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Isolation and characterisation of two lytic bacteriophages against non-typhoidal *Salmonella*

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Abstract

With the increasing rate of antibiotics failure, attention has been drawn to the bacteriophage as an alternative to control multidrug resistant bacteria. Herein, two *Salmonella* bacteriophages SH1 and SH2, capable of infecting multiple *Salmonella* serovars, were isolated and characterised. The phages were isolated from chicken fillet samples using the double agar overlay plaque assay by using *Salmonella* Hvittingfoss as a host bacterium. SH1 and SH2, the double-stranded DNA phages which belong to the family *Siphoviridae*, were found to have broad host range due to the capability of inhibiting 37 and 38 out of 58 *Salmonella* strains, which included 17 and 16 different serovars, respectively. Both phages were found to have the optimal multiplicity of infection of 1 with good pH tolerance (pH 5 - 11) and thermal stability (4 - 50°C). For the reproduction cycle, it was discovered that SH1 and SH2 have the latent periods of 20 and 10 min with average burst sizes of 59 and 52 PFU/cell, respectively. A total of 0.31 ± 0.01 and 0.32 ± 0.04 log CFU/mL viable cells of *Salmonella* host were significantly reduced from the initial count after treatment with SH1 and SH2 for 24 h, respectively. Moreover, SH1 and SH2 were discovered to have an effective eradication effect towards *S.* Hvittingfoss with a percentage of 99.74 ± 0.17% and 89.40 ± 0.29%, respectively, after 24 h treatment. The present work suggests that SH1 and SH2 could provide a good prevention and control effect against planktonic antibiotic-resistant *Salmonella* and its biofilms. The present work also describes the potential of both bacteriophages to be used for biocontrol of *Salmonella* in food owing to their features, particularly the stability and broad host range.

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Introduction

Bacterial antimicrobial resistance (AMR) is one of the leading global threats affecting public health. AMR is a biological phenomenon that occurs as a result of changes in bacteria over time causing no respond to drugs. Infections caused by multidrug-resistant (MDR) pathogenic bacteria are increasingly difficult to treat, further causing disease spread, severe illness, and death (WHO, 2021).

Recently, a widespread use of MDR in foodborne pathogens, such as *Salmonella*, is of particular concern as they were found to be resistant to clinically critical antibiotics such as fluoroquinolones and β-lactams, which could present difficulties in human salmonellosis treatment (Collignon, 2013; Xu *et al*., 2019). In addition, the World Health Organization (WHO) reveals an increase in reported cases of blood infections by resistant *Salmonella* in 2020 by at least 15% compared to rates in 2017, based on data reported by 87 countries (WHO, 2022). In Malaysia, the Malaysian National Surveillance on Antimicrobial Resistance (NSAR) reported an increase in the resistance rates of *Salmonella* in blood samples from 2019 to 2022, the resistance to ampicillin increased from 11.9 to 16.6%, co-trimoxazole from 3.4 to 5.6%, chloramphenicol from 3.0 to 7.2%, ceftriaxone from 0.6 to 2.4%, and ciprofloxacin from 1.3 to 3.5% (NSAR, 2022).

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Due to the emergence of MDR *Salmonella*, effective control measure as an alternative to antibiotics is needed. Biocontrol method by using bacteriophages is one of the promising approaches for controlling MDR *Salmonella* with several advantages such as efficiency, specificity, safety, and environmentally friendly, making it potentially attractive over antibiotics (Bao *et al.*, 2015; Pereira *et al*., 2016).

Bacteriophages are naturally occurring viruses that infect and replicate only in bacterial cells. They are harmless to humans and animals, as well as environment, making them an ideal candidate to be used as biocontrol and bio-preservation agents (Garvey, 2020).

Bacteriophages have drawn interest in controlling foodborne pathogens because of their highly species-specific feature, which targets only a single bacterial species or even specific strains within a species of host bacteria, regardless of their MDR characteristic, as the phages mechanism of action differs from that of antibiotics (Kasman and Porter, 2022; Aranaga *et al*., 2022). Bacteriophages destroy bacteria through their lytic replication mechanism which results in the lysis of the bacterial membrane, and the release of phage progeny, which consequently kill the bacteria (Au *et al*., 2021).

Salmonella bacteriophage was commonly isolated from environmental samples such as sewage, wastewater treatment plant, farm ditch, and chicken market effluent (Huang *et al*., 2018; Sonalika *et al*., 2020; Zhang *et al.*, 2020; Tayyarcan *et al*., 2022); *in-vitro* characterisation indicated the capability of these phages to reduce the growth of their respective *Salmonella* host, displaying notable bacterial killing activity.

In addition, *Salmonella* is capable of biofilm formation which gives rise to the hygienic and economic issues due to the contamination of different food batches in processing line (Borges *et al*., 2018). Several studies have highlighted the capability of *Salmonella* to adhere and form biofilm on various surface materials such as plastic, glass, and stainless steel that are commonly used in food processing establishments (Ćwiek *et al*., 2019). Most conventional methods of biofilm elimination are using chemicals that lead to the development of antimicrobial resistance (Roy *et al*., 2018). This issue raises interest in the usage of biological tools such as bacteriophages for attacking biofilms. Several studies have reported the reduction of biofilm mass produced by *Salmonella* following treatment with their respective bacteriophages such as *S*. Enteritidis (SE_2 phage) (Tiwari *et al*., 2013) and *S*. Typhimurium (P22 phage) (Karaca *et al*., 2015).

Considering the efficacy of bacteriophages in controlling foodborne pathogens, the present work aimed to (i) isolate and characterise lytic *Salmonella* bacteriophages from chicken fillet samples, and (ii) determine the antibacterial and antibiofilm activity of isolated bacteriophages against *Salmonella*.

Materials and methods

Isolation of bacteriophages

Salmonella strain

An isolate of *Salmonella* Hvittingfoss was employed as the host for bacteriophage isolation. This isolate was recovered from Indian pennywort (*Centella asiatica*, locally known as *pegaga*, isolate code WP 10), and characterised as MDR carrying 13 types of virulent traits as described in previous study by Haslinda *et al*. (2022; 2023). The isolate was stored at -80°C until further use.

