forming units (CFU) ml^{-1} for all succeeding procedures and analysis, unless stated otherwise.

Isolation and Purification of Bacteriophages

Two hundred fifty (250) mL of the sewage samples were collected from two public markets in Sampaloc and Quiapo, Manila City, Philippines. The pH and temperature parameters were recorded for each sample before processing for isolation. Initial sample enrichment was performed based on the methods by Santos and Papa (2020). A working culture of *Salmonella* Typhimurium was transferred into double-strength Tryptic Soy Broth (TSB) to which filtered sewage samples were added. The setup was incubated 12 hr at 37°C. After incubation, the setups were centrifuged at 3,500 rpm for 10 min and were filtered using 0.45 µm syringe filters. To determine the presence of phages in the enriched samples, a spot test using the double-layer agar method was performed following the procedure of Clokie and Kropinski (2009). The presence of phages in the filtrates was indicated by the appearance of a clearing against the bacterial lawn. Purification of bacteriophages was performed through repeated plaque assay to obtain isolated plaques (Clokie & Kropinski, 2009). To produce monoclonal phage stocks of the isolated bacteriophages, at least two more rounds of plaque picking and purification from previously isolated plaques were done. When the resulting plaques from the rounds of picking and assay have identical morphology and growth—traits characteristic of the original population, the phage stock is considered monoclonal (Storms & Sauvageau, 2014).

Determination of Phage Titer

The phage titer expressed as Plaque Forming Units per mL (PFU/mL), was determined by counting the number of plaques that formed on the lawn. The following formula by Clokie and Kropinski (2009) was used to calculate the phage titer:

$$PFU\ mL^{-1} = \text{number of plaques} / (\text{dilution factor} \times \text{volume plated})$$

All phages were maintained at 10^8 PFU ml^{-1} for all succeeding procedures and analysis, unless stated otherwise.

Determination of Phage Lytic Activity

Isolated phages were subjected to various temperature and pH ranges, as well as host range testing to assess their lytic activity. Their stability at different temperatures was evaluated by incubating the phages at 30°C to 60°C, in 5°C increments. For pH stability, the isolates were incubated at pH levels 3, 5, 7, 9, and 11. The phages were exposed to these conditions for 30, 60 and 90 mins. After exposure, the phages were spot-tested against a pre-plated *Salmonella* Typhimurium seeded agar plates and were incubated overnight at 37°C. Similarly, the host range of phages was determined through a spot test method using different strains and serovars of *S. enterica*. After incubation, the results of the spot test were recorded based on the criteria for clearing, as illustrated in Figure 1.

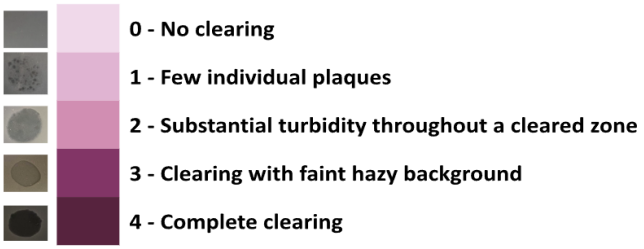

Figure 1. Reference chart for measuring the degree of lysis adapted from Yu et al. (2013).

Morphological Characterization

For the morphological characterization, the phages were sent to the Department of Materials Science and Engineering, NYCU, Taipei, Taiwan, for viewing under transmission electron microscopy (JEOL JEM-2000 EXIII). Briefly, the phages were fixed onto a copper Forvar grid and were negatively stained using 3-5% uranyl acetate (UA). The morphology of the phages was determined and classified based on the reported structural presence and measurements by Ackermann (2009).

In Vitro Biocontrol testing of *Salmonella* Typhimurium Phages

The procedures performed were based on the methods by Guenther et al. (2009). A culture of *Salmonella* Typhimurium was diluted to a final concentration of $1{\cdot}10^5$ CFU/mL, while the starting concentration of the phage isolates was at $1{\cdot}10^8$ PFU/mL to achieve a multiplicity of infection (MOI) of 1,000. The experiment had two treatments: (1) phage monoculture, and (2) phage cocktail, with SM buffer as a negative control. The phage cocktail mixture consisted of three phage isolates with a $1{\cdot}10^9$ PFU/mL titer and was prepared by combining the three phages in equal volumes. The phage-host mixture was cultured for 4 days in TSB at 4°C and 30°C. From day 1 to day 4, daily phage counts, and viable host cells were done through plaque assay to quantify phages and spread plate method using Xylose-Lysine-Deoxycholate (XLD) agar to quantify *Salmonella* Typhimurium which appeared as black colonies against the XLD plate. Both plaque assay and spread plate were performed in triplicates, and the results were expressed as PFU mL^{-1} (for phage titers) and CFU mL^{-1} or bacterial concentration.

The bacterial concentration was determined using the formula below:

> CFU mL^{-1} = number of colonies x dilution factor / volume plated

Phage Cocktail Applications in *Salmonella* Typhimurium-infected Bacon Samples

Using ISO 6579:2017, which comprises pre-enrichment, enrichment, and plating on selected media, bacon samples were tested for *Salmonella* spp. Only samples devoid of *Salmonella* were utilized in the experiment. Similar to the *in vitro* biocontrol, a multiplicity of infection (MOI) of 1,000 was applied in these procedures. Briefly, 1 mL of $1{\cdot}10^5$ CFU/mL culture was added to 8g of the bacon samples by direct pipetting and incubated at room temperature (RT) for 15 min to allow the bacteria to adhere to the samples. After this, 1 mL of the prepared phage cocktail was added. This was then incubated at 4°C and 30°C for 4 days (Guenther et al., 2009). As a negative control, one set-up per condition was inoculated with 1mL of SM buffer, totaling the set-ups utilized to four: (1) 4°C phage cocktail, (2) 30°C phage cocktail, (3) 4°C control, and (4) 30°C control. Phage recovery and bacterial load determination were performed through plaque assay and spread plate method. Similarly, all analyses were performed in triplicates and were expressed as PFU mL^{-1} and CFU mL^{-1}.

Data Analysis

Statistical analysis was done using Paleontological Statistics (PAST) statistical software (Hammer & Harper, 2001) and Microsoft Excel. Different statistical tests were performed for the *in vitro* biocontrol testing of *Salmonella* Typhimurium phages and phage cocktail applications bacon samples (including Shapiro-Wilk's test, Spearman Rank correlation, One-way ANOVA, Tukey's pairwise test for parametric samples, and Kruskal-Wallis and Dunn's post hoc tests for non-parametric samples). A p-value of < 0.05 and confidence level of 95% was used for all statistical tests.

