Poultry Science

Isolation and characterization of multidrug-resistant *Salmonella*-specific bacteriophages and their antibacterial efficiency in chicken breast

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ABSTRACT The use of phages as biocontrol agents against antibiotic-resistant strains of *Salmonella* spp. is gaining attention. This study aimed to isolate lytic bacteriophages specific for multidrug-resistant *Salmonella* enterica serovars Typhimurium; it also evaluated the bactericidal effect of isolated phages (STP-1, STP-2, STP-3, and STP-4) from sewage sample against *S.* Typhimurium as host strains. Moreover, a current study evaluated the efficacy of a bacteriophage cocktail against *S.* Typhimurium cocktail in chicken breast meat. The 4 phages were classified under the *Caudoviricetes* class by morphology characterization. On host range testing, they exhibited lytic activities against *S.* Typhimurium, *S.* Enteritidis, and *S.* Thompson. In the stability test, the phages exhibited resistance to heat (above 70°C for 1 h) and pH (strongly alkaline for 24 h). Additionally, the phages had comparable adsorption rates (approximately 80% adsorption in under 5 min). Additionally, the latent periods ranged from 30 to 50 min, with respective burst sizes of 31, 218, 197, and 218 PFU/CFU. In vitro, bacterial challenge demonstrated that at a multiplicity of infection (**MOI**) of 10, each phage consistently inhibited *S.* Typhimurium growth at 37°C for 24 h. In the food test, the phage cocktail (MOI = 1,000) reduced *S.* Typhimurium in artificially contaminated chicken breast meat stored at 4°C by 0.9 and 1.2 log CFU/g after 1 and 7 d, respectively. The results point toward a promising avenue for addressing the challenge of multidrug-resistant *S.* Typhimurium in the food industry through the use of recently discovered phages. Notably, the exploration of phage cocktails holds significant potential for combating *S.* Typhimurium in chicken breast products in the times ahead.

Key words: *Salmonella*, chicken breast meat, contamination, bacteriophage, food safety

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INTRODUCTION

Foodborne illnesses pose social and economic burdens and present an unforeseen hazard to human health (Parra et al., 2018; Ashrafudoulla et al., 2019). Consuming contaminated food or water is the most common way to get nontyphoidal salmonellae, including S. Typhimurium. Even though the stomach's acidity acts as a strong defense against this bacterium, eating a sufficient amount of contaminated food or consuming it with a meal that has a buffering effect may cause a productive infection that progresses to overt sickness (Galán, 2021). Approximately 153 million cases of gastroenteritis and 57,000 fatalities globally are attributed to nontyphoidal *Salmonella* annually. *S.* Typhimurium and *S.* Enteritidis are the 2 *Salmonella* serotypes that most frequently cause gastroenteritis in humans worldwide (Edris et al., 2013). *S.* Typhimurium potentially induces pathogenesis in various hosts, unlike other serovars (Galán, 2021). Almost 95% of human salmonellosis cases are related to the consumption of various foods, such as meat, poultry, eggs, milk, seafood, and fresh produce (Jung et al., 2018).

Chicken is a common source, and more recently attention has been focused on deciphering how widely *Salmonella* is present across different stages of the chicken production chain (Golden et al., 2021) Among the most critical sources of cheap protein, poultry products constitute one of the most popular because poultry meat is more affordable than red meat (Wahyono and Utami, 2018). The poultry industry's ability to meet growing consumer demand for antibiotic-free "natural" products for chicken and poultry products is greatly hampered by microbial contamination (Marmion et al., 2021). Due to its high perishability, chicken meat has a relatively short shelf life due to cross-contamination among chicken carcasses or from the manufacturing environment (Cerveny et al., 2009; Song et al., 2021). There were 298

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salmonellosis outbreaks linked to chicken eating between 1998 and 2017, resulting in 7,881 infections and 4 death (Centers for Disease Control and Prevention, 2020).

To prevent *S.* Typhimurium contamination of foods, thereby enhancing food safety, a number of treatments based on physical and chemical techniques have been formulated. Nonetheless, disadvantages exist, such as food-matrix effects chemical materials remaining; moreover, taste, flavor, and texture all impact quality (Deng et al., 2020). In the food sector, biological food treatment is emerging as a workable strategy for overcoming these shortcomings (Byun et al., 2022). Growing interest is being shown in using bacteriophages as biocontrol agents to effectively eliminate harmful bacteria from food without changing food quality (Coughlan et al., 2016).

Bacteriophages (phages) are bacterial viruses, and they constitute the most diverse and abundant biological life form. Phage has emerged as promising biocontrol for controlling foodborne pathogen due to their ubiquitous existence, specificity, and bactericidal effect (Azeredo et al., 2021). The initial interest in phage bactericidal properties gradually decreased with the discovery of penicillin and its antibiotic properties. According to the Center for Disease Control and Prevention (CDC, 2020) report, since the 1940s, when penicillin initially proved its potent effectiveness in the human body, several nations have utilized antibiotics in hospitals and farm production. Nevertheless, the overuse/misuse of antibiotics has triggered the emergence and rapid spread of multidrug-resistant (**MDR**) bacteria (English and Gaur, 2010). Phage research and application were largely neglected over time until the early 1980s when Smith and Huggins (1982) conducted their research, reigniting interest in phages. In recent years, recognition of the advantages of phages in combating MDR bacteria has grown, particularly through their ability to proliferate on a large scale using a host. Furthermore, phages generally have minimal impact on microbial ecology and are considered relatively safe organisms (Kortright et al., 2019; Walker et al., 2021).

More phage products are now commercially available for disease management than ever before owing to the United States Food and Drug Administration's 2006 approval of ListShield, a phage cocktail (a combination of several aggressive phages), as a food preservative. Health organizations in various nations, including Israel, Canada, China, Switzerland, Australia, New Zealand, and the European Union, have approved the use of phage products in food (Alomari et al., 2021). However, studies on bacteriophage use to lower *S.* Typhimurium contamination in Korean cuisine are limited (Hong and Chang, 2013). Therefore, in this study, *S.* Typhimurium lytic phage was isolated from sewage samples and its host range, morphology, and viability were evaluated at various temperatures and pH to confirm its effectiveness as a biological control agent for MDR *S.* Typhimurium.