Sample preparation

A total of 66 chicken fillet samples were purchased from different wet markets in Terengganu, Malaysia. Briefly, 10 g of each sample was weighed in a 50 mL sterile centrifuge tube, then 10 mL of salt magnesium (SM) buffer (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM pH 7.5 Tris-HCl, and 0.01% gelatine [Nacalai Tesque, Japan]) was added into the sample. The mixture was shaken at 150 rpm for 15 min in an orbital shaker (SI-600, Lab Companion, USA), and centrifuged at 10,000 *g* for 5 min to pelletise large particles. The supernatant was filtered through a 0.22 µm pore size syringe filter (Millipore, Ireland) into a new sterile centrifuge tube to obtain 5 mL of filtrate. The filtrate was then mixed with 200 µL of log-phase *Salmonella* host culture (OD600 nm = 0.4 - 0.6), and incubated at 37°C, 150 rpm overnight. After incubation, bacterial cells were pelletised by centrifugation at 10,000 *g* for 5 min, and the supernatant was filtered with a 0.22 µm syringe filter.

Detection of bacteriophage

Screening for the presence of bacteriophage was done using spot-titre method according to Beck *et al*. (2009). The filtrate was diluted ten-fold (through 10^{-6}) by using SM buffer. Each of these

dilutions was spotted in 10 µL on a double-layer agar plate with bacterial host. The plate was allowed to dry for 30 min, and then incubated at 37°C for 18 - 24 h. The highest dilution filtrate, which showed a clear lysis zone on the spot, was selected for isolation of bacteriophage.

Isolation and purification of bacteriophage

Bacteriophage was isolated by using the double agar overlay plaque assay as described by Kropinski *et al*. (2009). Briefly, 100 µL of selected dilution phage filtrate was transferred into a tube containing 3 mL of warm (45°C) overlay medium (LB agar 0.6%, 1st BASE, BIO-4000, Singapore), then 100 µL of log-phase bacterial host culture was immediately added and gently mixed. The mixture was then poured evenly onto LB agar base plate and allowed to solidify for 30 min prior to incubation at 37°C for 18 - 24 h. After incubation, a single plaque with clear lysis appearance was picked up using a sterile inoculation loop, and resuspended in 5 mL of SM buffer with 200 µL of log-phase host culture. The mixture was incubated at 37°C, 150 rpm for 18 - 24 h. After incubation, the sample was centrifuged at 10,000 *g* for 5 min, and filtered through a 0.22 µm pore size syringe filter. A sample from this plaque-stock was further plated by the double-layered agar method as described earlier. Plaque-purification was repeated three times for the discovered phage in order to purify a single homogenous phage (van Charante *et al*., 2019). The final suspension was stored at 4°C, and considered to be a pure phage suspension for further characterisation purposes.

Characterisation of bacteriophage

Transmission electron microscopy (TEM)

The morphology of bacteriophage was visualised by a transmission electron microscope (TEM). A drop of bacteriophage was placed on a carbon film coated 400 mesh copper grid, and left for 3 min to allow phage attachment to the film. The film was then negatively stained with 2% (w/v) uranyl acetate for 1 min. The excess sample was wicked to dry with a filter paper. The phage morphology was then viewed with a Zeiss Libra 120 TEM (Carl Zeiss, Germany) at 120-kV.

Bacteriophage genome analysis

The bacteriophage nucleic acid was extracted using EasyPure® Viral DNA/RNA kit (Transgenbiotech, China). For the type of nucleic acid determination, 10 µL of extracted nucleic acid was treated separately with 3 µL of DNase I (1 U/µL) (Thermoscientific, USA) and 3 µL of RNase A (10 mg/mL) (PureLink™, Invitrogen, USA) for 35 min at 37°C (Al-Razem *et al*., 2022). After treatment, the sample was electrophoresed through 0.7% (w/v) agarose gel at 80 V for 40 min with comparison to the 1 kb DNA marker, and viewed under luminescent image analyser (LAS-4000, FUJIFILM, Japan).

Host range specificity

The overlay method (Viazis *et al*., 2011) was used to determine the host range of bacteriophage within the group of 63 bacterial strains, including 57 *Salmonella* strains isolated from salad vegetables in previous study (Haslinda *et al*., 2022), reference culture *Salmonella* Typhimurium ATCC 14028, and five reference cultures of non-*Salmonella* strains (*Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 13932, *Staphylococcus aureus* ATCC 25923, *Pseudomonas fluorescens* ATCC 13525, and *Shigella flexneri* ATCC 12022). Twenty microliters of bacteriophage were spotted on a double-layer agar plate seeded with each of the bacterial strain; the agar plate was allowed to dry for 30 min before being incubated at 37°C for 18 - 24 h. The presence of lysis zone in correspondence with the spot was considered evidence of bacterial susceptibility toward the bacteriophage. Based on the degree of clarity, the observed results of lysis plaque were differentiated into three classes: (+++) clear plaque, (++) clear plaque with colonies, (+) turbid plaque, and (-) no plaque.

Optimal multiplicity of infection (MOI)

Multiplicity of infection (MOI) represents the ratio of the number of bacteriophage particles to the number of host bacteria at the time of initial infection (Sinha *et al*., 2018). The MOI of bacteriophage was analysed according to Li *et al*. (2021a) with some modifications. The bacteriophage suspension was mixed with log-phase bacteria host at different MOIs (0.001, 0.01, 0.1, 1, and 10 PFU/CFU) and incubated at 37°C for 3.5 h. After incubation, the sample was centrifuged at 10,000 *g* for 5 min, and filtered with a 0.22 µm syringe filter. The filtrate was then plated onto a double-layer agar to determine the bacteriophage titre. Bacteria-free suspension and bacteriophage-free suspension were included as

control samples. The MOI ratio with the highest bacteriophage titre was determined as the optimal MOI of the bacteriophage.

One-step growth curve

The latent period and burst size of bacteriophage was determined from one-step growth curve, and the analysis was performed following the protocol described by Tan *et al*. (2021). Based on the optimal MOI (MOI = 1), bacteriophage suspension was mixed with 1 mL of log-phase host bacteria, and incubated at 37°C, 150 rpm for 10 min to allow the adsorption of bacteriophages to the host cells. The mixture was then centrifuged at 10,000 *g* for 30 s, and the pellet was suspended in 10 mL of TSB. The suspension was further incubated at 37°C for 3 h with shaking at 150 rpm. Next, 100 µL of sample was withdrawn at 10 min intervals, and enumerated by double agar overlay plaque assay. The latent period was determined as intersection between the initial horizontal line and slope of the curve, while the burst size was calculated as: $B_s = N_t / N_i$, where N_t was the phage titre at the end of lysis, and N_i was the phage titre at the initial stage of infection (Yang *et al*., 2023).

