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Bacteriophage-based biocontrol of *Salmonella enterica* ser. Enteritidis in selected ready-to-eat food

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Salmonella sp. is one of the major causes of food-borne illnesses in the world, causing serious threat to human health. Innovations are constantly being explored to lessen the incidence of food-borne pathogens because the current approaches are still ineffective. One alternative solution to mitigate the problem is the use of bacteriophages. In this study, three phages A3CE, PF01, and, PF02 were partially characterized based on morphology, host range, and infectivity under different pH and temperature conditions. All three phages have contractile tails and belong to the Family *Myoviridae*. Results of phage stability assays showed that the three phages were able to retain infectivity at pH 5-9 and 30°C – 50°C and were able to infect a few other serotypes of *Salmonella.* All three phages were used to reduce *Salmonella* ser. Enteritidis in Ready to eat (RTE) food. Pepper pastrami, chicken ham, bologna, bean sprouts, and lettuce were spiked with *Salmonella* ser. Enteritidis (1 x 10^5 CFU/ml) and were treated with (a) Phage A3CE, (b) Phage PF01 and PF02 cocktail, (c) Phage A3CE, PF01, and PF02 cocktail at a multiplicity of infection (MOI) of 1000 and were incubated for 4 days at 4 and 25°C. Results showed that phage treatment resulted in a significant reduction of *Salmonella* ser. Enteritidis in RTE foods, with the phage cocktail (A3CE, PF01, and PF02) treatment showing up to 5 log reductions. Overall, the results of the study demonstrate the potential of virulent phages in controlling *Salmonella* in food.

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INTRODUCTION

Salmonella is a gram-negative, rod-shaped, facultative anaerobic bacilli and is one of the most common food-borne pathogens worldwide (Scallan et al. 2011; Yang et al. 2015; Shao et al. 2011). The disease caused by *Salmonella* is called salmonellosis, and symptoms include diarrhea, nausea, vomiting, and even septicemia in serious cases (McLandsborough 2005; Grassl and Finlay 2008). The prevalence of *Salmonella* in food products in the Philippines was investigated in several studies. Del Rosario and Rivera, (2012) reported a 30.63% incidence of *Salmonella enterica* in raw and processed meats from select wet markets, while another study by Calayag et al., (2017) showed a 44% incidence of *Salmonella* in slaughtered swine. Meanwhile, Elumba et al., (2017) reported a 34% incidence of *Salmonella* in chicken meat.

The steady increase of several food related diseases caused by bacteria such as *Campylobacter, Listeria*, *Escherichia coli, Salmonella,* and other bacterial pathogens that impact public health prove that the current technologies and techniques being employed to kill bacterial pathogens in food are ineffective (Garcia et al. 2008). There are approximately 600 million people around the world who get infected with food-borne pathogens annually, and an estimated 420,000 deaths (World Health Organization 2019). One possible solution to combat foodborne pathogens is the use of bacteriophages. Bacteriophages are viruses that infect bacterial cells and can multiply within them. Biocontrol of bacteria in food during industrial food processing

can be done by the application of phages directly on the surface of food. The control of food-borne pathogen using phages has moved beyond the laboratory, and regulatory approvals have been granted for commercial phage products that are currently available to the food industry (Hudson et al. 2010).

There are various ways to control foodborne pathogens using bacteriophages from the food production to product consumption. These include phage therapy for the reduction of colonization of bacteria, biosanitation for the disinfection, biocontrol of pathogens from raw materials, carcasses and ready-to-eat (RTE) food, and biopreservation of the final product (Garcia et al. 2008). These methods have been applied in several studies. A study by Thung et al, (2017) has shown Phage SE07 was able to exhibit 2 log reductions of *Salmonella* Enteritidis on fruit juice, eggs, beef, and chicken samples. Phage P22 showed 2-3 log reductions of *Salmonella* Typhimurium on liquid eggs, energy drinks, whole and skimmed milk, apple juice, chicken breast, and chicken mince (Zinno et al. 2014). The application of bacteriophage F01-E2 showed 3 log reductions of *Salmonella* Typhimurium on chocolate milk and turkey deli while it showed 2 log reductions on hotdogs and seafood (Guenther et al. 2011). The use of a cocktail of two phages exhibited a 1.5 log reduction of *Salmonella* on broccoli sprouts (Pao et al. 2004). Phage Felix 01 resulted in a 1.8 log reduction of *Salmonella* Typhimurium on chicken frankfurters (Whichard et al. 2003). These studies showed that bacteriophages are a viable alternative to antibiotics in controlling foodborne pathogens, especially now that antibiotic resistant bacteria are on the rise. According to Thapa, Shreshta, & Anal (2019), antibiotic resistant bacteria (ARB) can infect humans either indirectly by consuming ARB contaminated food or directly through direct contact with ARB-infected animals.