MATERIALS AND METHODS

Bacterial Strain

Table 1 contains a list of the strains employed in this investigation. The Ministry of Food and Drug Safety (**MFDS**) has 20 wild *Salmonella* Enteritidis and Typhimurium samples. Additionally, 1 strain originates from the National Culture Collection for Pathogens and 7 from the American Type Culture Collection (**ATCC**). A stock culture that contained 10^8 to 10^9 CFU/mL was pipetted into 10 mL of tryptic soy broth (**TSB**; Difco, Detroit, MD) and incubated for 24 h at 37°C under aerobic conditions. Further incubation was initiated by pipetting 100 μL of the incubated solution into 10 mL of fresh TSB. By diluting it with 0.1% peptone water (**PW**; Oxoid, ants, UK) and plating it on xylose lysine deoxycholate (**XLD**) agar (Difco), cell density was ascertained.

Isolation, Purification, and Propagation of S. Typhimurium-Specific Bacteriophages

The sewage sample used to isolate *S.* Typhimurium-specific bacteriophages was obtained from a sewage treatment facility (Anseongsi, Gyeonggido, South Korea). Bacteriophage isolation was conducted as previously described by (Kim et al., 2020). The wastewater sample was divided into 25-mL portions to ensure proper mixing in a 50-mL tube. The tubes were subsequently centrifuged at 10,000 × *g* for 10 min at 4°C. Thereafter, the obtained 20 mL of supernatant was mixed with 20 mL of 2× TSB, and each bacterial strain (10^8 CFU/mL) was combined with 50 μL of the mixture. The resulting solution was subsequently subjected to incubation at 150 rpm and 37°C for 24 h. Following incubation, centrifugation was performed at 10,000 × *g* for 10 min at 4°C, followed by filtration using a 0.22-μm filter (Merck Millipore, Carrigtwohill, Ireland). The resulting sample was subsequently used in the plaque assay without further dilution. To isolate a single phage, a syringe needle was prepared and used to gently detach 1 plaque formed in the soft agar. The single plaque was immersed in a 1.5-mL Eppendorf (**EP**) tube containing 900 μL of sodium chloride−magnesium sulfate (**SM**) buffer and cultured at 4°C for 24 h. This phage-isolation process was repeated at least thrice using the plaque assay method. Thereafter, for the lysate step, 5 mL of SM buffer was distributed across the soft agar layer, covering the plaques. The plate was wrapped in foil to exclude light and subsequently incubated at 100 rpm for 4 h at room temperature. After incubation, the lysate was filtered through a 0.22-μm filter and stored at 4°C. For bacteriophage propagation, 500 μL (10^8 CFU/mL) was added to a 30% glucose solution and filtered using a 0.45-μm filter into 100 mL of TSBC solution. After adding 500 μL of the host bacterial strain to the TSBC solution, it was incubated at 37°C for 1 h. Thereafter, 5 mL of the lysate solution was added to the culture, and incubation was continued at 37°C for 5 h. Following

Table 1. Host range test of isolated *Salmonella* Typhimurium-specific bacteriophages.

Host strain		Refs.	No.	Isolated phages			
				STP-1	STP-2	STP-3	STP-4
S. Typhimurium Gram (−)	Wild-type 1	Kim et al. (2020)	1	-	+0.5	+1	+2
	Wild-type 2		2	+4	+0.5	-	+0.5
	Wild-type 3		3	+4	+1	+3	+4
	Wild-type 4		4	+4	-	+1	+4
	Wild-type 5		5	+4	+0.5	+4	+3
	Wild-type 6		6	+4	+4	+3	+4
	Wild-type 7		7	+4	+4	+4	+4
	Wild-type 8		8	+4	+4	+4	+4
	Wild-type 9		9	+4	+3	+0.5	+4
	Wild-type 10		10	+4	+4	+0.5	+4
	ATCC 14028		11	+4	+4	+3	+4
	ATCC 19585		12	+4	+1	+4	+4
	NCCP 14772		13	+	-	+3	+4
S. Enteritidis Gram (−)	Wild-type 1	Kim et al. (2020)	14	+4	+0.5	+4	+0.5
	Wild-type 2		15	+4	+0.5	+4	+0.5
	Wild-type 3		16	+4	+1	+	+1
	Wild-type 4		17	+4	-	+	+4
	Wild-type 5		18	+4	+0.5	+	+1
	Wild-type 6		19	+4	+1	+	+0.5
	Wild-type 7		20	+4	-	+4	+0.5
	Wild-type 8		21	-	+0.5	+	+1
	Wild-type 9		22	-	-	+	+2
	Wild-type 10		23	-	-	+	+3
	ATCC 13076		24	+3	-	+3	+1
S. Thompson	Wild-type 4021	Nahar et al. (2022)	25	-	+4	-	-
	Wild-type 4024		26	+2	+3	-	-
	Wild-type 6817		27	+2	+3	-	-
Gram (−)	*E. coli* ATCC10536		28	-	-	-	-
Gram (+)	*S. aureus* ATCC 6538		29	-	-	-	-
Gram (−)	*C. sakazakii* ATCC 12868		30	-	-	-	-

Host infection level: +4 complete clearing; +3 clearing throughout but with faintly hazy background; +2 substantial turbidity throughout the cleared zone; +1 a few individual plaques; +0.5 very small and hard individual plaques; - but you may see a spot where the pipette tip touched the agar.

incubation, the solution was filtered using a 0.22-μm filter, and the filtered solution was stored at 4°C for future use in the experiment. In this study, the 4 phages were STP-1, STP-2, STP-3, and STP-4.

Antibiotic-Susceptibility Testing

The antibiotic-resistant phenotypes of the 4 host strains (*S.* Typhimurium) of isolated bacteriophages were detected using the disk-diffusion assay according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020) and the method proposed by Thai et al. (2012) , with slight modifications. Agar diffusion assays were performed on Muller−Hinton agar (**MH** agar) using disks containing 24 antibiotic agents (Oxoid, UK). Briefly, 2 mL of bacterial suspension was dried on MH agar for 1 min and removed the remained suspension using a pipette and dried for 15 min on a clean bench. Thereafter, the antibiotic disk put and incubated for 24 h at 37°C. According to the CLSI guidelines (CLSI, 2010), the following interpretative categories: "sensitive(S)," "intermediate (I)," and "resistant (R)" were applied.