Temperature and pH stability

Temperature and pH stability test of bacteriophage were conducted following a previously described protocol by Tan *et al*. (2021). For the temperature stability, bacteriophage suspension in SM buffer was incubated at different temperatures (4, 10, 25, 37, 50, 60, and 70°C) for 2 h, meanwhile, for pH stability, bacteriophage was suspended in SM buffer at different pH ranges (pH 2, 3, 5, 7, 9, 11, and 12), and incubated for 2 h at 37°C. Bacteriophage suspension kept at 4°C in the SM buffer (pH 7.5) was used as a control. After incubation, bacteriophage titre for each of treated sample was determined by double agar overlay plaque assay. The bacteriophage's survival rate was calculated by dividing the bacteriophage titre after treatment (PFU/mL) with the initial bacteriophage concentration (PFU/mL).

In-vitro lytic activity

In-vitro lytic activity of bacteriophage was analysed by referring to the method described by Tan *et al.* (2021). Log-phase *Salmonella* host culture was mixed with bacteriophage suspension at the optimum MOI (MOI = 1), and incubated immediately at 37°C. Then, 50 µL of sample from the mixture was withdrawn at 0, 2, 4, 6, and 24 h for spread plating onto a CHROMagar™ *Salmonella* plate (CHROMagar™, France). The plate was then incubated at 37°C for 18 - 24 h for the enumeration of *Salmonella*. *Salmonella* host culture without bacteriophage served as control.

Antibiofilm activity

A colorimetric method using 96-well flat bottom microplate (Jet Biofil, Spain) as described previously by Hosny *et al*. (2023) with some modifications was used to quantitatively determine the effectiveness of bacteriophage to destruct the formed *Salmonella* biofilm.

Biofilm formation and assay

Individual wells of sterile microplate were filled with 200 µL of the log-phase *S*. Hvittingfoss (10^8 CFU/mL) host culture, and incubated at 37°C for 72 h to develop the highest density of cells and strong biofilm (OD > 4 × ODc). After incubation, the contents of each well were removed by gentle tapping, then washed with 200 µL of phosphate buffer saline (GeneTex, USA) three times to remove planktonic cells. The formed biofilm was fixed and dried by incubation at 80°C for 30 min. Then, 200 µL of 0.5% crystal violet solution (Merck, Germany) was added to each well, and allowed to react at room temperature for 1 min. After removing the supernatant, each well was washed twice with sterile water and air dried. The retained crystal violet was eluted with 200 µL of 95% ethanol, and biofilm mass was measured at 590 nm (Spectrophotometer, MULTISKAN GO, Thermoscientific, USA) (Agarwal *et al*., 2011).

Assessment of biofilm destruction

Following the biofilm formation, 200 µL of 10^8 PFU/mL bacteriophage lysate was added to the established biofilm, and incubated at 37°C for 0, 3, 6, 9, and 24 h to assess the time-dose effect of phage application on biofilms. After incubation, wells were rinsed three times with sterile PBS prior to air drying. The remaining biofilm cells were stained as previously described to determine the final biofilm density. Negative control wells contained established *Salmonella* biofilm inoculated with 200 µL of SM buffer without bacteriophage.

Statistical analysis

All the experiments were performed in triplicate. The data obtained were analysed by One-way analysis of variance (ANOVA) and independent-samples *t*-test with 95% confidence interval using SPSS Statistics 25 (IBM, New York, USA). The results were presented as mean ± standard deviation. Statistical differences between the mean values were defined at a significance level of $p < 0.05$.

Results

Isolation, morphology, and genome of bacteriophage

Two bacteriophages (SH1 and SH2) were isolated from different chicken fillet samples by using *Salmonella* Hvittingfoss as a host. These phages produced clear lysis zones on the double-layer agar plate (Figures 1a - 1d).

Based on TEM, SH1 and SH2 possessed icosahedral head diameter of 86.15 ± 2.57 and 65.27 ± 2.63 nm with long, non-contractile, thin tail length of 205.32 ± 61.91 and 172.85 ± 34.36 nm, respectively. This morphology indicated that SH1 and SH2 resembled *Siphoviridae* family (Figures 2a and 2b).

The nucleic acids extracted from both SH1 and SH2 were found sensitive to DNase digestion and resistant to RNase. This revealed that the phages genome type was double stranded deoxyribonucleic acid (dsDNA).