The use of bacteriophages to control the growth of bacterial pathogens in food is still relatively recent compared to their use in therapeutics and diagnostics. The use of phages as biocontrol agents in food have been primarily studied in fresh produce, dairy products, and meat products, whereas most of the research concerning spoilage causing bacteria had been largely focused on meat (Hudson et al. 2010; Garcia et al. 2009). There is also limited information on the use of bacteriophages to control foodborne pathogens like *Salmonella* in the Philippines. In this study, we evaluated the potential of three bacteriophages as biocontrol agents against *Salmonella* ser. Enteritidis in different ready-to-eat (RTE) food. The phages were able to significantly reduce the number of *Salmonella* ser. Enteritidis on the different food models, particularly the cocktail treatment. The phages were also stable at pH 5-9 and 30°C – 50°C, meaning it can survive many storage and preparation conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Salmonella ser. Enteritidis, obtained from the Collection of Microbial Strains, University of Santo Tomas (USTCMS), Manila, was used for the experiment. Other *Salmonella* strains and bacteria used for the determination of phage host range was obtained from the Research Institute for Tropical Medicine (RITM) and USTCMS (Table 1). Working cultures of the bacteria were routinely grown on Tryptic Soy Broth (TSB) and Tryptic soy Agar (TSA) at 37 °C and were stored at 4 °C.

Bacteriophage isolation and purification

Bacteriophages lytic for *Salmonella* ser. Enteritidis were isolated and purified from soil samples collected from farms in Pulilan, Bulacan using the methods described by Twest and Kropinski (2009), Bigot et al. (2011), and Clokie and Kropinski (2009). A previously isolated bacteriophage from agricultural soil samples in Marilao, Bulacan, phage A3CE (Santos et al, 2014) was also used and included in this study.

One (1) ml of an overnight culture of *Salmonella* ser. Enteritidis culture was transferred to a 250 ml flask containing 100 ml TSB. Five (5) grams of the soil sample were added to the flask containing TSB and *Salmonella* ser. Enteritidis. These were then incubated in a rotary shaker at 160 rpm for 24 hours at room temperature and were centrifuged at 7,500 rpm for 10 minutes. The supernatant was then filtered using a 10 ml syringe with 0.22 and 0.45 µm Acrodisc syringe filter (Sartorius). The filtrates were then tested for the presence of bacteriophage through spot plate assay. Five hundred microliters of a five-hour old *Salmonella* ser. Enteritidis broth culture was added to three mL molten 0.7% soft Tryptic Soy Agar (TSA) and the mixture was poured on a TSA base plate. Twenty microliters of the filtrate were spotted on the TSA plate. The plate was incubated for 24 hours at 37˚C and was observed for the appearance of a clear spot (presence of bacteriophage).

The plaques of bacteriophages on positive filtrates were obtained using the Agar Overlay Method. The filtrates were serially diluted (10-fold dilutions) 8 times (10^{-1} – 10^{-8}) using Phosphate Buffered Saline Solution (PBS). Five hundred microliters of a 5-hour old *Salmonella* ser. Enteritidis broth culture and 500 µl of the filtrate were added to a three ml molten 0.7% soft TSA. The mixture was then poured on a TSA base plate. The plates were incubated overnight at 37 ˚C and were examined for the presence of plaques. Isolated plaques of different morphological types were picked and mixed separately with 10 ml of a five- hour old broth culture of *Salmonella* ser. Enteritidis. These were then incubated on a rotary shaker at 160 rpm for 24 hours at room temperature. After incubation, the mixture was centrifuged at 7,500 rpm for 10 minutes. The supernatants were then filtered using a 10 ml syringe with 0.22 and 0.45 µm Acrodisc filter. The filtrates were again subjected to Agar Overlay Method to determine phage purity (until all plaques exhibit uniform morphology). Purified bacteriophage filtrates were stored at 4 °C until further use.

Bacteriophage characterization

Phage Morphology by TEM

A high titer phage stock (Clokie & Kropinski 2009) of 10^8 PFU/mL was prepared by adding 10 ml of the phage filtrate to a flask containing 90ml five-hour old broth culture of *Salmonella* ser. Enteritidis. This phage-host mixture was incubated and filtered as described earlier. The high titer phage stock was then sent to the Pathology Department of the Research Institute for Tropical Medicine (RITM) for Transmission Electron Microscopy (TEM). The samples underwent negative staining using the phosphotungstate (PT) reagent and the electron micrographs were taken using Jeol 1220 TEM under 40,000X and 60,000X magnification.

Phage Host Range

Host range of the isolated phages was determined by spot inoculating the bacteriophage filtrate (1 x 10^8 PFU/ml) to agar overlays of different serovars of *Salmonella* sp. (*Salmonella enterica* serovar Stpaul, *S.* Typhimurium, *Salmonella* sp. 1, and *Salmonella* sp. 2) and other food pathogens (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*). After overnight incubation at 37˚C, the presence of clearing zones was observed and recorded.

Phage thermal and pH stability test

One (1) ml of the phage filtrates (1 x 10^8 PFU/ml) was mixed with nine ml phage buffer. Each phage suspension was subjected to different temperatures (30, 40, 50, 60, and 70 °C) and pH

values (3, 4, 5, 6, 7, 8, 9, and 10). At certain time intervals (0, 30, 60, and 90min), 10 µl of each phage filtrate was spotted to agar overlays of five hour-old culture of *Salmonella* ser. Enteritidis and were incubated for 24 hrs. The presence of clearing zones on the plates was observed and the degree of clearing was noted as: no clearing, turbid, or clear (Langlet et al. 2007; Feng et al. 2003).