Characterization of Isolated Phages

Morphology Five microliters of the lysate solution were placed onto a carbon-coated grid and observed for 1 min to allow for attachment. Subsequently, each sample was stained with 2% uranyl acetate for ≤15 s and then analyzed for morphology using electron field-transmission electron microscopy (**EF-TEM**) (Byun et al., 2022).

Host Range Test Host range testing of isolated phages was performed as previously described by Byun et al. (2022). Briefly, by applying the spot test, 300 μL of each bacterial strain was mixed with soft agar, poured onto a layer of hard agar, and allowed to dry for 15 min. Once the soft agar had solidified, 10 μL of the lysate solution was added as spots onto the surface of the solidified soft agar. After ≥15 min, the plate was incubated at 37°C for 12 h. The transparency of the clear zone, where the phage was placed, was used to determine the degree of lysis zone against the host strain, following a standard scoring system (Ackermann, 2009).

Adsorption Rate and 1-Step Growth Curves Phage-adsorption investigation was performed according to the method proposed by Kim et al. (2020), with several modifications. The bacteria−bacteriophage culture was inoculated at a multiplicity of infection (**MOI**) of 0.0001 and subsequently incubated at 37°C. At specific time points, the culture was removed and centrifuged at 12,000 rpm for 2 min to separate the supernatant. Thereafter, the plaque assay was performed using the supernatant to measure the number of unattached phages.

The phage's latent period and burst size were determined using a 1-step growth curve from an earlier study (Sadekuzzaman et al., 2018), and a graph of the released phage number by time was constructed. To determine the phage's latent period and burst size, we defined the

latent period as the time interval between adsorption and the commencement of the first burst. Burst size was calculated by dividing the final number of phage units by the initial number of infected host cells at the beginning of the experiment.

Temperature and pH Level Stabilities Various temperature and pH condition tests were conducted using 10^9 to 10^{10} log PFU/mL of phage. The phage solution was placed in a 1.5-mL EP tube for heat-stability testing. The samples were maintained at various temperature points (from 60°C to 90°C) for up to 1 h (at 10, 20, 30, and 60 min). At each time point, the samples were removed from the water bath, and the plaque assay was conducted to assess phage activity. For the pH-stability test, 5 mL of TSB was added to a 15-mL tube, and the pH was adjusted accordingly (from 2 to 13) using sodium hydroxide or hydrochloric acid. At each pH condition, 900 μL of the adjusted solution was added to separate EP tubes. Thereafter, 100 μL of the phage solution was added to each tube. The tubes were subsequently incubated at 37°C for 24 h, and the plaque test was used to detect the presence of live phages thereafter.

Bacterial Challenge Assay

Bacterial challenge assays were performed via lysis kinetics according to the protocol outlined by Byun et al. (2022). To investigate phage impact on the viability of *S.* Typhimurium in vitro, a population of each host strain at a concentration of 7 log CFU/mL was prepared. Subsequently, 100 μL of each phage was combined with the same volume of the designed MOI in a 96-well plate. The negative control was noninoculated TSB, whereas the positive control comprised host strains in TSB with no inoculated phage. The incubation time points were set at hourly intervals up to 12 h, with the final data point recorded at 24 h. During this incubation, the host bacteria were exposed to different phage concentrations, with varying MOI values of 0.1, 1, and 10.

Efficiency Test of Bacteriophage Cocktail in Chicken Breast Meat

A modified version of the method described by Kim et al. (2020) was employed. Equal parts of the 4 phages (STP-1, STP-2, STP-3, and STP-4) were combined to create a cocktail with a concentration of 108 PFU/mL. A mixed suspension of *S.* Typhimurium strains, namely, ATCC14028, ATCC 19585, wild-type 4, and wild-type 6, was prepared at the same ratio as a 10^6 CFU/mL concentration, serving as the host strain for each phage.

Chicken samples were purchased from a grocery market in Gyeonggi-do, South Korea, and cut with a sterilized knife into square pieces (2 × 2 cm, approximately 5 g). The meat pieces were subsequently inoculated with 100 μL of the cocktail host *Salmonella* strain suspension (10^6 CFU/mL) and dried at room temperature for 2 h to facilitate bacterial attachment to the meat surface. Subsequently, the samples were inoculated with 100 μL of the phage cocktail at 10^7, 10^8, and 10^9 PFU/mL concentrations, corresponding to MOIs of 10, 100, and 1,000, respectively. The samples were stored at 4°C. The SM buffer was used in the negative control. After 0, 1, 3, 5, and 7 d of storage, the samples were placed in 45 mL of 0.1% PW and homogenized using a paddle blender (speed: 4, time: 2 min). To enumerate the *S.* Typhimurium residents, diluted samples were plated onto XLD agar plates and incubated overnight at 37°C. After incubation, the colonies were counted to determine the population of *S.* Typhimurium.

Statistical Analysis

The experiments were performed in triplicates. Log numbers were used to reflect the average microbial populations. Microbial reductions in food owing to phage cocktail treatments were compared using Duncan's multiple range test after 1-way analysis of variance was used to identify any significant differences. SPSS (version 26; SPSS Institute Inc.,Chicago, IL) was used for all statistical analyses, and statistical significance was set at $P < 0.05$.

RESULTS

Phage Isolation, Purification, and Propagation

Four phages were isolated from a sewage sample (STP-1, STP-2, STP-3, and STP-4 using *S.* Typhimurium wild-type 4, wild-type 6, ATCC 19585, and ATCC 14028, respectively). The smallest bacteriophage size was <2.5 mm. Extensive bacteriophage propagations have titers of $>10^{11}$ PFU/mL.