Bacteriophage isolation

Three lytic bacteriophages were isolated from sewage water samples obtained at wet public markets in Manila, Philippines, with *Salmonella* Typhimurium strain as the host. The isolates were named *svBEATS-5* (nomenclature: s for *Salmonella*, v for virus, and bacteriophage identifier code), *svBEATS-6*, and *svBEATS-9* based on ICTV guidelines for writing species epithet (Turner et al., 2023). The samples' thermal and pH conditions were recorded at the time of their collection (Table 1).

Table 1. Environmental conditions of the sampling sites and the isolated *Salmonella* Typhimurium bacteriophages from each sample.

Sewage Samples	Sampling Site	Recorded Parameters		Bacteriophage Isolates
		pH	Temperature (°C)	
QM2A	Quiapo, Manila	6.75	24.7	*svBEATS-9*
TB2A	Sampaloc, Manila	7.67	28.1	*svBEATS-5 and svBEATS-6*

Bacteriophage characterization

The bacteriophages were characterized based on plaque and virion morphologies, host range, and thermal and pH stability. The plaque morphologies of the isolated *Salmonella* bacteriophages are shown in Figure 2. Each isolated bacteriophage exhibited similar clear plaque morphologies within 24h of incubation with apparent differences in their diameters. *svBEATS-9* (Figure 2C) and *svBEATS-5* (Figure 2A) created larger clear plaques with average diameters of (n= 34) 1.90mm and (n=40) 1.60mm, respectively. In contrast, *svBEATS-6* (Figure 2B) produced smaller plaques with an average diameter (n=31) of 0.90mm.

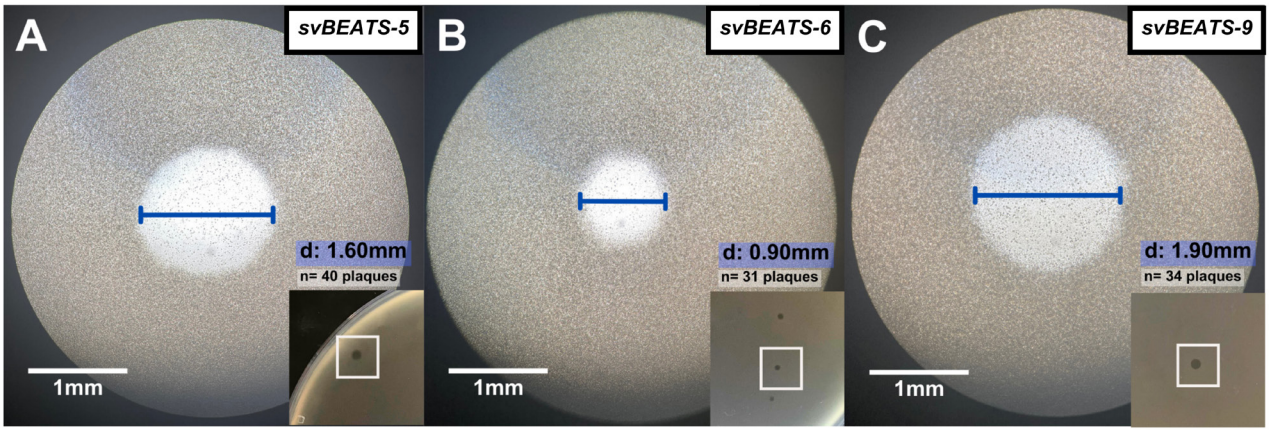

Figure 2. Plaque morphologies of the isolated phages. Clear plaques of (A) *svBEATS-5*, (B) *svBEATS-6*, and (C) *svBEATS-9* show the average diameter (d) of the plaques (n) in millimeters. The scale bar is 1mm.

The transmission electron microscopy of *svBEATS-5* (Figure 3) revealed that it was a podovirus classified under class *Caudoviricetes* based on the presence of its short, non-contractile tails (King et al., 2011; Turner et al., 2023). Measurement of virion structures showed that svBEATS-5 has a capsid diameter of ±73.46nm and a tail length of ±24.50nm. Aside from the presence of the tail structure and icosahedral head, the length of the regions play a part in morphological classification. Podophages have a tail length range of 10 - 20 nm and a capsid size range of 50 - 145 nm (Ackermann, 2003).

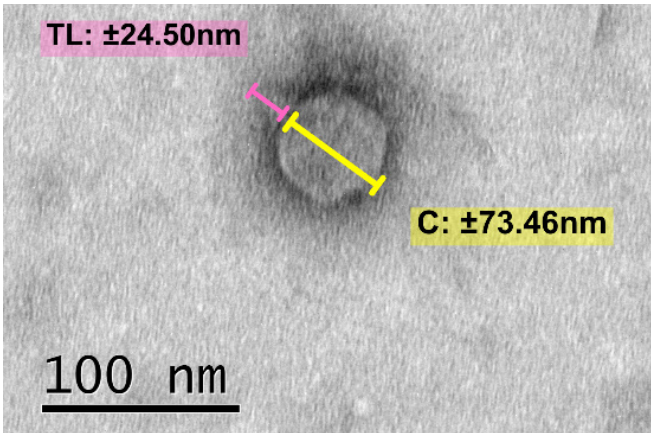

Figure 3. Representative transmission electron micrograph of podovirus *svBEATS-5* showing the capsid (C) and tail length (TL) measurements in nanometers. The scale bar is 100nm.

Analysis of the ability of phages to infect and lyse S. ser. Typhimurium and streptomycin-resistant Choleraesuis OU7526 revealed that all the isolated phages (n=3) were able to lyse both *S. enterica* serovars (Table 2).

Table 2. The lytic ability of the isolated phages against different *S. enterica* serovars.

Sewage Samples	*Salmonella enterica* serovar	
	Typhimurium	Choleraesuis OU7526
svBEATS-5	+	+
svBEATS-6	+	+
svBEATS-9	+	+

Note: (+) denotes that the phage could infect and lyse the corresponding *Salmonella* serovar.

Thermal and pH stability are presented as heatmaps (Figure 4-5) and are evaluated based on their degree of lysis (Figure 1). Under these conditions, *svBEATS-5* and *svBEATS-6* showed similarly high levels of lytic activity at pH levels 3 to 11 after 30 to 90 min of exposure. Furthermore, both phages were stable under 30°C to 50°C after 30 to 90 min of incubation. However, *svBEATS-9* is more susceptible to acidic conditions, as exhibited by its inactivation at pH 3 to 5. However, it showed more potent lysis of the host at pH 7 and a slightly decreased lytic activity from pH 9 to 11. Its thermal stability was relatively similar to *svBEATS-5* and *svBEATS-6* but with decreased lytic activity under prolonged exposure to 30°C to 45°C. All of the phages have a significant reduction in their lytic activity at higher temperatures, 55°C and 60°C. After 90 min of incubation at 60°C, all phages were inactivated, producing negative results in the spot test.