Biocontrol of *Salmonella* ser. Enteritidis by the phages in Tryptic soy broth

Overnight cultures of *Salmonella* ser. Enteritidis were diluted until an absorbance of 0.08-0.1 (0.5 MacFarland Standard, 1.5 x 10^8 CFU/ml) was achieved when measured at 600 nm (A600). These were then diluted to achieve a final concentration of 1 x 10^5 CFU/ml. The bacteriophage, with a concentration of 1 x 10^8 PFU/ml, was added. There were four treatments used in the experiment: (1) monoculture of phage A3CE, (2) cocktail of phage PF01 and PF02, (3) cocktail of phage A3CE, PF01, and PF02, and (4) PBS as the negative control. MOI of 1000 was used for all treatments and the phage-host mixture was incubated at 4°C and 25 °C for five days. *Salmonella* ser. Enteritidis were enumerated everyday up to five days using the spread plate method wherein the host-phage mixture was serially diluted (10-fold dilutions) nine (9) times (1 x 10^{-1} – 1 x 10^{-9} dilution) on PBS. One hundred (100) µl of each diluted tube were spread evenly onto Salmonella Shigella Agar (SSA) plates. The colonies of *Salmonella* ser. Enteritidis were then counted after incubation at 37 °C for 24 hours (Guenther, et al., 2011).

Food Sample Acquisition and Preparation

Five different RTE food products were chosen for this study: pepper pastrami, chicken ham, bologna, lettuce, and bean sprouts. All food samples were purchased from local grocery shops in Manila and were screened for the presence of *Salmonella* following ISO 6579-1:2017. Samples were pre-enriched in buffered peptone water, were enriched in Rapapport-Vassiliadis broth and Tetrathionate broth, and were plated on selective media (SSA and XLDA) (International Organization for Standardization 2017). Only samples free of *Salmonella* were used for the experiment.

Biocontrol of *Salmonella* ser. Enteritidis on food models using phages

Sample Inoculation and treatment

Overnight cultures of *Salmonella* ser. Enteritidis were diluted until an absorbance of 0.08-0.1 (0.5 MacFarland Standard, 1.5 x 10^8 CFU/ml) was achieved when measured at 600 nm (A600). These were then diluted to achieve the target cell density of 1 x 10^5 CFU/ml. Eight grams of each sample were placed in sterile Ziploc bags and one ml of the 1 x 10^5 CFU/ml *Salmonella* ser. Enteritidis culture was added by evenly spreading the culture over the samples. Spiked samples were incubated at room temperature for 20 minutes to allow the bacteria to adhere to the samples. The spiked samples were inoculated by adding one ml of the bacteriophage filtrates (1 x 10^8 PFU/ml). One set-up was inoculated with one ml of PBS instead of the bacteriophage and served as the negative control. The samples were then sealed and incubated at 4 and 25 °C for four days (Guenther et al. 2011).

***Salmonella* ser. Enteritidis recovery**

Daily enumeration of *Salmonella* ser. Enteritidis was determined from Day 0 up to Day 4. After incubation, samples were homogenized in 90 ml Buffered peptone water using a masher. One ml of the homogenized sample was serially diluted nine times in a 1:100 ratio (1 x 10^{-1} – 1 x 10^{-9} dilution) in PBS. One hundred microliters of each diluted sample were spread evenly onto Salmonella Shigella Agar (SSA) plates. *Salmonella* colonies that appear colorless with black centers on SSA were counted after 24 hours of incubation at 37 ˚C and the CFU/g was computed (Guenther et al. 2011; Zinno et al. 2014).

Data Analysis

Bacterial counts were determined using the spread plate method and three trials were performed. Results were presented as mean values and standard deviations of the means were represented as error bars. One-way ANOVA and Tukey post hoc tests were used to determine the significance of the cell count differences between the control samples and samples treated with phages with an alpha level of 0.05.

RESULTS AND DISCUSSION

Phage Morphology

The morphological characteristics of the isolated phages were determined. All three phages A3CE, PF01, and PF02 belong to order *Caudovirales*, family *Myoviridae* because of the presence of a contractile tail based on TEM analysis (Figure 1) (Kutter and Sulakvelidze 2005). The morphological characteristics and measurements of the three phages were summarized in Table 2, with phage A3CE having the largest head diameter at 128 nm and phage PF01 having the longest tail length at 180 nm. Many of the phages used in studies for biocontrol of *Salmonella* in foods are under the Family *Siphoviridae.* Some of the *Siphoviridae* phages include phage wksl3 (Kang et al. 2013), ΦSH17 and ΦSH18 (Hooton et al. 2011), Φ st1 (Wong et al. 2013), and SSP6 (Kocharunchitt et al. 2008). There are only a few *Myoviridae* phages used in studies for biocontrol of *Salmonella* in foods like phages PA13076 and PC2184 (Bao et al. 2015), and ΦSH18 (Hooton et al. 2011). All three phages are lytic, shown by their clear plaques, indicating the ability to lyse and kill the host bacteria they came in contact with and according to Furfaro et al. (2018), the main type of phages used in phage therapy.