Morphological Characteristics

The EF-TEM images of the isolated phages are illustrated in Figure 1. All the isolated phages (STP-1, STP-2, STP-3, and STP-4) were characterized as members of the *Caudoviricetes* class, presenting a long noncontractile tail. Their head diameters and tail lengths were 74.09 ± 1.52 and 122.48 ± 2.21 nm for STP-1 (Figure 1A), 73.86 ± 5.44 and 249.29 ± 15.89 nm for STP-2 (Figure 1B), 77.27 ± 1.97 and 236.22 ± 3.74 nm for STP-3 (Figure 1C), and 65.23 ± 7.13 and 256.71 ± 3.69 nm for STP-4 (Figure 1D), respectively.

Host Range Test

The 4 bacteriophages were tested against a variety of *Salmonella* serovars, including 13 distinct *S.* Typhimurium, 11 *S.* Enteritidis, and 3 *S.* Thompson serovars, as well as one each of *E. coli* ATCC10536, *S. aureus* ATCC 6538, and *C. sakazakii* ATCC12868. STP1, STP2, STP3, and STP4 were able to lyse 22 (81%), 20 (74%), 23 (85%), and 24 (89%) different *Salmonella* spp. strains, respectively. Other bacterial genera could not be lysed by any of the phages discovered in this study (Table 1).

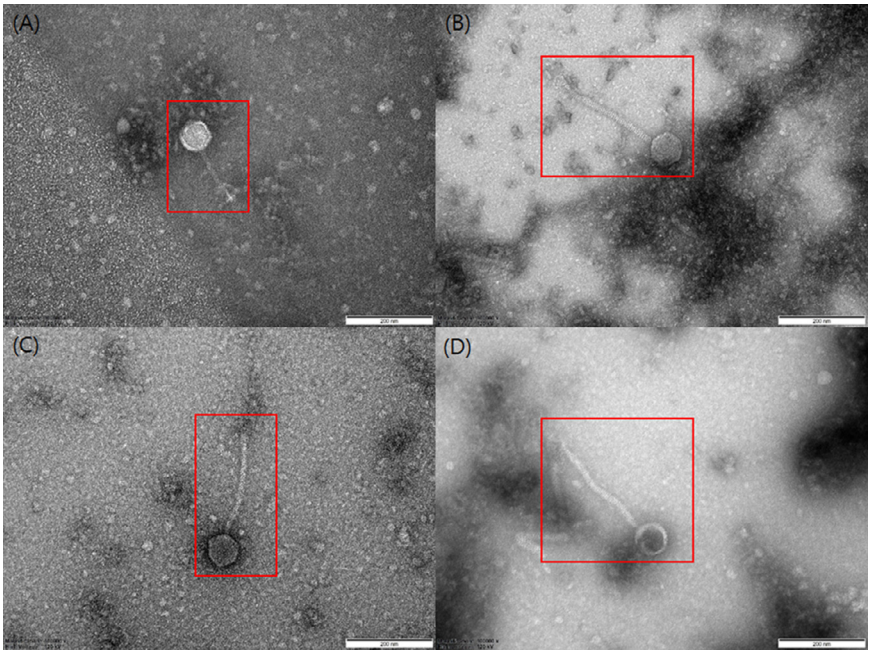

Figure 1. Transmission electron micrographs (TEMs) of isolated *Salmonella* bacteriophages; (A) STP-1, (B) STP-2, (C) STP-3, and (D) STP-4.

Antibiotic-Susceptibility Patterns of S. Typhimurium

The antibiotic sensitivity of the 4 host *S.* Typhimurium strains, that is, the 2 wild-type and 2 ATCC strains, was estimated against a range of 24 antibiotics (Table 2). The antibiotic resistance of the 2 *Salmonella* ATCC strains was less than 50%, however, the isolated wild-type 4 and wild-type 6 strains exhibited high resistance rates of 54 and 75%, respectively. Wild-type 6 strains displayed the lowest sensitivity (12.5%). Surprisingly, every examined bacterial strain was determined to be MDR as it demonstrated resistance to at least one antibiotic from more than 3 distinct classes.

Adsorption and 1-Step Growth Curves

The adsorption process of all phages to the host cells was remarkably rapid. STP-1 demonstrated that over 50% of the phages were adsorbed within 3 min, reaching approximately 100% within 18 min (Figure 2A). STP-2 achieved adsorption within 5 min, reaching approximately 100% within 30 min (Figure 2B); STP-3 within 1 min, reaching approximately 100% within 9 min (Figure 2C); and STP-4 within 1 min, reaching approximately 100% within 10 min (Figure 2D).

The 1-step growth curves provided insight into phage mechanics. With burst sizes of roughly 31 and 218 PFU/-cell, respectively, STP-1 and STP-4 had latent periods of

Table 2. Antibiotic-resistance spectrum of *Salmonella* Typhimurium host strains used in this experiment.

No.	Antibiotic	Antibiotic contents per disk (μg)	Wild-type 4	Wild-type 6	ATCC 19585	ATCC 14028
1	Amikacin (AN)	30	I	R	S	R
2	Ampicillin (AM)	10	R	R	I	R
3	Cefepime (FEP)	30	S	S	S	S
4	Cefotaxime (CTX)	30	R	R	R	R
5	Ceftazidime (CAZ)	30	R	R	R	R
6	Ceftriaxone (CRO)	30	R	R	S	S
7	Chloramphenicol (C)	30	S	R	S	S
8	Ciprofloxacin (CIP)	5	I	S	I	I
9	Clindamycin (CC)	2	R	R	R	R
10	Doxycycline (D)	30	R	R	S	S
11	Erythromycin (E)	15	R	R	R	R
12	Gentamicin (GM)	10	I	R	R	R
13	Imipenem (IPM)	10	I	R	I	I
14	Kanamycin (K)	30	R	R	R	I
15	Levofloxacin (LVX)	5	S	S	S	S
16	Meropenem (MEM)	10	R	R	I	R
17	Minocycline (MI)	30	R	R	R	R
18	Nalidixic acid (NA)	30	R	I	I	I
19	Penicillin (P)	10	R	R	R	R
20	Polymixin B	300	I	I	I	R
21	Sulfamethoxazole-Trimethoprim (SXT)	25	S	I	S	S
22	Tazobactam-Piperacillin (TZP)	110	R	R	R	R
23	Ticarcillin/clavulanic acid (TIM)	85	S	R	S	S
24	Tobramycin (NN)	10	I	R	S	I