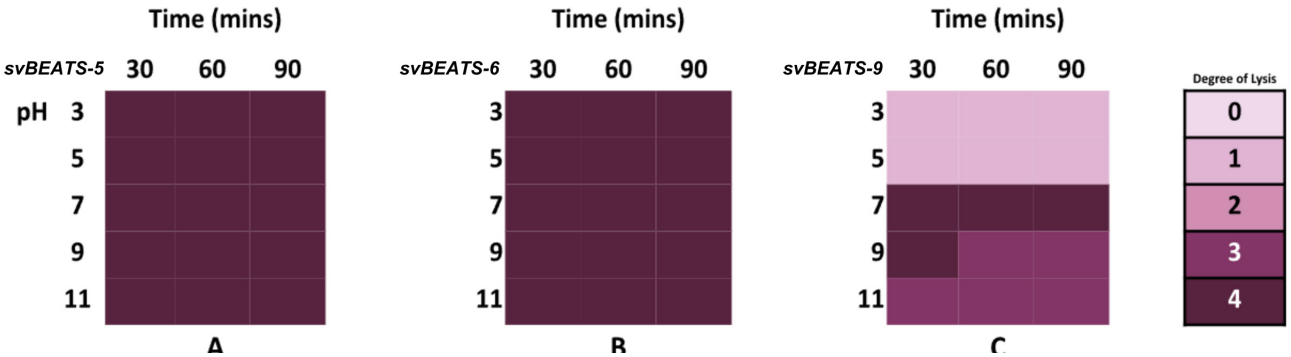

Figure 4. Stability of phages under different pH levels and times. (A) *svBEATS-5,* (B) *svBEATS-6*, and (C) *svBEATS-9*.

Note: The degree of lysis is measured as prescribed by Yu et al. (2013): 0 - No clearing, 1 - few individual plaques, 2 - substantial turbidity throughout a cleared zone, 3 - clearing with faint hazy background, and 4 - complete clearing.

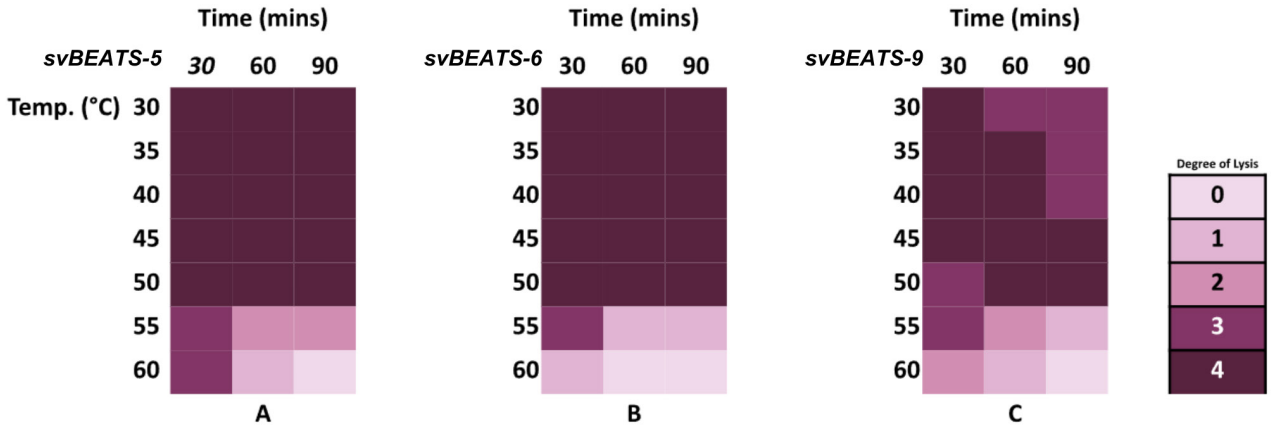

Figure 5. Stability of phages under different temperatures and times. (A) *svBEATS-5,* (B) *svBEATS-6*, and (C) *svBEATS-9*.

In vitro biocontrol of *Salmonella* Typhimurium

The lytic ability of the isolated phages as monophage treatments to reduce the concentration of *Salmonella* Typhimurium *in vitro* at 4°C and 30°C for four days is presented in Figure 6. The reduction in *Salmonella* Typhimurium *in vitro* at 4°C was significantly reduced in concentrations below the untreated control sample after 3 days of treatment ($p < 0.05$). All monophage treatments were able to reduce the bacterial population at 4°C lower than that of the initial concentration, 5 Log_{10} CFU/mL, with phage *svBEATS-6* having the highest reduction by 1.32 Log_{10} CFU/mL. Conversely, at 30°C, higher bacterial concentrations were observed during the treatment compared to the initial inoculum at day 0. After four days of biocontrol, the number of *Salmonella* Typhimurium was significantly reduced compared to the control. *svBEATS-5* has the highest reduction at 0.46 Log_{10} CFU/mL compared to the bacterial concentration of the untreated sample on the 4th day of treatment.