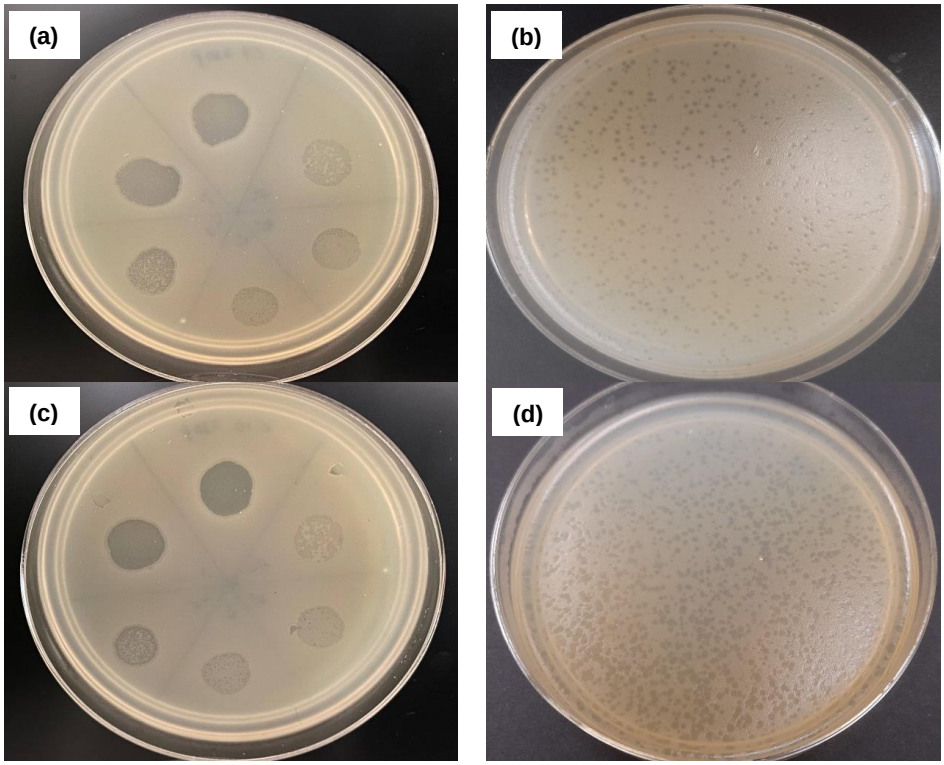

Figure 1. Bacteriophages plaque on lawn of *Salmonella* Hvittingfoss. **(a)**: Spot plaque of SH1 in different dilutions (1:10 dilutions, 0 to -5); **(b)** plaque of SH1 on double agar overlay; **(c)**: spot plaque of SH2 in different dilutions (1:10 dilutions, 0 to -5); and **(d)** plaque of SH2 on double agar overlay.

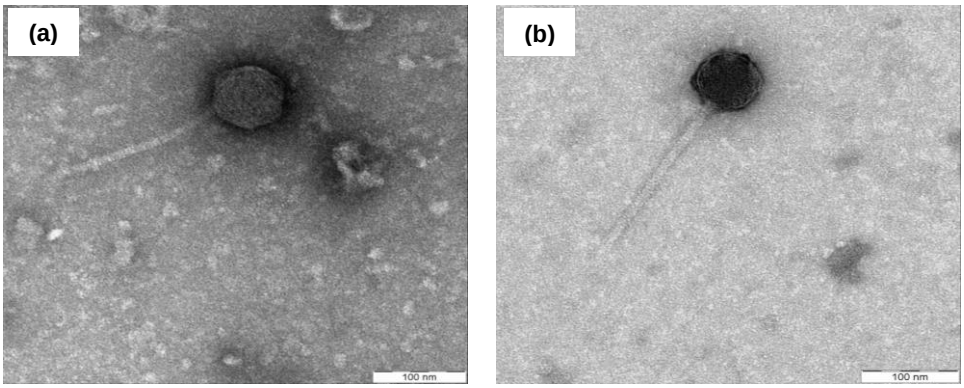

Figure 2. Transmission electron micrographs of **(a)** bacteriophage SH1, and **(b)** bacteriophage SH2.

Host range specificity

For the host range determination, bacteriophage SH1 and SH2 were found to produce lysis zone on 65.5% (*n* = 38/58) and 63.8% (*n* = 37/58) of the double layer agar plates seeded with *Salmonella* strains, respectively. In addition, no lysis zones were observed on all non-*Salmonella* (*n* = 5/5) bacteria lawn agar plates tested with both phages. With regard to the serovars, both phages showed similar host range specificity where they were able to lyse 75, 33.3, 100, 75, and 100% of *S*. Weltevreden, *S*. Albany, *S*. Hvittingfoss, *S*. Aberdeen, and *S*. Poona isolates, respectively. Furthermore, they were able to lyse at least one isolate of several other *Salmonella* serovars tested, as presented in Table 1. In total, SH1 and SH2 showed the capability of inhibiting 17 and 16 types of *Salmonella* serovars tested in the present work, respectively.

Table 1. Host range specificity of bacteriophages SH1 and SH2.

No.	Isolate	Code	Plaque formation			
			SH1	No. of positive (%)	SH2	No. of positive (%)
1	*Escherichia coli*	ATCC 25922	-	0 (0)	-	0(0)
2	*Listeria monocytogenes*	ATCC 13932	-		-	
3	*Staphylococcus aureus*	ATCC 25923	-		-	
4	*Pseudomonas fluorescens*	ATCC 13525	-		-	
5	*Shigella flexneri*	ATCC 12022	-		-	
6	*Salmonella* Typhimurium	ATCC 14028	-		-	
7	*Salmonella* Weltevreden	WSE1	+++	9 (75)	+++	9 (75)
8	*Salmonella* Weltevreden	WSE2	++		++	
9	*Salmonella* Weltevreden	WUR1	+++		+++	
10	*Salmonella* Weltevreden	WUR2	+++		+++	
11	*Salmonella* Weltevreden	WT1	+++		+++	
12	*Salmonella* Weltevreden	WSE3	+++		+++	
13	*Salmonella* Weltevreden	WSE4	+++		+++	
14	*Salmonella* Weltevreden	WBB1	++		+++	
15	*Salmonella* Weltevreden	FIW1	-		-	
16	*Salmonella* Weltevreden	FIW2	++		++	
17	*Salmonella* Weltevreden	FIW3	-		-	
18	*Salmonella* Weltevreden	FSO1	-		-	
19	*Salmonella* Albany	WP5	-	2 (33.3)	-	2 (33.3)
20	*Salmonella* Albany	WP6	-		-	
21	*Salmonella* Albany	WUR3	-		-	
22	*Salmonella* Albany	WUR4	++		+	
23	*Salmonella* Albany	WUR5	-		-	
24	*Salmonella* Albany	WBB3	++		++	
25	*Salmonella* Hvittingfoss	WBB2	+++	4 (100)	+++	4 (100)
26	*Salmonella* Hvittingfoss	WSE9	+++		+++	
27	*Salmonella* Hvittingfoss	WSE10	+++		+++	
28	*Salmonella* Hvittingfoss	SP1	+++		+++	
29	*Salmonella* Aberdeen	WBB5	+	3 (75)	+	3 (75)
30	*Salmonella* Aberdeen	WP11	-		-	
31	*Salmonella* Aberdeen	WSE6	+++		+++	
32	*Salmonella* Aberdeen	WSE7	++		++	
33	*Salmonella* Poona	WP7	++	3 (100)	++	3 (100)
34	*Salmonella* Poona	WP8	++		+++	
35	*Salmonella* Poona	WP9	+		+	
36	*Salmonella* Corvallis	WP12	-	0 (0)	-	0 (0)
37	*Salmonella* Corvallis	WP13	-		-	