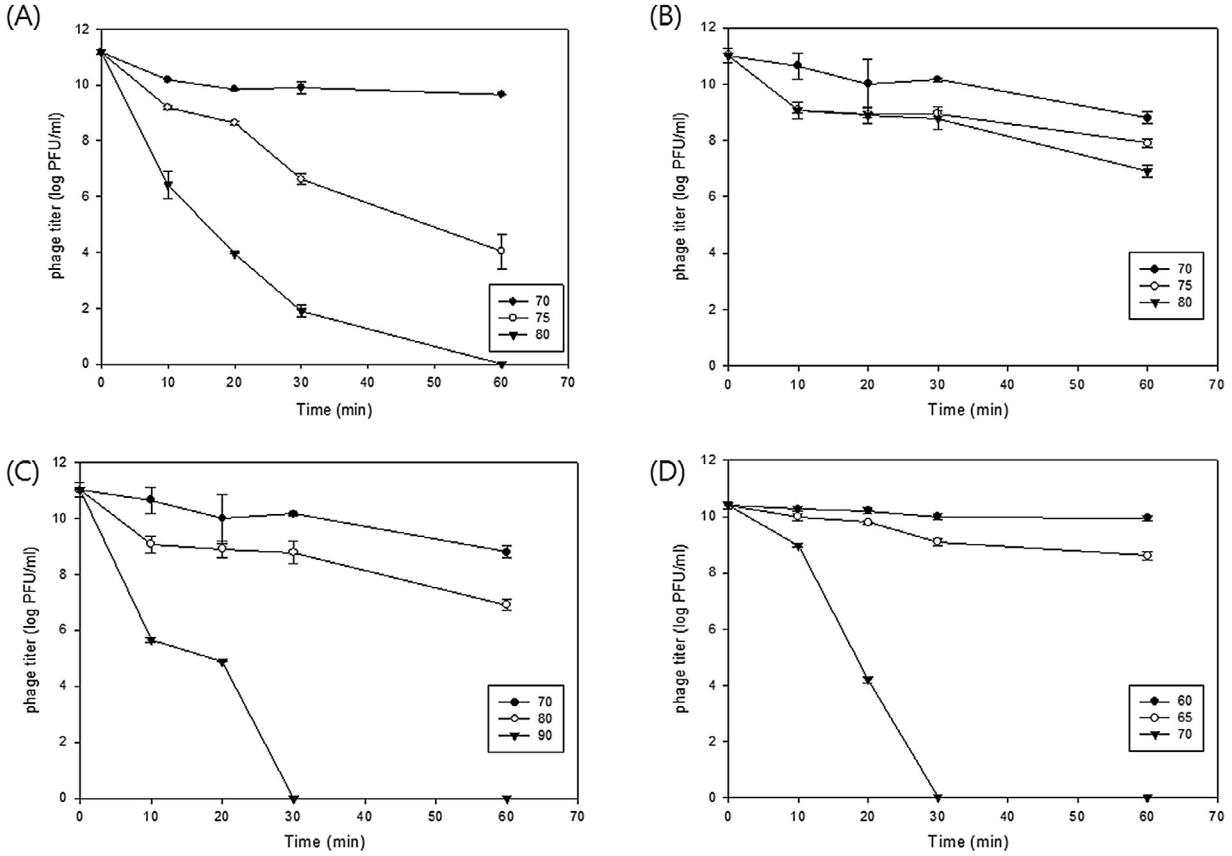

Figure 2. Adsorption of isolated *Salmonella* bacteriophages to their host strain at 37°C; (A) STP-1, (B) STP-2, (C) STP-3, and (D) STP-4.

approximately 40 min each (Figure 3A and D). Figure 3B and C shows the latent periods and burst sizes for STP-2 and STP-3, which were approximately 50 and 30 min, respectively.

Temperature and pH Level Stability

Figures 4 and 5 show phage capability in various temperature and pH ranges. According to the pH-stability test, STP-1 exhibited stability from pH 5 to 11 at 10.09 ± 0.13 log PFU/mL (Figure 4A). STP-2, STP-3, and STP-4 displayed activity within the alkaline range up to pH 12, with minimal decline after pH 12 (Figure 4). In particular, STP-3 (Figure 4C) exhibited relative stability between pH 5 and 11 but declined gradually at lower or higher pH values. STP-1 and STP-2 (Figure 4A and B) demonstrated stability at 70°C and 75°C for 1 h, respectively, and STP-2 and STP-3 both exhibited stability at 80°C. However, STP-1 was observed to gradually decline at 80°C at all points, and the phage was eventually undetectable at 60 min. STP-3 displayed stability at high temperatures, among other phages that demonstrated activity at 90°C for 30 min. However, STP-4 (Figure 4D) exhibited stability for 1 h at 60°C and 65°C; nevertheless, it only showed activity for 30 min at 70°C.

In Vitro Bacterial Challenge Assay

The inhibitory effect of isolated phages on host strains was tested in TSB medium. As shown in Figure 6, the $OD_{600\ nm}$ values after single-phage treatment (STP-1, STP-2, STP-3, or STP-4) could inhibit *S.* Typhimurium growth. All 4 phages inhibited *S.* Typhimurium growth at MOIs of 0.1, 1, and 10. In the case of STP-1, the difference between MOIs began to appear after 4 h, and at MOI 10, it gradually decreased and remained below 0.2 $OD_{600\ nm}$ for up to 24 h. STP-2 exhibited a clear difference between MOIs after 12 h, and MOI 10 treatment yielded approximately half the absorbance of the positive control. In the case of STP-3, *S.* Typhimurium growth decreased after 1 h at MOI 0.1, 1, and 10; in particular, it remained almost constant from 3 h at MOI 10. STP-4 displayed a similar pattern between MOI and the positive control and yielded similar results at MOI 0.1 and 1 for up to 24 h; however, MOI 10 exhibited approximately half of the positive control.