Table 1: Bacterial strains used in the study.

Bacterial Strain	Source
Salmonella ser. Enteritidis	USTCMS
Salmonella ser. Typhimurium	USTCMS
Escherichia coli	USTCMS
Pseudomonas aeruginosa	USTCMS
Salmonella ser. Stpaul	RITM
Salmonella sp. 1	RITM
Salmonella sp. 2	RITM
Listeria monocytogenes	RITM

Table 2: Measurements of head and tail structures of phages A3CE, PF01 and PF02.

Characteristics	Phage A3CE	Phage PF01	Phage PF02
Head diameter	128 nm	122 nm	116 nm
Tail length	112.5 nm	180 nm	78 nm
Sheath length (Contracted)	92 nm	102 nm	44 nm
Sheath diameter (Contracted)	14 nm	26 nm	18 nm
Tail pin length	20 nm	78 nm	34 nm
Tail pin diameter	6 nm	12 nm	10 nm
Contractile tail	yes	yes	Yes

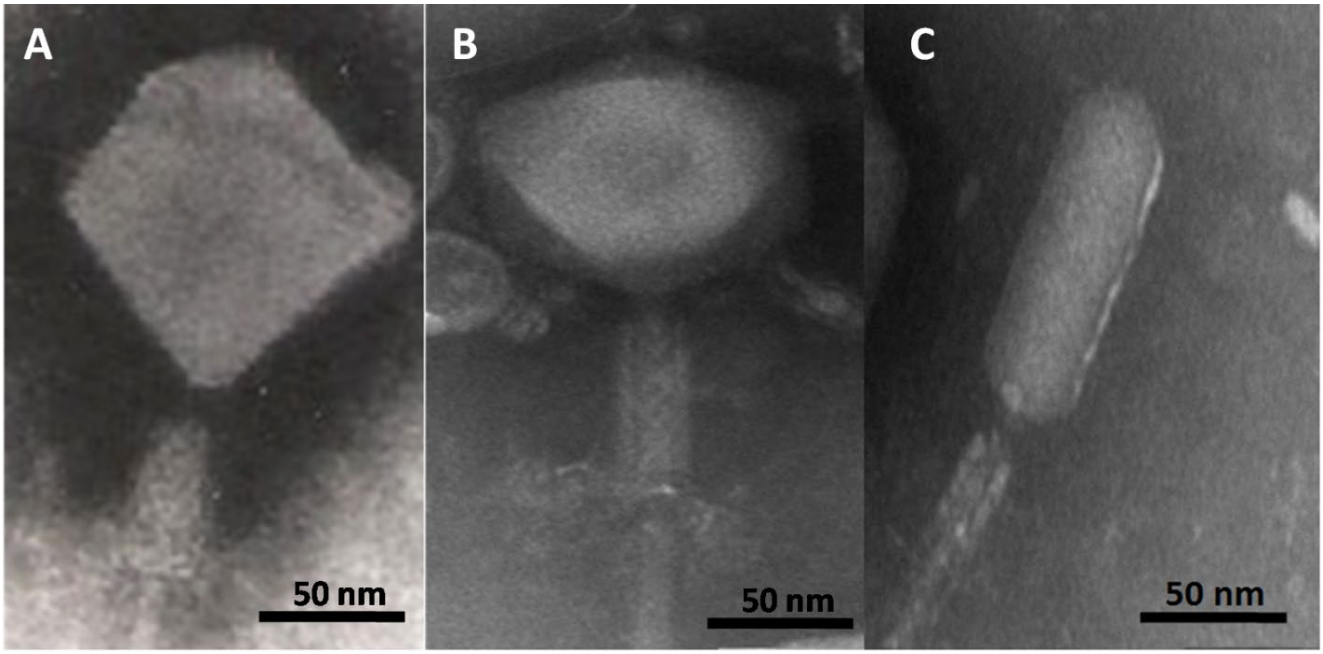

Figure 1: Transmission Electron Micrograph of Phage A3CE (A), Phage PF01 (B), and Phage PF02 (C). All three phages have an icosahedral head and a contractile tail, with Phage PF02 exhibiting an elongated head. ***Electron micrographs taken by the RITM at 302,000x magnification.***

A

Phage Treatment

pH	A3CE				PF01 + PF02			
	Exposure time (min)				Exposure time (min)			
	0	30	60	90	0	30	60	90
3								
4								
5								
6								
7								
8								
9								
10								

B

Phage Treatment

Temp-erature	A3CE				PF01 + PF02			
	Exposure time (min)				Exposure time (min)			
	0	30	60	90	0	30	60	90
30 °C								
40 °C								
50 °C								
60 °C								
70 °C								

Legend: Clear/ Unaffected; Turbid/ Partially Inactivated; No Clearing/ Inactivated

Figure 2: Effect of A. pH and B. Temperature on phage infectivity

Table 3: Host Range of Phage A3CE, PF01 and PF02.