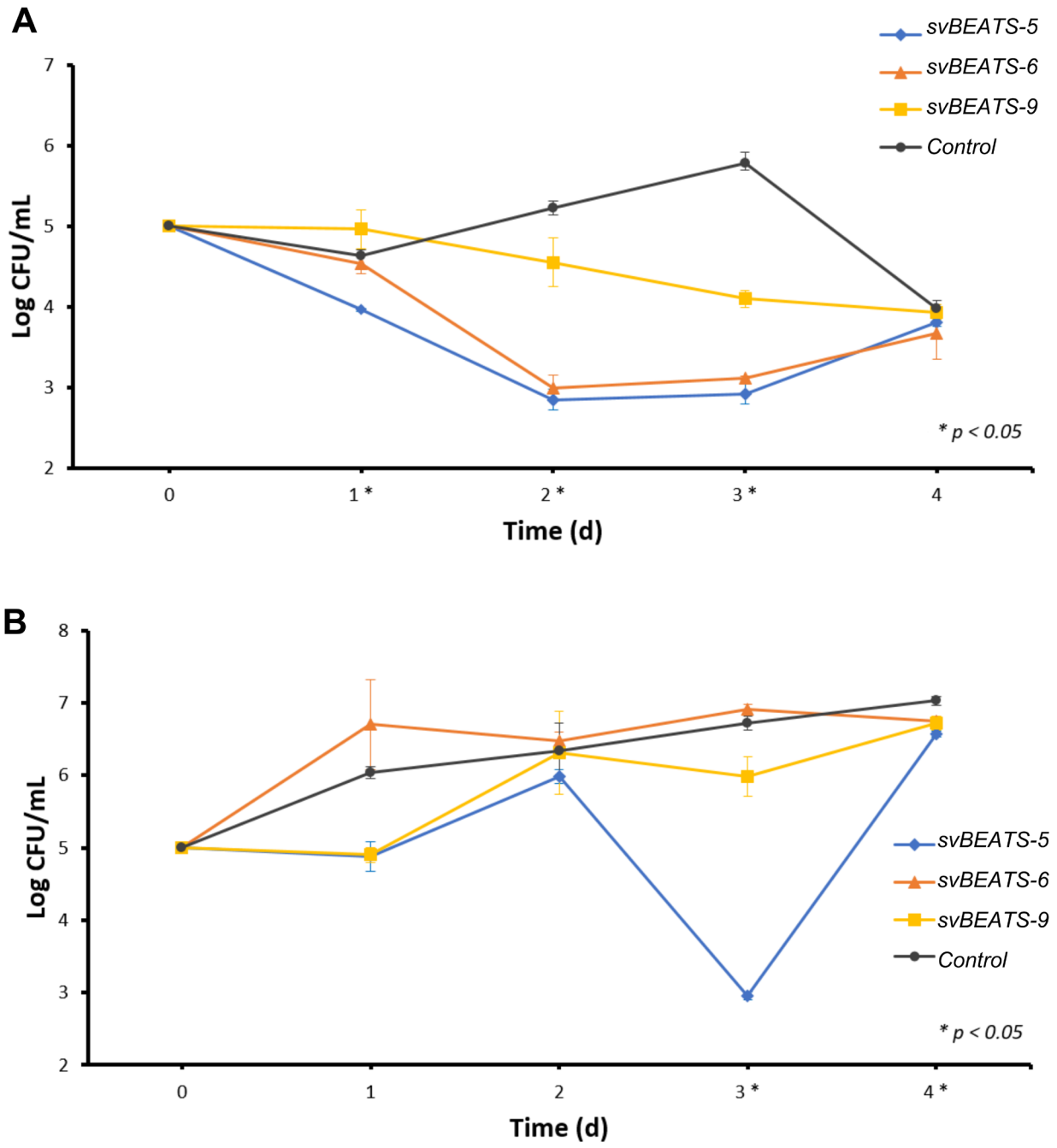

Figure 6. Effect of monophage treatments in *Salmonella* Typhimurium concentration at (a) 4°C and (b) 30°C for 4 days.

To further assess the efficacy of the biocontrol application of the treatments *in vitro* at 4°C and 30°C, the phage titers were monitored simultaneously during the 4-day treatment, as represented in Figure 7. At 4°C, none of the phages could increase their concentration compared to the initial dose administered at the start of the treatment, 8 Log_{10} PFU/mL. In addition, *svBEATS-6* at day 3 had its titer significantly closest to the initial phage titer at 6.45 Log_{10} PFU/mL ($p < 0.05$). At 30°C, the first two days of treatment similarly showed a significant reduction in the phage concentrations below the initial concentration ($p < 0.05$). However, the phage titers increased after the 3rd day of the treatment. *svBEATS-6* has the highest concentration at day 4 with a 9.64 Log_{10} PFU/mL titer.