38	*Salmonella* Corvallis	WBB4	-		-	
39	*Salmonella* Saintpaul	WSE12	+++		+++	
40	*Salmonella* Saintpaul	WP4	++		+++	
41	*Salmonella* Newport	WUR6	++		++	
42	*Salmonella* Newport	WP3	+		+	
43	*Salmonella* Stanley	WKB1	++		+++	
44	*Salmonella* Stanley	WT2	+		+	
45	*Salmonella* Rissen	WP2	-		-	
46	*Salmonella* Rissen	SP2	-		-	
47	*Salmonella* Lexington	SSE1	+++		+++	
48	*Salmonella* Lexington	SUR3	+++		+++	
49	*Salmonella* Augustenborg	FUR1	-		-	
50	*Salmonella* Augustenborg	FUR2	+		+	
51	*Salmonella* Virchow	WSE11	++	17 (68)	++	16 (64)
52	*Salmonella* Kentucky	WSE13	-		-	
53	*Salmonella* Rubislaw	WP1	+++		+++	
54	*Salmonella* Virginia	WSE5	-		-	
55	*Salmonella* Bareily	WSE8	-		-	
56	*Salmonella* Muenchen	WKB2	+		+	
57	*Salmonella* Agona	WBB6	+++		+++	
58	*Salmonella* Heidelberg	WBB7	-		-	
59	*Salmonella* Hindmarsh	WUR7	-		-	
60	*Salmonella* Braenderup	SUR2	+		+	
61	*Salmonella* Mountpleasant	SUR1	+++		+++	
62	Subsp. Ii Ser47:enx, z15:1,6	FIW5	+++		-	
63	Subsp. Iiib Ser 47; C; Z35	FIW4	+++		+++	
	Total		**38**		**37**	

(+++): clear plaque formation; (++): clear plaque formation with colonies; (+): turbid plaque formation; and (-): no plaque formation. All *Salmonella* strains were isolated in previous study by Haslinda *et al*. (2022).

Multiplicity of infection (MOI)

At an MOI of 1, the progeny titre of bacteriophages SH1 and SH2 were the highest, with the average of 8.40 ± 0.29 and 7.85 ± 0.16 log PFU/mL, respectively (Figures 3a and 3b). Therefore, the MOI ratio of 1 was considered the optimal MOI for both phages.

One-step growth curve

The results of phage titre in 10 min intervals for the two bacteriophages were plotted in Figure 4 (4a and 4b) to produce one-step growth curve. From the curve, SH1 and SH2 were observed to have latent period of 20 and 10 min, with an average burst size of 59 and 52 PFU per cell, respectively.

Temperature and pH stability

Bacteriophages SH1 and SH2 were found to remain relatively stable under temperatures of 4, 10, 25, 37, and 50°C, with survival rate ranging from 83.13 to 100.00% (Table 2). However, at 50°C, a significantly higher survival percentage was observed in SH2 as compared to SH1, which demonstrated a significant decrease in survival. Furthermore, at 60°C, further decrease in survival rates to 65.64 and 63.43% was observed in SH1 and SH2, respectively. At a temperature of 70°C, the phages stability was dramatically reduced with survival rates of only 1.85 and 4.76% for SH1 and SH2, respectively. From the pH stability test results, both phages were much stable at pH 5 to 11 with survival percentage of 92.35 to 100.93% (Table 3). Aside from that, at pH 3, it was noticed that SH1 and SH2 survived at a significantly low percentage of 3.53 and 10.28%, respectively. Under strong acid and strong alkali (pH 2 and 12), the phages were completely denatured. These results indicated a remarkable stability of bacteriophages SH1 and SH2 at temperature below 50°C and pH 5 to 11.

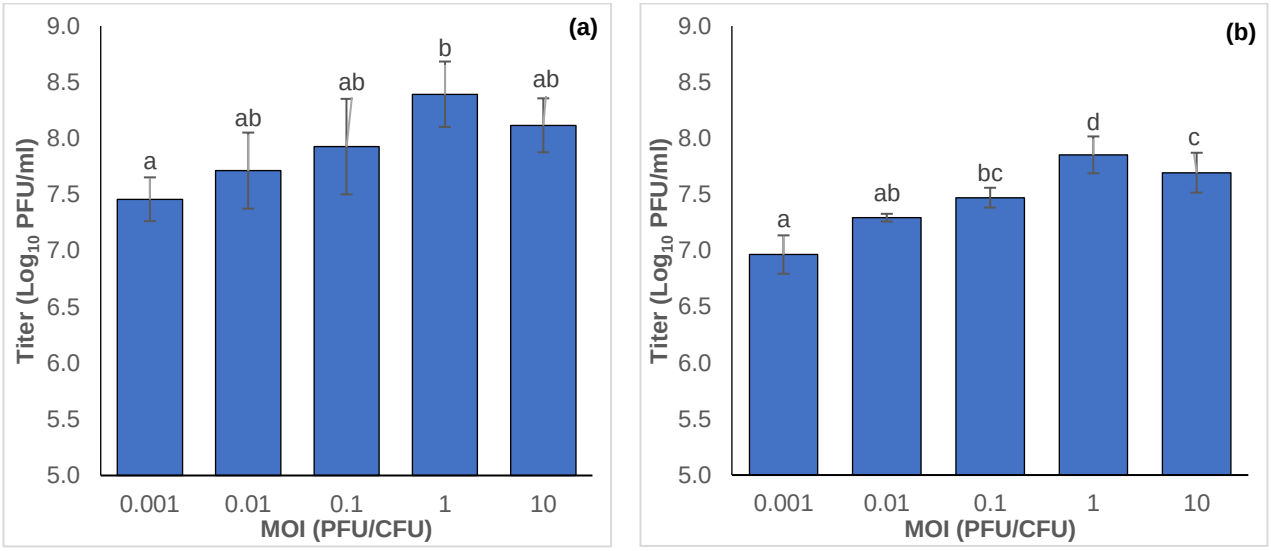

Figure 3. Multiplicity of infection of bacteriophage SH1 **(a)** and bacteriophage SH2 **(b)**. Error bars indicate standard deviations. Different lowercase letters above the bars indicate statistically significant difference ($p < 0.05$).