Host	Phage A3CE	Phage PF01 + PF02
Salmonella ser. Enteritidis	+	+
Salmonella ser. Typhimurium	+	+
Salmonella ser. Stpaul	+	+
Salmonella sp. 1	+	+
Salmonella sp. 2	+	-
E. coli	-	-
P. aeruginosa	-	-
L. monocytogenes	-	-

Host range

The phages were tested to determine whether they can infect bacteria other than *Salmonella* ser. Enteritidis. Results of host range assay showed that phage A3CE was able to lyse five different *Salmonella* isolates while the combination of phages PF01 + PF02 infected four *Salmonella* isolates and can possibly infect even more serotypes (Table 3). All three phages were unable to infect the three non-*Salmonella* hosts tested. All three phages were able to infect *Salmonella* serotypes other than *Salmonella* ser. Enteritidis, which makes them good candidates when creating phage cocktails (in conjunction with other *Salmonella* phages) intended to control foodborne *Salmonella* serotypes since they can take the place of several phages only capable of infecting one serotype. According to De Jonge et al. (2018), every bacteriophage has their own host range with some having a narrow host range (most studied type), only capable of completing their life cycle on only one host (species) of bacteria, while others have a broad host range, capable of infecting and completing their life cycle on multiple taxonomically distinct hosts (several species/strains). All phages used in this study are broad host range according to the definition of De Jonge et al. (2018) since the three phages were able to infect *Salmonella* serotypes other than *Salmonella* ser. Enteritidis. There are several factors that determine the host range of phages, such as the type of host binding proteins, presence of prophages and specific plasmids, phage resistance, and biochemical processes during infection (Ross et al. 2016). The phages being able to lyse more than one serotype of *Salmonella* suggest that the three phages can be good candidates for phage therapy and biocontrol. Phage A3CE even showed effectivity in controlling *Salmonella* ser. Havana in another study (Santos et al. 2014)

Effect of pH and temperature on phage infectivity

The ability of the isolated phages to retain infectivity under different pH and temperature was examined. All phages were spotted onto lawns of *Salmonella* ser. Enteritidis after exposure to a range of pH and temperature. The range used was based on the conditions where *Salmonella* can grow (pH: 4-9, temperature 5 °C - 47 °C). The stability of phages at these conditions was determined by their ability to lyse their hosts through the clarity of the spots. The presence of clear spots indicates that the phages were able to lyse their host and are therefore unaffected by the conditions. The presence of turbid spots indicates partial lysis, wherein some of the phages were inactivated and are unable to lyse its host, indicating reduced activity. Spots with no clearing indicate that the phages were inactivated by the growth conditions, rendering them unable to lyse its host.

For the effect of pH, Phage A3CE was stable at pH 5-8, partially inactivated at pH 4 and 9, and was inactivated at pH 3 and 10. On the other hand, Phage PF01 + PF02 was stable at pH 5-9 and was partially inactivated at pH 3, 4, and 10 (Figure 2A). For the effect of temperature, Phage A3CE was stable at 30˚C - 50˚C and was partially inactivated at 60˚C and 70˚C. Phage PF01 + PF02 were stable at 30˚C - 60˚C and was only partially inactivated at 70˚C (Figure 2B).

The stability of phages from pH 5 to 9 depends on their protein capsids (Adams 1959; Kutter and Sulakvelidze 2005) while the formation of disulfide cross links in the proteins of the capsid stabilize them from denaturation caused by heat (Jurczak-Kurek et al. 2016). The titer and infectivity of phages are reduced significantly by low pH and high temperatures (Nobrega et al. 2016; Jończyk et al. 2011; Meyer et al. 2017; Leverentz et al. 2001) and therefore, it is important to know whether the phages can still be effective in reducing the number of food pathogens at these conditions since many RTE foods are processed at conditions unfavorable to the growth of phages (Garcia et al. 2009; Ly- Chatain 2009). The results showed that the phages were able to tolerate and retain infectivity at pH 5-8 and 50-60 ºC and can therefore potentially survive processing and packaging conditions. The results are comparable with Phage Z (Jamal et al. 2015), which is stable at pH 5-11 and temperatures up to 70 ºC.

The stability of phages to different physical conditions is generally an integral part of their characterization, most especially when they are used for application in therapy or biocontrol since the hosts are also exposed to various environments where phages can be rendered inactive. The food manufacturing process employ methods that modify pH and temperature since microbial growth and contamination is influenced by the pH and temperature (Kim et al. 2019). For phage products being used in the food industry, one of the self-limiting criteria is that phages are susceptible to various factors such as phage resistance (Intralytix 2016; Polaska and Sokolowska 2019), which can decrease their titer, hence also decrease their capability to control food pathogens. This action, though, is true for all antimicrobial agents, may it be a phage or an antibiotic. Stability is usually stated for the specific duration of infectivity.