Effect of the Phage Cocktail on the Viability of Salmonella Typhimurium in Chicken Breast

We investigated the antibacterial efficacy of the phage cocktail (STP-1 + STP-2 + STP-3 + STP-4) by assessing the effects of MOI 10, 100, and 1,000 on the *Salmonella* cocktail (4 host bacteria) in chicken breast at 4°C for 7 d (Figure 7). Before the experiments, we checked for naturally occurring *Salmonella* spp. on the XLD agar that had not been detected in the samples. The *S.* Typhimurium counts in non−phage-treated chicken breast were 6.04 ± 0.04, 5.93 ± 0.02, 6.22 ± 0.04, 5.80 ±

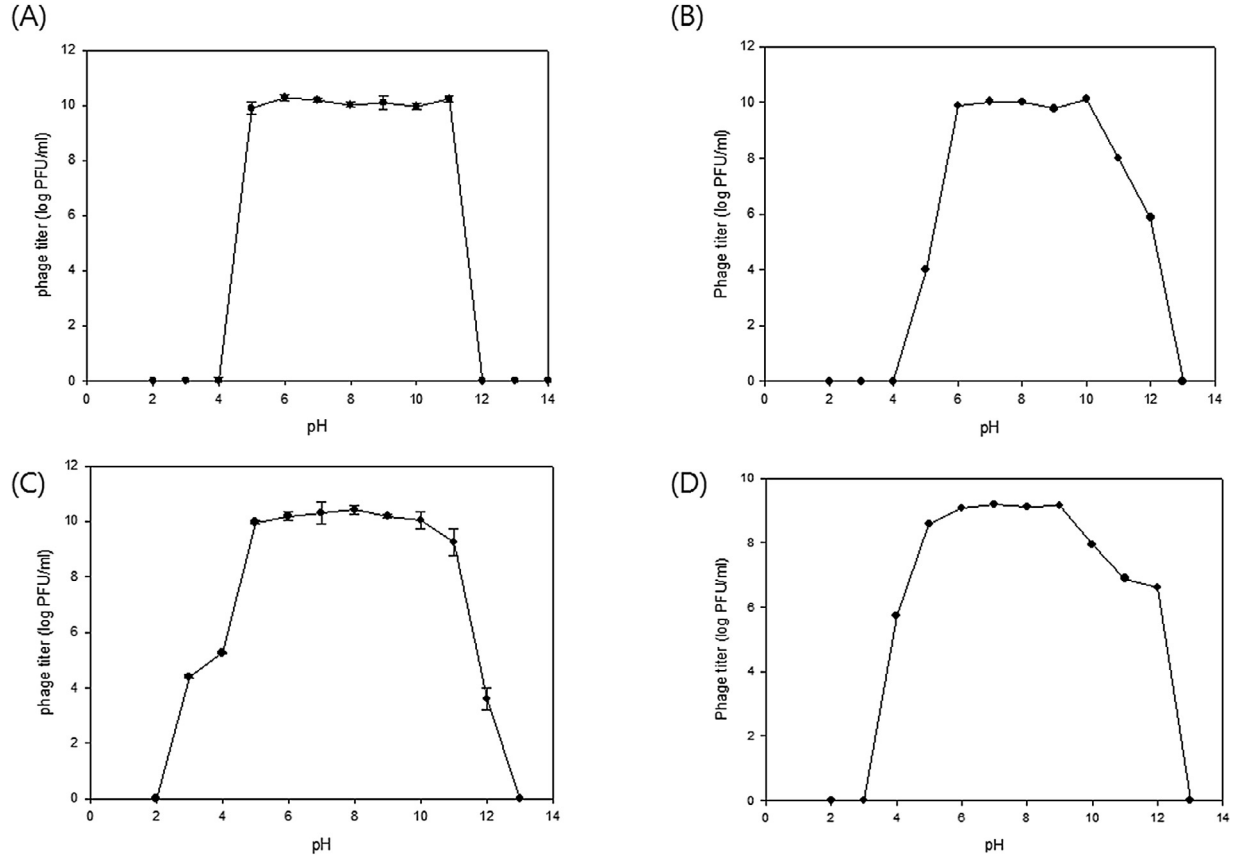

Figure 3. One-step growth curve of isolated *Salmonella* bacteriophages on their host strain at 37°C; (A) STP-1, (B) STP-2, (C) STP-3, and (D) STP-4. Results are presented as the mean ± standard deviation.