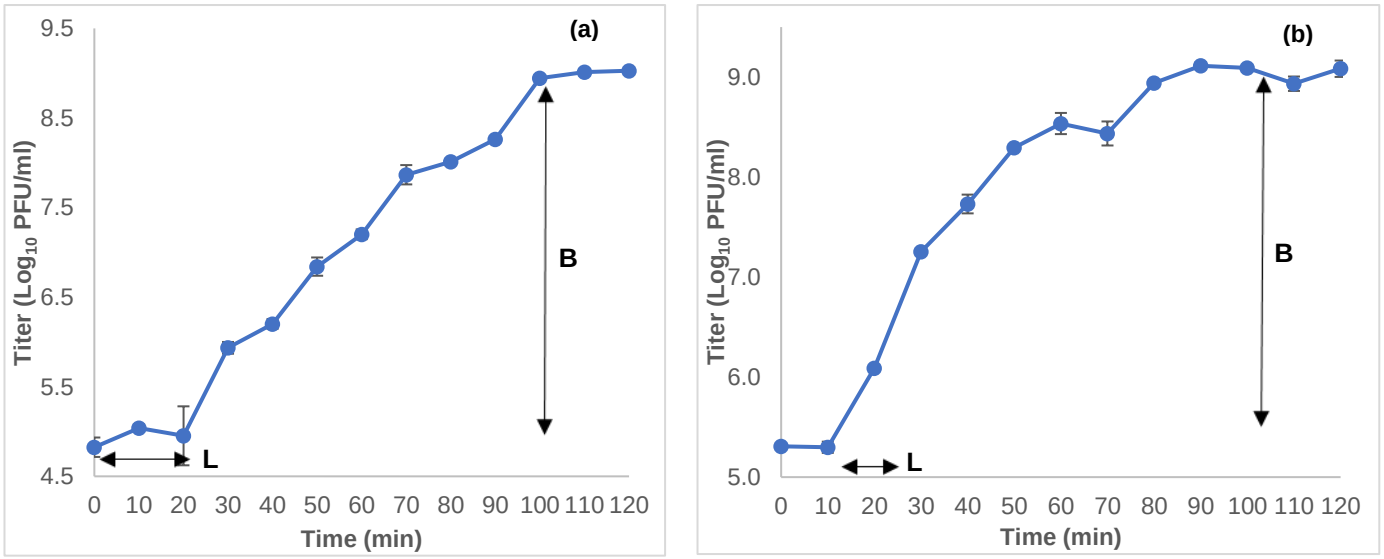

Figure 4. One-step growth curve for bacteriophage SH1 **(a)** and bacteriophage SH2 **(b)**. (L): Latent period, and (B) burst size. Error bars indicate standard deviation.

Table 2. Temperature stability of bacteriophages SH1 and SH2.

Temperature (°C)	Percentage of survival (%)	
	SH1	SH2
4	93.83 ± 4.46^{Acd}	97.45 ± 3.34^{Ac}
10	95.47 ± 5.83^{Ad}	99.66 ± 2.41^{Ac}
25	96.50 ± 4.99^{Ad}	100.00 ± 2.04^{Ac}
37	96.30 ± 4.28^{Ad}	99.49 ± 3.18^{Ac}
50	83.13 ± 4.11^{Ac}	98.30 ± 1.28^{Bc}
60	65.64 ± 2.79^{Ab}	63.43 ± 2.99^{Ab}
70	1.85 ± 1.24^{Aa}	4.76 ± 1.28^{Ba}

Values are mean ± standard deviation. Means within the same row with different uppercase superscripts are significantly different ($p < 0.05$). Means within the same column with different lowercase letters are significantly different ($p < 0.05$).

Table 3. pH stability of bacteriophages SH1 and SH2.

pH	Percentage of survival (%)	
	SH1	SH2
2	0.00^{Aa}	0.00^{Aa}
3	3.53 ± 1.77^{Aa}	10.28 ± 3.37^{Bb}
5	92.35 ± 4.60^{Ab}	98.44 ± 3.28^{Ac}
7	98.63 ± 4.41^{Ab}	99.38 ± 3.57^{Ac}
9	97.26 ± 2.96^{Ab}	100.93 ± 3.74^{Ac}
11	96.67 ± 5.34^{Ab}	100.31 ± 1.17^{Ac}
12	0.00^{Aa}	0.00^{Aa}

Values are mean ± standard deviation. Means within the same row with different uppercase superscripts are significantly different ($p < 0.05$). Means within the same column with different lowercase letters are significantly different ($p < 0.05$).

In-vitro lytic activity

Results of *in-vitro* lytic activity test showed that bacteriophages SH1 and SH2 had good inhibitory effect toward the *S.* Hvittingfoss host culture as compared to the untreated control sample. Lytic capacity of both SH1 and SH2 were found similar ($p > 0.05$) over the incubation period. Both phages demonstrated a significant lytic activity after 4 h incubation at 37°C. However, no further significant lytic activity was observed after 6 h interaction (Figure 5). After 24 h interaction with SH1 and SH2, the total reduction of *S.* Hvittingfoss from initial count were 0.31 ± 0.01 and 0.32 ± 0.04 log CFU/mL, respectively. In addition, the recoverable of *S.* Hvittingfoss count after 24 h as relative to the phage free control were 2.37 ± 0.04 and 2.38 ± 0.05 log CFU/mL for SH1 and SH2, respectively.

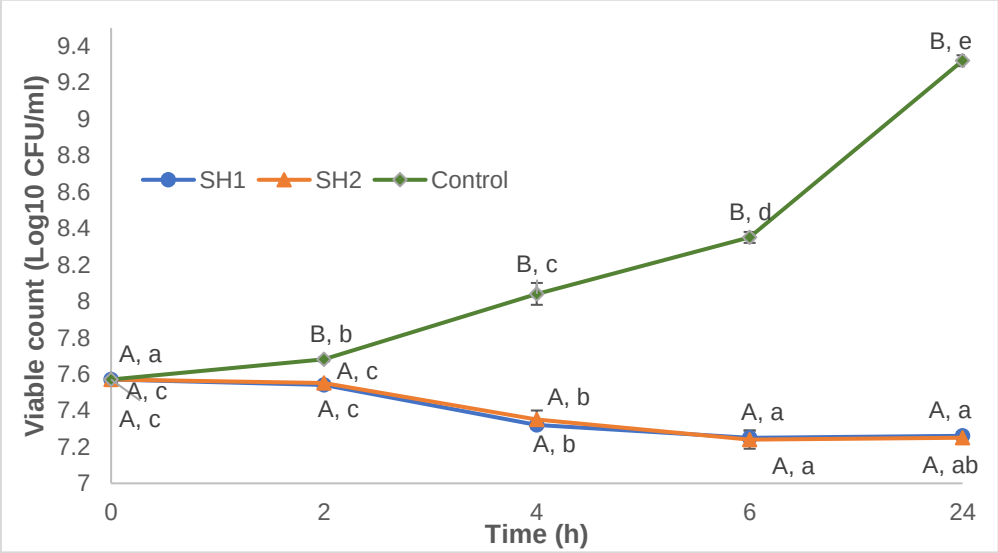

Figure 5. Effects of bacteriophage SH1 and SH2 on viability of *S.* Hvittingfoss. Error bars indicate standard deviations. Values with different uppercase letters within the same *y*-axis are significantly different ($p < 0.05$). Values with different lowercase letters within the same *x*-axis are significantly different ($p < 0.05$).