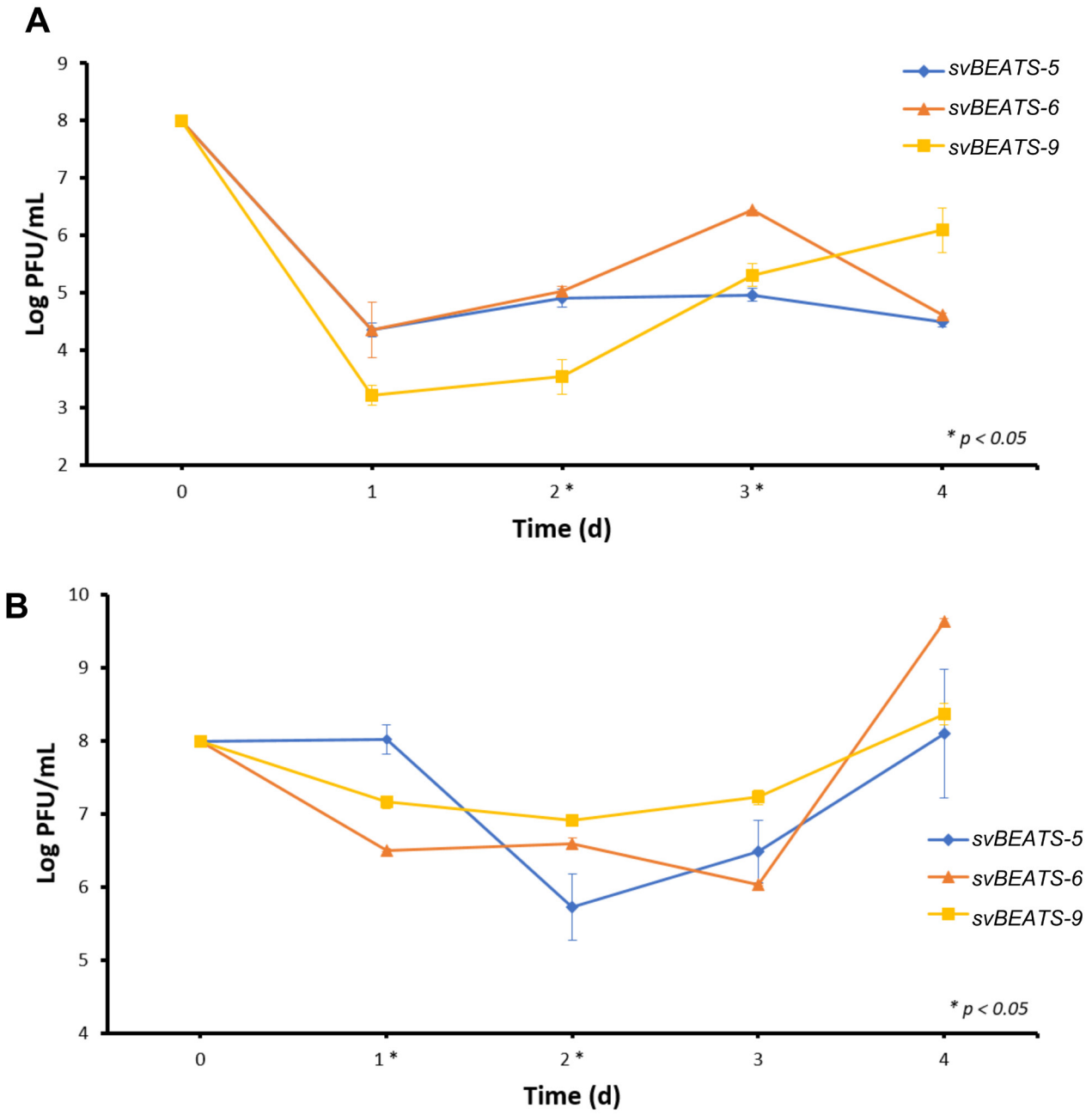

Figure 7. Phage titers in the 4 days of in vitro biocontrol at 4°C and 30°C.

Biocontrol of *Salmonella* Typhimurium in bacon samples

The efficacy of the phage cocktail formulation in reducing *Salmonella* Typhimurium in bacon at 4°C and 30°C was assessed (Figure 8). At 4°C, the phage cocktail significantly reduced the *Salmonella* Typhimurium concentration below the untreated bacon samples from days 2 to 4 of the treatment ($p < 0.05$). Even though the bacterial concentration of the treated meat sample significantly increased on day 3, its concentration is still significantly lower compared to the untreated bacon, with a reduction of 0.69 Log_{10} CFU/g. The treatment also consistently reduced bacterial concentrations below the initial inoculum of 5 Log_{10} CFU/g. At 30°C, the *Salmonella* Typhimurium concentrations showed a similar trend to its *in vitro* counterpart. The untreated and treated bacon samples have higher bacterial concentrations than the initial inoculum. However, after three days of the treatment, the phage cocktail significantly reduced the bacterial count below the control ($p < 0.05$). After 4 days, the treated sample had a 0.31 Log10 reduction in its *Salmonella* Typhimurium concentration compared to the control. Although the phage cocktail reduced the bacterial concentration at both temperatures, the reduction at 30°C was less than that of the treated meat at 4°C.

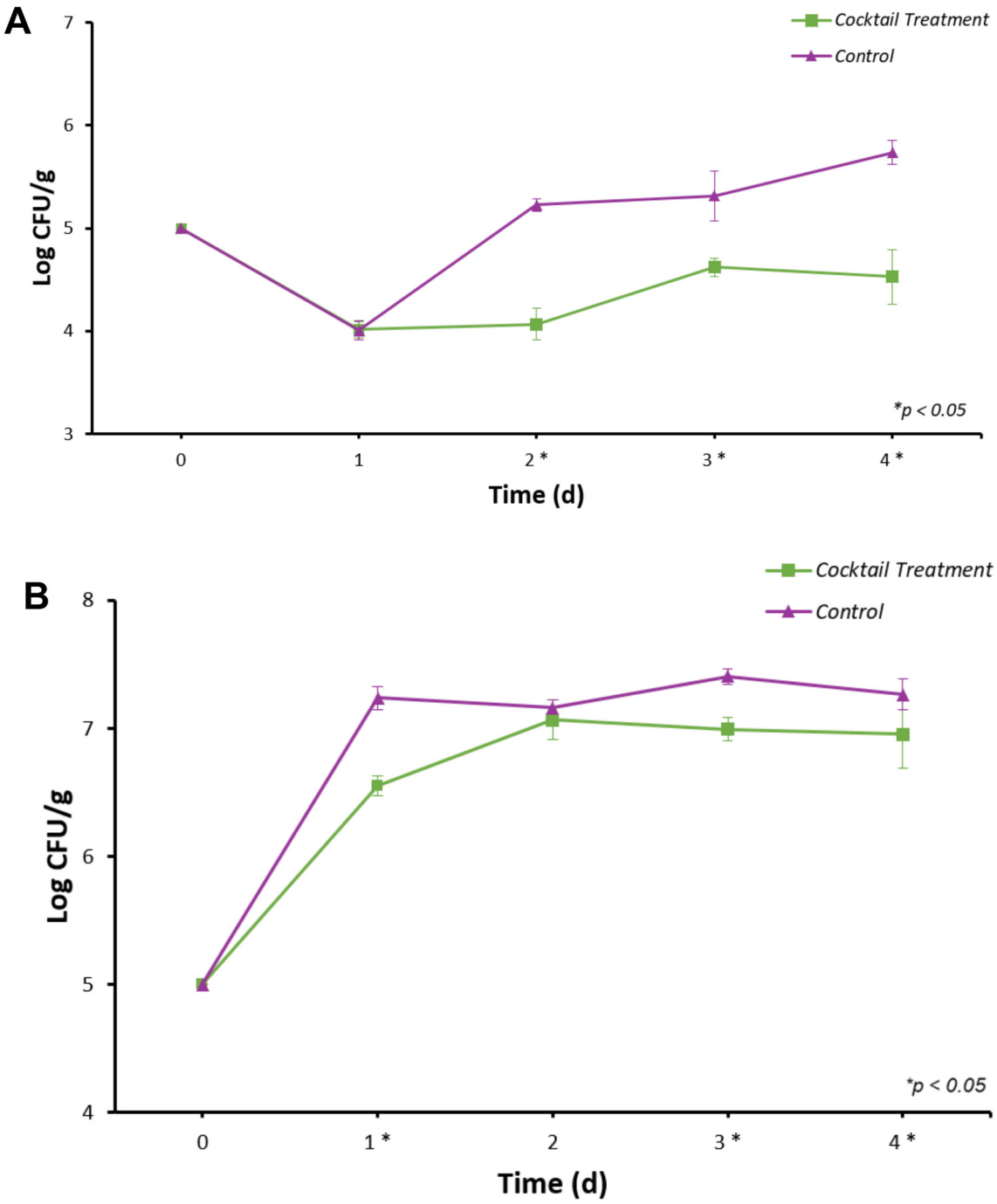

Figure 8. Effect of phage cocktail treatment in *Salmonella* Typhimurium concentration at (a) 4°C and (b) 30°C for 4 days.