Biocontrol of *Salmonella* ser. Enteritidis in Tryptic soy broth

The reduction in the numbers of *Salmonella* ser. Enteritidis grown in TSB by the phages was investigated. At 4ºC, no *Salmonella* ser. Enteritidis was recovered after three days for phage A3CE and cocktail treatment, and also for phage PF01 + PF02 treatment after five days. All three phage treatments showed significant reductions ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control starting at day three (Figure 3A). The number of *Salmonella* ser. Enteritidis on samples treated with phage A3CE and cocktail treatments were significantly lower ($P < 0.05$) compared to phage PF01 + PF02 from Days 3-4, though by Day 5 there were no significant difference among treatments as all the treatments reduced *Salmonella* ser. Enteritidis to undetectable levels.

At 25°C, the numbers of *Salmonella* ser. Enteritidis did not decrease significantly for all treatments ($P > 0.05$). Only phage A3CE and cocktail treatment showed significant reductions ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control. The numbers of *Salmonella* ser. Enteritidis increased for the control treatment starting at day two.

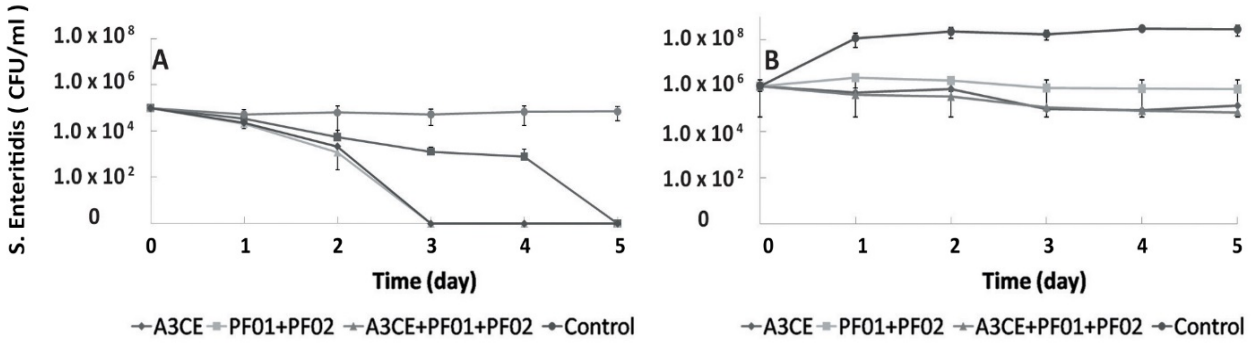

Figure 3: Number of recoverable *S.* Enteritidis in TSB incubated at 4 °C (A) and at 25 °C (B)