Antibiofilm activity

Results of antibiofilm activity as presented in Table 4 indicate that the biofilm mass of *S.* Hvittingfoss was significantly inhibited with a capability of 67.94 and 72.92% after 3 h interaction with SH1 and SH2, respectively. The percentage of biofilm reduction by SH1 and SH2 were further increased until 24 h by 99.74 and 89.40%, respectively. In comparison between both bacteriophages, SH1 showed a significantly higher destruction capacity toward biofilm at 6 and 9 h, then reached a comparable ($p > 0.05$) total reduction with SH2 after 24 h treatment.

Table 4. Effects of bacteriophage SH1 and SH2 on biofilm produced by *S*. Hvittingfoss in 96-well plates.

Incubation time (h)	Biofilm mass, OD_{590} (percent reduction, %)		
	Control	SH1	SH2
0	1.34 ± 0.30^{Aa}	1.40 ± 0.11 (0)^{Ac}	1.31 ± 0.06 (0)^{Ac}
3	1.41 ± 0.29^{Ba}	0.44 ± 0.13 (67.94 ± 11.57)^{Ab}	0.35 ± 0.07 (72.92 ± 5.01)^{Ab}
6	1.24 ± 0.09^{Ca}	0.08 ± 0.03 (94.65 ± 1.64)^{Aa}	0.31 ± 0.10 (76.32 ± 8.04)^{Bb}
9	1.44 ± 0.13^{Ca}	0.02 ± 0.01 (98.57 ± 0.43)^{Aa}	0.26 ± 0.22 (80.34 ± 2.50)^{Bab}
24	1.35 ± 0.19^{Ba}	0.004 ± 0.003 (99.74 ± 0.17)^{Aa}	0.14 ± 0.01 (89.40 ± 0.29)^{Aa}

Values are mean ± standard deviation. Means within the same row with different uppercase superscripts are significantly different ($p < 0.05$). Means within the same column with different lowercase letters are significantly different ($p < 0.05$).

Discussion

Bacteriophages are highly ubiquitous in the environment, and recognised as the most numerous biological entities on earth (Suttle, 2013). They can be readily isolated from the environment such as lake sediment, poultry farm, seawater, sewage, and soil (Kim and Park, 2023). The isolations of bacteriophages from fresh foods were relatively less reported, even though they can also be found inside plants and animals. According to Greer (2005), bacteriophages can be successfully isolated from food where the target bacteria were present in relatively high concentrations. Non-typhoidal *Salmonella* (NTS) is a common foodborne pathogen originated and transmitted by poultry products, particularly chickens (Cogan and Humphrey, 2003; Cox *et al*., 2005; Nadi *et al*., 2020). Due to this, there are possibilities of isolating *Salmonella* bacteriophages from chickens. Several studies have reported the isolation of *Salmonella* bacteriophages from chicken-related sources such as meat, skin, gizzard, and faeces (Hungaro *et al*., 2013; Duc *et al*., 2018; Li *et al*., 2021b; Al-Razem *et al*., 2022; Mhone *et al*., 2022). The presence of bacteriophage in chicken was further proved by the finding in the present work, which isolated two *Salmonella* bacteriophages (SH1 and SH2) from different chicken samples collected from wet markets.

TEM is one of the best direct imaging methods for the purpose of recognition and classification of bacteriophage families. Based on TEM, bacteriophages SH1 and SH2 were located in the *Caudovirales* order, *Siphoviridae* family, which consists of icosahedral-isometric head with thin, long, and non-contractile tail. Other studies have also documented the isolation of local *Salmonella* bacteriophages from various families such as SE07 belonging to the *Podoviridae* family (Thung *et al*., 2017), Φ st1 belonging to the *Siphoviridae* family (Wong *et al*., 2014), and ST02 belonging to the *Myoviridae* family (Thung *et al*., 2019). Genome analysis of bacteriophage SH1 and SH2 revealed that they encapsulate DNA genome as their genetic material. This finding agreed with Aprea *et al*. (2015), who mentioned that tailed bacteriophages are commonly constituted of double stranded DNA. Besides that, it is known that tailed bacteriophages in the order of *Caudovirales* with a double stranded DNA genome account for 96% of bacteriophages found, and it is suggested to be the predominant biological entity on earth (Zinke *et al*., 2022).

Host specificity is one of the vital aspects that should be considered in the selection of bacteriophages for pathogen detection and control. For example, a narrow host range is more suitable to be applied in pathogen detection as the bacteriophage detection assay requires higher specificity, thus increasing the probability of successful detection. Furthermore, a narrow host-specific bacteriophage is also preferred for application in the gastrointestinal tract as the bacteriophage only infects the host species or their subset of strains without affecting other endogenous bacteria (Viazis *et al*., 2011). On the other hand, bacteriophage with broader host range is desirable for biocontrol applications due to the broader range of targeted bacterial pathogens which resulted in a wider effect of pathogen control. As presented in Table 1, bacteriophages SH1 and SH2 were found to be able to infect several serovars of *Salmonella enterica* (17 and 16 serovars, respectively) other than their host serovar (*S*. Hvittingfoss). Due to this characteristic, both bacteriophages were classified as broad host range. As described by Fong *et al*. (2021), bacteriophages that can infect multiple strains of the same species of

bacteria may be characterised as broad host range. No infection by both bacteriophages SH1 and SH2 were detected against other species of bacteria tested, suggesting that these phages exhibited species-specific host range spectrum.