The phage titers were evaluated during the biocontrol of *Salmonella* Typhimurium in bacon at 4°C and 30°C (Figure 9). At 4°C, the titer of the phage cocktail significantly decreased from its initial concentration by 4.1 Log_{10} PFU/g ($p<0.05$). However, the phage cocktail significantly stabilized its concentration without undergoing a huge decline from days 2 to 4 of the treatment, exhibiting that the phages may have been acclimated under refrigerated conditions, retaining their infectivity ($p < 0.05$). After four days at 4°C, the phage cocktail titer was 3.88 Log_{10} PFU/g below its initial dose of 8 Log_{10} PFU/g. On another note, the phage cocktail's concentration at 30°C in bacon demonstrated that it could be stable despite significant reductions in its titer at specific time points. Compared to the concentrations at 4°C, the phage titers are significantly higher at 30°C ($p < 0.05$). The bacterial and phage counts in the treated bacon at 4°C and 30°C show that the phage cocktail remained infective against *Salmonella* Typhimurium throughout the treatment.

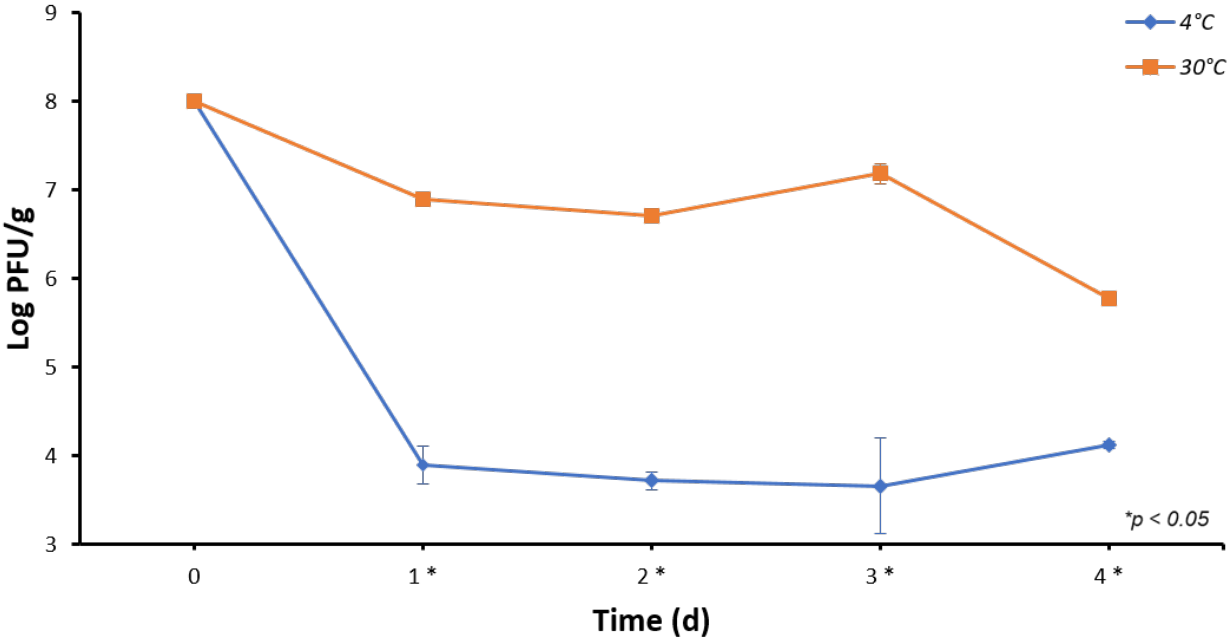

Figure 9. Phage titers in the 4-day biocontrol of *Salmonella* Typhimurium in bacon samples at 4°C and 30°C.

Discussion

This study was conducted to determine the efficacy of phage cocktail as biocontrol agents against Salmonella enterica ser. Typhimurium in artificially-infected bacon samples. Additional parameters were also tested to establish the biocontrol activity of the formulated phage cocktail at 30°C and 4°C. The temperatures were selected to simulate the storage temperatures of the selected meat sample. In relation to the selected meat sample, bacon has the fourth highest prevalence of *Salmonella* contamination among retail meat samples from wet public markets in Metro Manila, Philippines (Santos & Papa, 2020). This raises concern because processed food products such as bacon contain additives and preservatives among its ingredients to inhibit the growth of foodborne pathogens. *S. enterica's* ability to persist and to colonize food products in the presence of chemical additives and preservatives is due to its well-regulated response against abiotic stresses from different manufacturing stages of preserved meat products (Mutz et al., 2020). Adaptation of *S. enterica* in processed food matrices enhances their pathogenicity, increasing the risk of exposing and contaminating cured meat products to highly virulent pathogenic cells. Furthermore, bacon is stored in ambient and refrigerated conditions (30°C and 4°C) at different food processing and manufacturing stages. Measuring the activity of the isolated bacteriophages under these conditions is essential in determining if they are viable biocontrol agents against *Salmonella* Typhimurium.

This study reports three *Salmonella* bacteriophages (*svBEATS-5, svBEATS-6,* and *svBEATS-9*) isolated from sewage samples obtained at wet public markets in Sampaloc and Quiapo, Manila City, Philippines. Sewage waters from public markets are a common source of bacteriophages infecting different bacterial species, including *Salmonella*, where fecal run-off and water used to wash raw meat may be present (Carey-Smith et al., 2006; Khan Mirzaei & Nilsson, 2015; Nair et al., 2015; Hyman, 2019).

Based on the plaque morphologies of the phages, they can be characterized as virulent or lytic bacteriophages due to their clear plaques (Gallet et al., 2011). In therapeutic use, obligate lytic phages are recommended due to their ability to lyse and their inability to induce transduction and potentially initiate lysogeny (Pirnay et al., 2015). Furthermore, lytic phages are preferably used because they effectively eliminate the bacterial host and simultaneously release multiple virulent phages that can infect other cells (Kazi & Annapure, 2016).

Host range is a critical criterion in selecting phages that will be used for biocontrol applications in the food industry (Fong et al., 2021). Our results showed that all isolated bacteriophages (n=3) could infect 2 strains of *Salmonella* Typhimurium, and streptomycin-resistant *Salmonella* Choleraesuis OU7526 (Chiu et al., 2010). Compared to *Salmonella* Typhimurium, serovar Choleraesuis preferentially causes human systemic infections, which are more

severe, but they still cause gastroenteritis (Chiu et al., 2004). Based on Sia et al. (2018), a fifteen-year study of *Salmonella* serotype distribution and antimicrobial resistance in the Philippines, *Salmonella enterica* serovars Typhimurium and Choleraesuis were among the most common non-typhoidal Salmonella in the country isolated from human specimens. The ability of a single bacteriophage to infect and lyse different serovars of the same bacterial species can be explained by their ability to use the same bacterial receptors to attach themselves to these different *Salmonella* serovars (Thanki et al., 2019). With this, the phages isolated in this study were able to infect two different *Salmonella enterica* serovars but further analysis and wider scope of host range testing is required to establish polyvalency. In the food industry, phages with a broad spectrum are preferably used due to their ability to infect different strains and serotypes of the host (Fong et al., 2017). However, expanded host range testing should be performed to establish this.