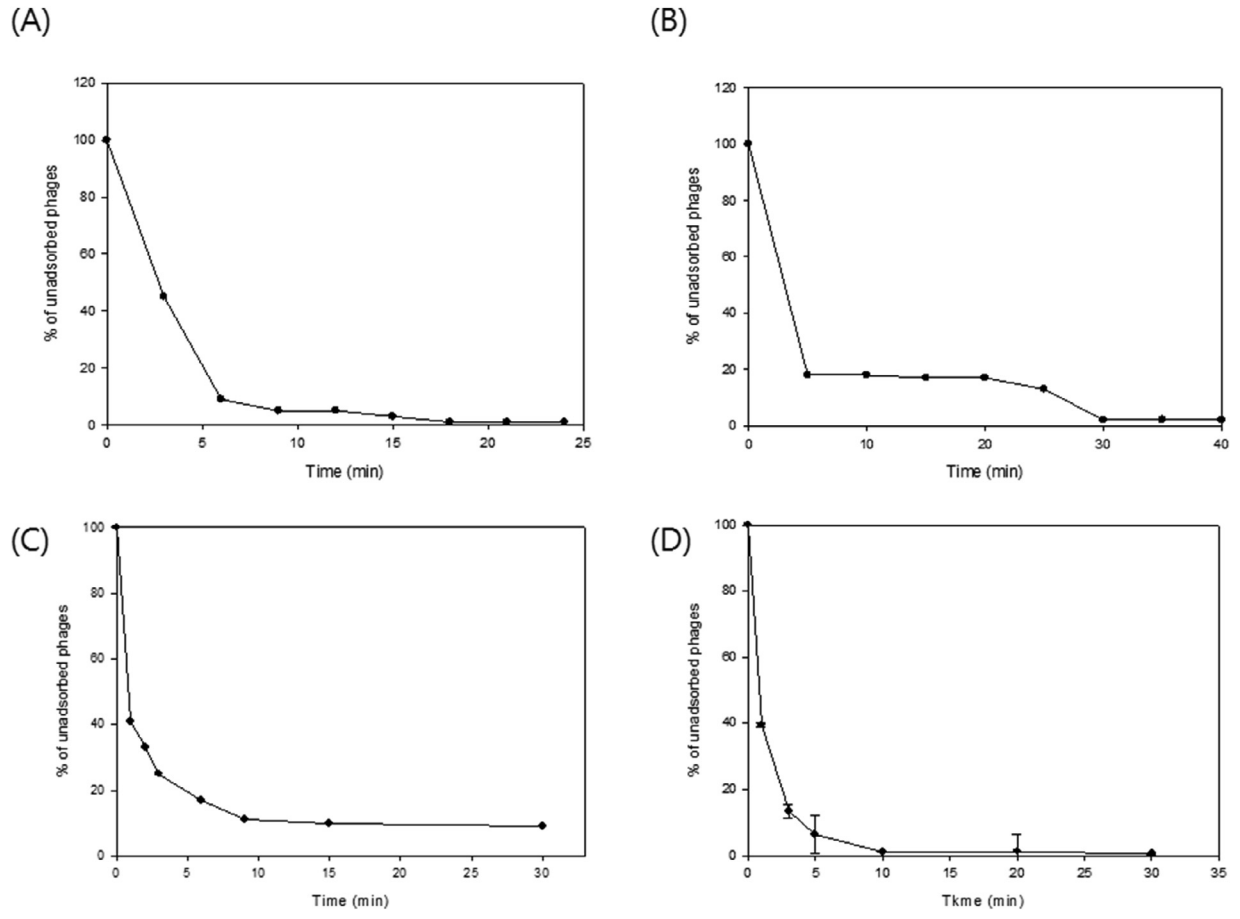

Figure 4. The pH-stability test of isolated *Salmonella* bacteriophages; (A) STP-1, (B) STP-2, (C) STP-3, and (D) STP-4. Results are presented as the mean ± standard deviation.

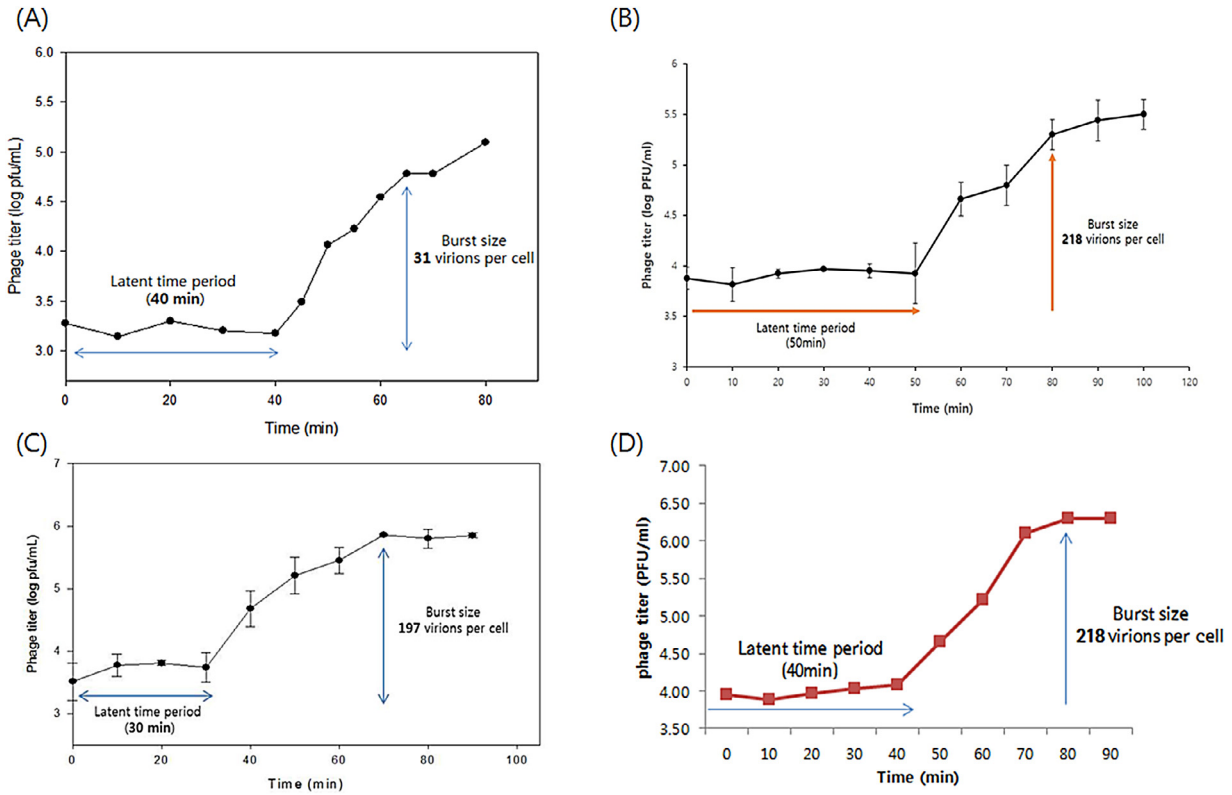

Figure 5. The effect of temperature on the stability of isolated *Salmonella* bacteriophages; (A) STP-1, (B) STP-2, (C) STP-3, and (D) STP-4. Results are presented as the mean ± standard deviation.