The optimal MOI for both bacteriophages SH1 and SH2 is 1. A value of MOI = 1 implies the condition where a single phage particle is available for infecting a single host cell in an infection medium (Sinha *et al*., 2018). The infection and killing process of bacteria depends on the chance of bacteriophage to meet with bacteria. Thus, to have a successful bacterial control, it is important to execute the infection process under optimal MOI. Different bacteriophages may infect and kill bacteria in different circumstances, thus having different optimal MOIs. For example, Wong *et al*. (2014) and Zhang *et al*. (2023) reported that the optimal Φst1 *Salmonella* and MOI of JNwz02 bacteriophages were 0.1 and 0.01, respectively.

One-step growth curve is developed based on the replication of bacteriophage by lysis process after it had infected the host bacteria. It is triphasic which consists of the latent, rise, and plateau phases (Liu *et al*., 2019). Bacteriophages SH1 and SH2 were found to have quite similar latent periods with other *Salmonella* bacteriophages such as SE-PH1 (10 min), SE-PH2 (20 min) (Tayyarcan *et al*., 2022), and S55 (10 min) (Ge *et al*., 2021). However, the latent periods were shorter than many other reported *Salmonella* bacteriophages such as STG5 (25 min) (Duc *et al*., 2018), CGG3-1 (59 min), CGG3-2 (65 min) (El-Dougdoug *et al*., 2019), and BPSELC-6 (30 min) (Li *et al*., 2020). Comparing with other *Salmonella* bacteriophages, SH1 and SH2 burst sizes were larger than bacteriophage S55 (40 PFU/cell) (Ge *et al*., 2021) and STG5 (49 PFU/cell) (Duc *et al*., 2018), but smaller than CGG3-1 (100 PFU/cell) and CGG3-2 (92 PFU/cell) (El-Dougdoug *et al*., 2019). Latent period and burst size are the important factors that influence the selection of a bacteriophage to be used as biocontrol agent. It is widely known that bacteriophages with short latent periods and large burst sizes are more effective in bacterial killing process, and provide more successful bacterial elimination (Gill and Hyman, 2010; Hyman, 2019; Yazdi *et al*., 2020). It has long been mentioned by Abedon (1989) that bacteriophage with a short latent period will lyse more bacteria in a certain time period, reflecting a desirable condition for biological control.

Temperature and pH stability are the crucial factors influencing the successful application of bacteriophages. In food, the usage of high stability (wide range of temperature and pH) bacteriophage is advantageous in order to maintain its titre during food processing, including storage and transportation, in which the temperature and pH conditions might vary (Vandenheuvel *et al*., 2015; Kering *et al*., 2020). In the present work, bacteriophages SH1 and SH2 possessed stability over wide range of temperatures (4 - 50°C) and pH (5 - 11), comparable with several other *Salmonella* bacteriophages such as ST02 (Thung *et al*., 2019), ZCSE6 (Abdelsattar *et al*., 2021), and MSP1 (Park *et al*., 2023).

Bacteriophages SH1 and SH2 started to show significant lytic activity toward *Salmonella* Hvittingfoss host after 4 h incubation at 37°C (Figure 5). This condition might be explained by the higher gap between phages and host concentration in the early stage of infection (0 to 4 h), which was reflected by the insignificant differences in *Salmonella* numbers. According to Huang *et al*. (2018), it takes some time for the bacteriophage to infect host cell and multiply in order to increase their concentration, after that, the host bacteria will be easily controlled, and show significant decrease in number. A similar finding was also reported by Pereira *et al*. (2016), who observed a significant reduction in *Salmonella* Typhimurium counts after 4 h treatment with each of phSE-1, phSE-2, and phSE-5 bacteriophage. Furthermore, Hungaro *et al*. (2013) also demonstrated a reduction in *Salmonella* concentration exposed to their bacteriophages after 2 to 4.5 h incubation, depending upon the conditions of the experiment like MOI and temperature. Bacteriophages SH1 and SH2 showed the ability to reduce and control the growth of *S*. Hvittingfoss, as compared to the untreated control (Figure 5). However, no further significant reduction of *S*. Hvittingfoss after 6 h interaction was observed for both bacteriophages, indicating bacterial resistance as previously described (Islam *et al*., 2020b; Li *et al*., 2021b; Alharbi *et al*., 2023). The development of resistance in the host bacteria could reduce the efficacy of phage treatment; in order to minimise this problem, some measures could be considered such as the use of bacteriophages with a broad host range in mixtures (cocktails). After 24 h, the total reductions of *S*. Hvittingfoss from the initial count were 0.31 and 0.32 log CFU/mL, respectively. This finding was

similar to the study by Sun *et al*. (2022), which reported less than 1 log CFU/mL reduction of *S.* Typhimurium after 24 h treated with PSDA-2 phage. The efficacy of lytic activity by bacteriophage is mainly associated with the optimum MOI, which leads to the increase in binding probability of bacteriophages to the host cells (Wong *et al*., 2014).

Biofilm formation is a strategy used by *Salmonella* to survive under environmental stress conditions such as in limited nutrient sources and fluctuations in temperature and pH, leading to a persistent source of contamination. To resolve this situation, bacteriophages offer a remarkably effective treatment for the eradication of *Salmonella* biofilm (Lamas *et al*., 2021). Results for antibiofilm activity study revealed that SH1 and SH2 were effective in reducing the established biofilms produced by *S.* Hvittingfoss by 99.74 and 89.40%, respectively, after 24 h treatment. Their effectiveness was comparable to a study by Korzeniowski *et al*. (2022), which exhibited capabilities of 84, 87, and 82% decrease in *S.* Enteritidis biofilm mass by UPWr_S1, UPWr_S3, and UPWr_S4 *Salmonella* bacteriophages, respectively. On the other hand, lower capabilities were also reported for bacteriophages LPST153 (31%) (Islam *et al*., 2020a), ST (~63%) (Kim and Park, 2023), and cocktails of LPSTLL, LPST94, and LPST153 (63%) (Islam *et al*., 2019) against *S.* Typhimurium biofilm.

Conclusion

In the present work, two *Siphoviridae Salmonella* bacteriophages SH1 and SH2 capable of infecting multi-serovars of *Salmonella* were isolated and characterised. Based on the results of characterisation tests, these phages showed great potentials to be utilised in the biocontrol of *Salmonella* and its biofilm in food processing establishments.

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