The stability of phages to different physical conditions is an integral part of their characterization since the hosts are exposed to various environments where phages can be inactive (Jończyk et al., 2011). Environmental conditions such as pH and temperature influence the physiological state of the host. This subsequently affects the host-phage interaction, which can, in turn, impact its efficacy in reducing foodborne bacteria (Fister et al., 2016). The results showed that both phages *svBEATS-5* and *svBEATS-6* are stable from pH 3 to 11 under prolonged exposure. Contrarily, *svBEATS-9* is highly susceptible to inactivation at pH levels 3 to 5 but is stable under pH 7 to 11 alkaline conditions. Inactivation of a phage under acidic conditions may be due to changes in the capsid, genome, and other virion structural components (Nobrega et al., 2016). Bacteriophage aggregation can be induced when hydrogen ion concentration is increased in an aqueous solution (Langlet et al., 2008). Evaluating their stability at various pH levels is important because the pH level of bacon can vary depending on various factors, such as the processing method, the amount of salt used, and the quality of the meat. However, the normal pH level of bacon generally falls within the range of 5 to 6. Furthermore, these findings suggest that the isolated Salmonella phages in this study are relatively stable in acidic and basic conditions. Based on the thermal stability results, the isolated bacteriophages exhibited potent lytic activity from 30°C to 50°C, with a noticeable decrease in efficacy at 55°C and complete inactivation at 60°C. Additionally, svBEATS-9 was slightly inactivated under 30°C - 40°C at more prolonged incubation, 60 to 90 min. The thermal stability of phages can be linked to the formation of disulfide linkages in their capsid proteins. This mechanism prevents the thermal denaturation of the phages when the temperature exceeds the optimum temperature (Jończyk et al., 2011). *Salmonella* Typhimurium can grow under temperatures 2°C to 54°C and pH 4 to 9.5 with optimal conditions of 37°C and a pH of 6.5 to 7.5 (Li et al., 2013). Additionally, *Salmonella* Typhimurium can persist in frozen meats for more than a year and months in an environment with low water activity (Muller et al, 2012; Kotzekidou, 1998). Exposure of *Salmonella* Typhimurium to extrinsic stress such as acidic conditions causes it to develop stronger resistance mechanisms against heat and high salt concentrations (Li et al., 2013). The pH and thermal stability of the phages reveal that their optimal infection parameters of the isolated phages were within the growing conditions of *Salmonella* Typhimurium at pH 7 and 37°C (Coburn et al., 2007; Keerthirathne et al., 2016). Jończyk et al. (2011) emphasized when the optimal infection and growth parameters of the phages and its host, respectively, will favor higher phage activity. The efficacy of biocontrol application of monophage treatments was assessed *in vitro* at 4°C and 30°C. Among the monophage treatments, *svBEATS-6* and *svBEATS-5* have the highest significant *Salmonella* Typhimurium reduction in vitro by 1.32 and 0.46 Log_{10} CFU/mL at 4°C and 30°C, respectively, after 4 days of treatment ($p < 0.05$). Testing the phages in *in vitro* setup can provide a baseline for their bacteriolytic ability, with phage *svBEATS-6* and *svBEATS-5* both exhibiting reductions in bacterial load after the 4 -day exposure. Likewise, the duration of the treatment is linked to the average growth cycle of *Salmonella* at both temperatures, where the bacteria have a significantly slower growth rate at lower temperatures (Xu et al., 2011; Petsong et al., 2019).

Phage titers were also determined for each treatment and temperature to assess the viability of the phages. Under extremely low temperatures, the phages will initially lose their titer (Abhisingha et al., 2020). However, phages are naturally resilient and adaptable to changes in temperature (Phothaworn et al., 2020). After the significant decrease in their number, it can be observed that the phages were able to stabilize and maintain their infectivity significantly ($p < 0.05$). This is further supported by the increase of phage titer on the second day of the treatment at 4°C ($p < 0.05$). At 30°C, the phage titers were higher compared to the concentrations at 4°C. This can be

attributed to its proximity to the average temperature of the phages' sampling sites, 26.4°C. Additionally, the thermal stability of the phages exhibited that they are highly lytic at this temperature. Moreover, all phages had a concentration higher than the initial concentration on the last day of the treatment.

In bacon, only the phage cocktail formulation containing three isolated phages (*svBEATS-5*, *svBEATS-6*, and *svBEATS-9*) was used as the biocontrol agent against *Salmonella* Typhimurium under the same temperatures as *in vitro*. Gu et al. (2012) emphasized that phage cocktails are ideal for therapeutic use against foodborne pathogens to delay the appearance of bacteriophage-resistant mutants. Consistent with the average reduction *in vitro*, the phage cocktail had larger reductions at 4°C compared to 30°C. Under refrigeration, microorganisms can maintain their physiological state, which is essential in phage-host interactions (Abhisingha et al., 2020). The ability of the phage cocktail to significantly reduce *Salmonella* Typhimurium at 4°C might indicate that the bacterial strain used in the study was able to adapt in this condition by lowering its metabolic rate, expressing cold-shock proteins, and maintaining the integrity of its cell membrane which enabled the phages to adsorb and cause cell lysis (Alvarez-Ordóñez et al., 2015). Several studies that applied phages as biocontrol agents against *Salmonella* in meat products did not determine the titers of the phages throughout their application. In this study, less fluctuation in the phage titers can also be observed in bacon compared to its concentration *in vitro*. This indicates that the phage cocktail sustained its infectivity and consistently reduced the *Salmonella* Typhimurium population in bacon. The lesser occurrence of severe fluctuations in the phage titer in bacon at both temperatures can be explained by the formulation of the cocktail being dominated by highly pH-stable phages.

The phage cocktail's *Salmonella* Typhimurium reductions in bacon at 4°C and 30°C were lower when compared to its in vitro application. The authors suggest that the larger reductions *in vitro* can be linked to the following factors: (1) composition of testing matrices, (2) phage-host interaction in solid substrates, and (3) presence of normal microbiota in tested samples.