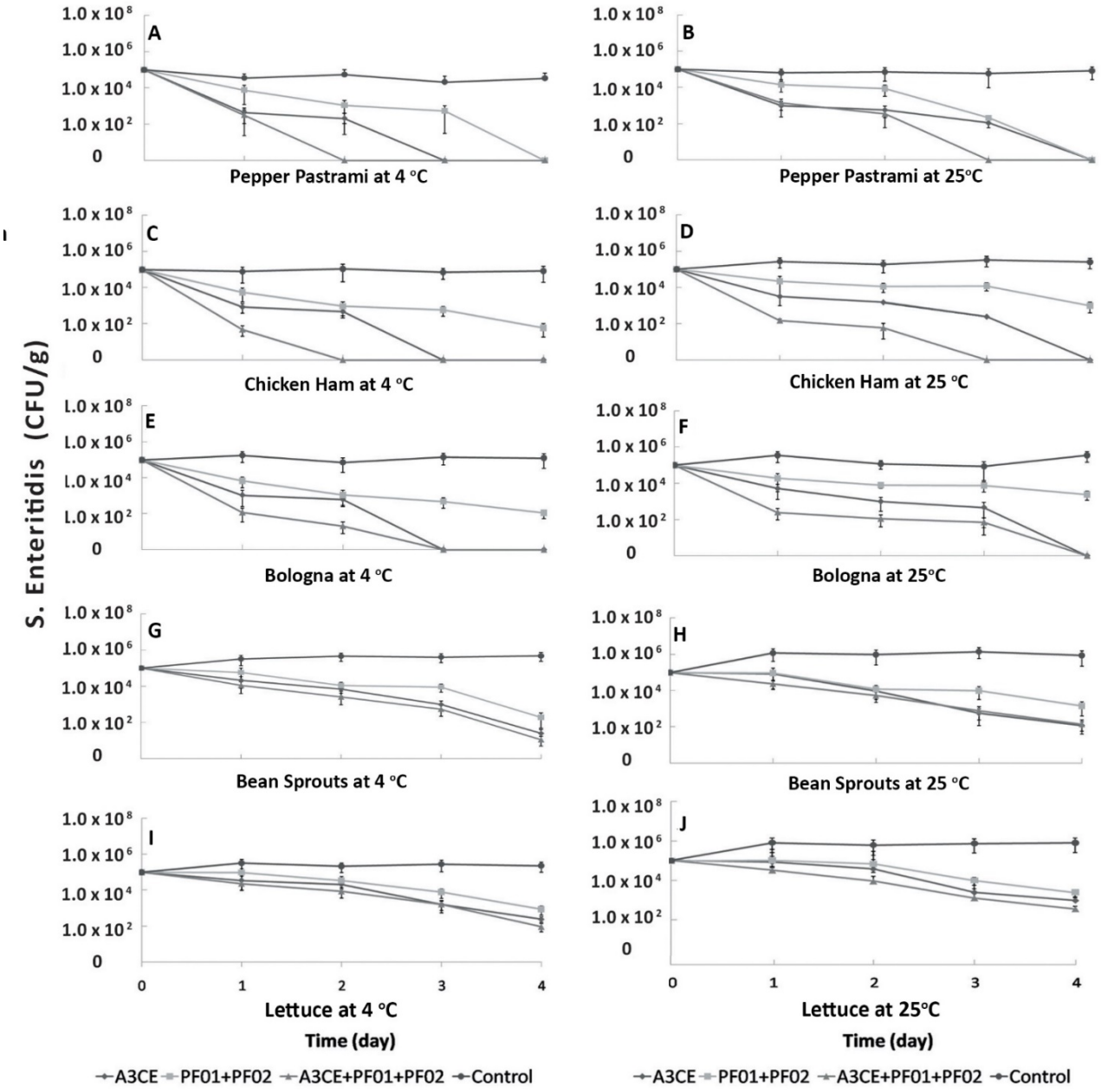

Figure 4: Number of recoverable *S.* Enteritidis on different RTE food treated with phage A3CE, PF01, and PF02 incubated at 4 °C and 25 °C.

Results showed that the phages were more effective in reducing the numbers of *Salmonella* ser. Enteritidis at 4°C than at 25°C as the numbers of *Salmonella* ser. Enteritidis were reduced to undetectable levels. Mesophilic bacteria like *Salmonella* ser. Enteritidis, grow much slower on low temperatures (< 20°C) (Pothakos et al. 2012), and in the case of *Salmonella*, growth is halted at temperatures < 7°C, although it still survives at even lower temperatures (Butler and Curtis 2009). Phages were stable at different conditions, which indicate their potential use in controlling food pathogens in refrigerated conditions.

Biocontrol of *Salmonella* ser. Enteritidis on selected RTE foods

The potential of the phages to reduce the numbers of *Salmonella* ser. Enteritidis on RTE foods was determined. In this experiment, four treatments with two incubation temperature conditions were used for the following RTE foods: pepper pastrami, chicken ham, bologna, lettuce, and bean sprouts. Three meat samples commonly available in local delis, and two vegetable products commonly used in fresh green salads, were included in this study.

For pepper pastrami at 4°C, no *Salmonella* ser. Enteritidis was recovered on Day 2 of the treatment for the cocktail, Day 3 for phage A3CE and Day 4 for phage PF01 + PF02 (Figure 4A). At 25 °C, no *Salmonella* ser. Enteritidis was recovered on Day 3 of the treatment for the cocktail and at Day 4 for Phage A3CE and PF01 + PF02 (Figure 4B). All three phage treatments showed significant reductions ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control for all time points in both temperature conditions. There was no significant difference ($P > 0.05$) in the efficiency of the three phage treatments after four days since all treatments reduced *Salmonella* ser. Enteritidis to undetectable levels.

For the chicken ham at 4 °C, no *Salmonella* ser. Enteritidis was recovered on Day 2 of the treatment for the cocktail, Day 3 for phage A3CE, and a 3 log reduction for phage PF01 + PF02 after 4 days (Figure 4C). At 25 °C, no *Salmonella* ser. Enteritidis was recovered on the Day 3 of the treatment for the cocktail, Day 4 for phage A3CE, and a 2 log reduction for phage PF01 + PF02 (Figure 4D). All three phage treatments showed significant reductions ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control for all time points in both temperature conditions. There was also a significant difference ($P < 0.05$) in the biocontrol activity of phage A3CE and cocktail treatment with the phage PF01 + PF02 treatment at Day 4.

For bologna at 4 °C, no *Salmonella* ser. Enteritidis was recovered on Day 3 of the treatment for the cocktail and phage A3CE, and a 3 log reduction for phage PF01 + PF02 (Figure 4E). At 25 °C, no *Salmonella* ser. Enteritidis was recovered on Day 4 of the treatment for the cocktail and phage A3CE and a 1.5 log reduction for phage PF01 + PF02 (Figure 4F). All three phage treatments on both incubation temperatures showed significant reductions ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control (for all time points). There was also a significant difference ($P < 0.05$) in the biocontrol activity of phage A3CE and cocktail treatment with the phage PF01 + PF02 treatment at Day 4.

For bean sprouts at 4°C, there was a 3 log reduction for cocktail treatment, a 2.6 log reduction for phage A3CE, and a 2 log reduction for phage PF01 + PF02 on Day 4 of the treatment (Figure 4G). At 25 °C, there was a 2.5 log reduction for cocktail treatment, a 2 log reduction for phage A3CE, and a 1.6 log reduction for phage PF01 + PF02 on Day 4 of the treatment (Figure 4H). All three phage treatments on both incubation temperatures showed significant reductions ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control (at all time points). There was also a significant difference ($P < 0.05$) among the biocontrol activity of the three treatments at Day 4.