First, there is a change in the physiological state of the *Salmonella* Typhimurium in a system with higher resource availability and accessibility, which the bacteria can capitalize on for their growth (Silva et al., 2014). In vitro experiment performed in this study allowed replication of the targeted host in a nutrient-rich media (ie. TSB). This, in turn, promoted phage infection due to the abundance and availability of the susceptible hosts (Gomez et al., 2015). On the other hand, restricted growth and colonization of *Salmonella* Typhimurium in the food matrix (i.e. preserved bacon) can be the cause for the declined bacteriolytic activity of the phages. This may be due to the composition of the curing process of bacon, which utilizes tertiary butyl hydroquinone (TBHQ) as an antioxidant and sodium nitrite as a preservative. Studies mentioned that TBHQ and its oxidation product tertiary butyl benzoquinone (TBBQ) have potent antimicrobial activity in bacterial cells by promoting the loss of membrane integrity (Ooi et al., 2013; Khezerlou et al., 2022). Furthermore, the salt concentration of bacon (11% w/w) is significantly higher than that of TSB used in vitro – thereby, hindering both the growth of the host and phage infection. Several studies demonstrated that high salt concentrations can affect the phages' ability to infect the host by breaking its head and tails due to osmotic pressure, increased thermal denaturation of phage proteins, and directly affecting the phage genome (Fennema, 1996; Joncyzk et al., 2011).

Additionally, one of the most significant challenges in using phages as biocontrol agents is maximizing the interaction between the bacteria and the phages in a solid or static matrix. Bull et al. (2018) have emphasized that the spatial structure plays an important role in the phage-bacterial dynamics. *In vitro* studies of biocontrol application of phages mainly use liquid media where the movement of phages and bacteria is extensive. Under these circumstances, they are more likely to interact, which is vital for efficient phage infections (Roucourt & Lavigne, 2009). In a solid food matrix such as bacon, diffusion of the phages is significantly more limited, and achieving a significant reduction in bacterial counts will take more time and a significantly higher number of phages (Hagens & Loessner, 2010). Aside from the titer of phages, it is also important to consider the delivery method of the agents unto the matrix. In this study, dipping method was done to introduce the phage cocktail into the artificially-infected samples. A more directed and targeted method for delivery should be considered to ensure the success of phage biocontrol application.

Lastly, presence of a diverse microbiota in the samples may also affect the efficacy of phage cocktails. Several studies have stated that the presence of other bacteria influences the development of phage resistance in the target host (Wang et al., 2019; Hasan & Ahn, 2022; McGee et

al., 2023). Reducing *Salmonella* using a phage cocktail will allow the other bacteria present in the environment to persist, creating competition between these bacteria to survive in the environment (Wang et al., 2019). Evolutionary dynamics of bacteria have shown that this competition will encourage *Salmonella* Typhimurium to develop phage resistance mechanisms by blocking the phage receptors used by the phages and having an extracellular matrix to protect themselves from infection (Labrie et al., 2010; Samson et al., 2013; Brockhurst et al., 2021; Hasan & Ahn, 2022).

Our results have shown that even though complete eradication of *Salmonella* Typhimurium in bacon was not achieved in the phage treatment, the reduction of the target host remained significant at 4°C and 30°C. Furthermore, processed meat products such as cured bacon present a unique challenge due to its high salinity content and presence of additives. Significant considerations mentioned in this paper must be evaluated to aid in designing effective phage therapies against foodborne pathogens in processed meat products.

Conclusion and Recommendations

This study proposed the use of Salmonella phage cocktail formulation (*svBEATS-5, svBEATS-6*, and *svBEATS-9*) against *Salmonella enterica* ser. Typhimurium – infected bacon samples. Decreased *Salmonella* reductions were observed using the phage cocktail compared to its in vitro application. Despite this, the phage cocktail maintained a consistent concentration without significant decline. Moreover, *Salmonella* Typhimurium in bacon was inhibited at levels lower than the untreated samples, with greater efficacy at 4°C than at 30°C. Looking into MOI optimization is a possible direction that future researchers can take to improve the reduction of *Salmonella Typhimurium* at 30°C. Once achieved, phage formulations will offer a promising alternative biocontrol agent to be used during the meat processing stage and storage phase, which are common food processing stages that are prone to bacterial contamination, including *Salmonella* Typhimurium.

Further studies can examine the use of *Salmonella* Typhimurium phages on other processed meat items and determine ways to keep phage concentrations constant for extended treatment periods to evaluate possible long-term effects. Genome analysis can be done to determine any potential deleterious genes such as lysogeny and virulence genes. The effect of additives can also be investigated to help fully understand how phages can reduce the target host in the food matrix. Likewise, developing cocktails with optimized concentrations to target serovars of *S. enterica* would be helpful for increased specificity and a more targeted approach. Determining the synergistic effects between phage isolates is also essential for an optimized treatment protocol. Lastly, future research can build on this work to develop a more useful biocontrol agent by examining how to maintain the phages' stability and efficacy under dynamic conditions (temperature and pH changes) and over more extended periods, such as those in the food processing chain.

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Conflict of interest

All authors declare no conflict of interest in the conduct of this study.

Authors' Contribution Statement

Jess Laurence Concepcion: Conceptualization, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration,

Funding acquisition. **Alonso Enrique Africa:** Conceptualization, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing. **Marty Bernard Matias:** Conceptualization, Formal analysis, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing. **Tracey Antaeus Gutierrez:** Formal analysis, Investigation, Resources, Data Curation, Writing - Review & Editing, Visualization, Supervision, Project administration. **Richard Rañeses:** Formal analysis, Investigation, Resources, Data Curation, Supervision, Project administration. **Ramon Arvin Noriel Santos:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Supervision, Project administration. **Gale Bernice Fungo:** Formal analysis, Investigation, Resources, Data Curation, Supervision, Project administration. **Carl Jay B. Bregente:** Investigation. **Jazon Harl D. Hidrosollo:** Investigation. Tran Thi Dieu Thuy: Investigation. **Cheng-Yen Kao:** Resources, Supervision. **Jose Bergantin Jr.:** Funding acquisition. **Donna May Papa:** Conceptualization, Writing - Review & Editing, Funding acquisition.

Ethics Statement

All experiments conducted in the research did not involve human and animal subjects. The research is the authors' own original work and has not been previously published in any other journals before its publication under the NRCP Research Journal. Consequently, this paper is not submitted and not being considered for publication in other journals elsewhere. The paper properly credits the viable contributions of co-authors. The results and analysis are completed and placed in a truthful and complete manner without any form of data manipulation. All the sources used in the paper are properly cited with the appropriate reference. Lastly, all authors provided their consent in the submission and publication of this manuscript containing their names and will take responsibility for its content.

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