For lettuce at 4 °C, there was a 4 log reduction for cocktail treatment, a 3.5 log reduction for phage A3CE, and a 2 log reduction for phage PF01 + PF02 on Day 4 of the treatment (Figure 4I). There was a significant difference ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control starting from Day 1 for the cocktail and phage A3CE treatment and day two for the phage PF01 + PF02 treatment. At 25 °C, there was a 3 log reduction for cocktail treatment, a 2 log reduction for phage A3CE, and a 1 log reduction for phage PF01 + PF02 on Day 4 of the treatment (Figure 4J). There was a significant difference ($P < 0.05$) in the number of *Salmonella* ser. Enteritidis when compared to the control starting from Day 1 for the cocktail treatment, Day 2 for the phage A3CE treatment and Day 3 for the phage PF01 + PF02 treatment. There was also a significant difference ($P < 0.05$) among the biocontrol activity of the three treatments at Day 4.

Table 4: Log reduction in the number of *Salmonella* ser. Enteritidis in RTE foods by using single and mixed phages.

Food Sample	A3CE		PF01 +PF02		A3CE +PF01 + PF02	
	4˚C	25 ˚C	4˚C	25 ˚C	4˚C	25 ˚C
Pepper Pastrami	>5	>5	>5	>5	>5	>5
Chicken Ham	>5	>5	3	2	>5	>5
Bologna	>5	>5	3	1.5	>5	>5
Bean Sprouts	2.6	2	2	1.6	3	2.5
Lettuce	3.5	2	2	1	4	3

Table 4 summarizes the log reductions of *Salmonella* ser. Enteritidis in different food models treated with phages and incubated at 4°C and 25°C after four days of incubation. The use of all three phage treatments resulted in the significant reduction of *Salmonella* ser. Enteritidis on all food samples and incubation temperatures, with consistent and steady reductions per day. The cocktail treatment exhibited highest reduction, where undetectable levels of *Salmonella* ser. Enteritidis in meat samples was observed as early as Day 3. There is a greater reduction for all food samples incubated at 4°C than those incubated at 25°C, which are consistent with the results obtained from the experiment using TSB. The difference, however, between RTE and TSB is that there is still a large reduction in the number of *Salmonella* on food samples incubated at 25°C (1 - > 5 log reductions), compared to TSB samples incubated at 25°C (0.1 -1 log reductions). The observed difference may be due to the fact that the nutrients present in TSB are simple and can be easily utilized by the host bacteria, therefore facilitating faster growth. This is in contrast with RTE foods because they are not easily utilized by the bacteria as they contain more complex nutrients than TSB. This means that the RTE foods have to be broken down first, slowing down bacterial growth (compared to growth on culture media), allowing the phages lyse them before they had a chance to quickly multiply and therefore resulting in greater reductions.

The reason for the lower reduction in the numbers of *Salmonella* ser. Enteritidis for lettuce and bean sprouts compared to the other samples may be due to the secondary compounds and metabolites (like phenolics and acetaldehyde) present in plant surfaces that are known to inactivate viruses, including bacteriophages (Guenther et al. 2009; Dawson et al. 2005). These results are comparable to the study of Snyder et al. (2016) that showed a 1-4 log reduction of bacteria on green pepper slices and spinach leaves, with higher reductions on samples incubated at 4 ºC than samples incubated at 25 ºC. Another reason is that there are no preservatives present on both of these samples, unlike the other three samples, that could help slow down the growth of *Salmonella* ser. Enteritidis and therefore yielding lower reductions.

CONCLUSION

In this study, treatment with a cocktail of three phages (A3CE, PF01, and PF02) resulted in the significant reduction of *Salmonella* ser. Enteritidis in both TSB and in different RTE foods incubated at 4°C and 25°C. All three phages remained infective on all pH and temperature conditions where *Salmonella* can thrive. The phages exhibited broad host range, capable of infecting a number of *Salmonella* serotypes. These results indicate the potential of the three phages for the development of a phage-based product for the biocontrol of *Salmonella* ser. Enteritidis on food. Further studies can be done to characterize the phages' stability to UV, chloroform, ether, and additional *Salmonella* serotypes can be included for the host range analysis. Other food samples and incubation temperatures can also be tested, and the synergistic effect of phage cocktails can also be investigated for the development of a bacteriophage cocktail that can be applied on food to combat foodborne pathogens.

An increasing number of consumers not only in the Philippines but also in the global market, are seeking natural and organic foods that are free of pathogens and are prepared in a safer way. The use of phages is one way to make these natural and organic foods free from contamination. Although, there some companies that have gathered regulatory approval of phage-based products, further studies are still needed in order to overcome challenges in using phages as biocontrol agents in food (different administration strategies, stability in different food matrices). Nevertheless, phages have the potential to be used in controlling food-borne pathogens in food.

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