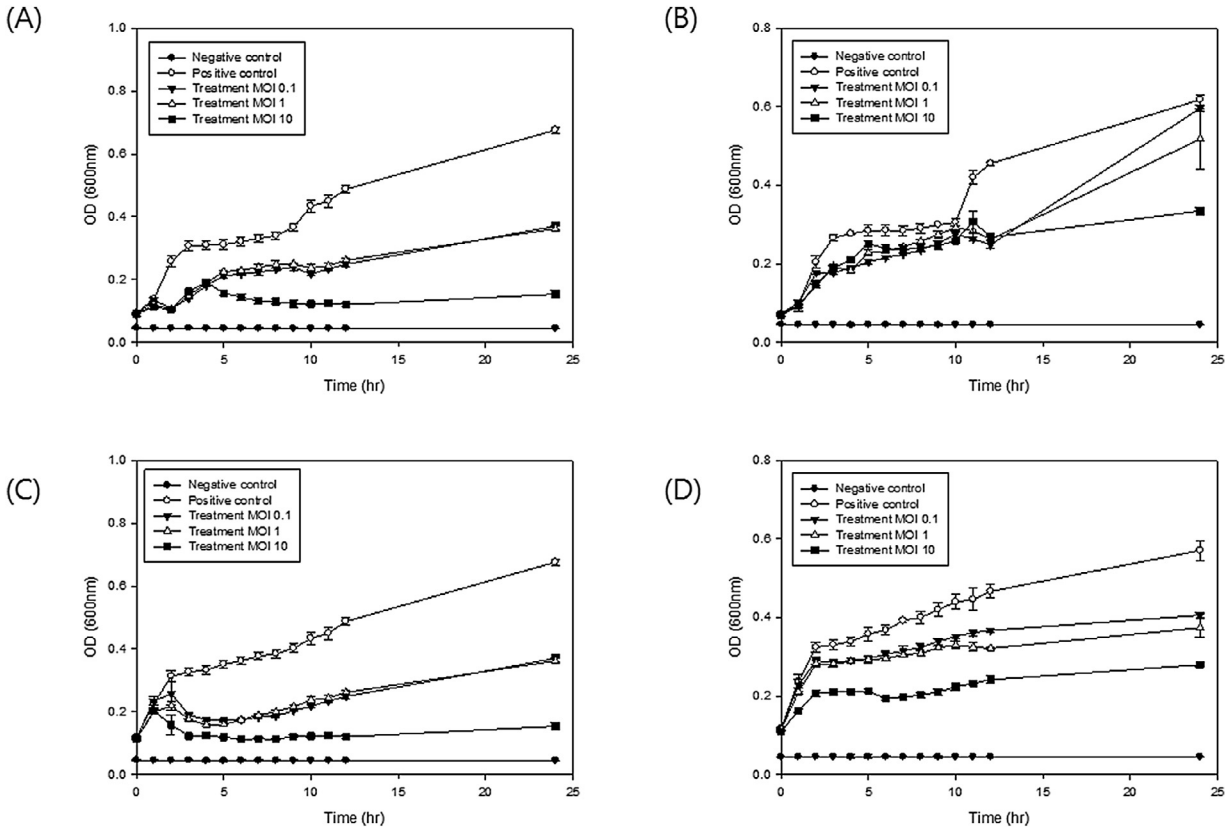

Figure 6. Bacterial challenge of isolated *Salmonella* bacteriophages; (A) STP-1, (B) STP-2, (C) STP-3, and (D) STP-4 at MOI 0.1, 1, and 10. Results are presented as the mean ± standard deviation.

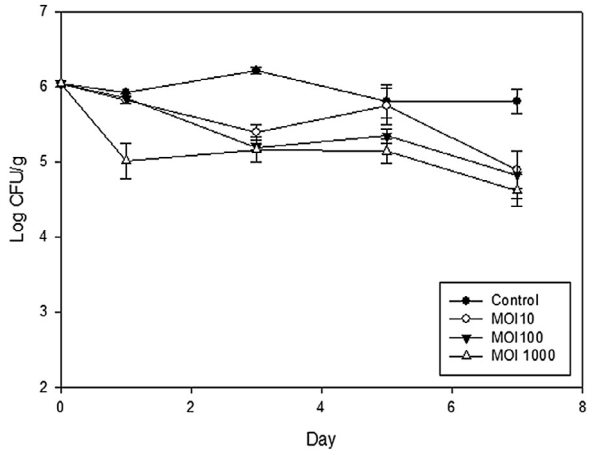

Figure 7. Bacterial reduction of a cocktail of *Salmonella* Typhimurium host strains by a cocktail of *Salmonella* bacteriophages in chicken breast meat at MOI 10, 10^2, and 10^3. Results are presented as the mean ± standard deviation.

0.22, and 5.81 ± 0.16 CFU/g on d 0, 1, 3, 5, and 7, respectively. On d 1, a significant difference ($P < 0.05$) in MOI 1,000 of 0.92 log compared with the control value without phage treatment was noted. On d 3, a significant difference ($P < 0.05$) was observed between the MOI 10 group, which decreased by 0.8 log compared with the control value, and the MOI 100 and 1,000 groups, which decreased by 1.0 log compared with the control value. Fifth day, results were 5.74 ± 0.24, 5.32 ± 0.09, and 5.14 ± 0.16 at MOI 10, 100, and 1,000, respectively. A significant difference ($P < 0.05$) was noted in the MOI 100 and 1,000 groups. Seventh day, results were 4.90 ± 0.25, 4.83 ± 0.31, and 4.62 ± 0.21 at MOI 10, 100, and 1,000, respectively. All treatment groups yielded significant differences ($P < 0.05$) from the control.

DISCUSSION

Nontyphoidal *Salmonella* is the major cause of human foodborne infections, posing a global threat to healthcare systems, particularly when combined with MDR strains. The use of phages as biocontrol agents is gaining attention as a method to control MDR strains that have emerged due to antibiotic misuse. Phages exist in various environments, resulting in an abundance of phage particles on Earth (Rohwer and Segall, 2015). *Salmonella*-targeted bacteriophages have already been approved for commercial use and can be applied to meat and poultry as well as fresh processed fruits and vegetables (Sharma, 2013). Moreover, previous studies have confirmed the effectiveness of bacteriophages as biocontrol agents, for example, SalmoFresh and PhageGuard S in poultry products, such as poultry parts (De Vegt et al., 2019) as well as skinless and skin-on poultry products (Hagens et al., 2018).

In this study, 4 bacteriophages were successfully isolated from sewage water, namely, phage STP-1, STP-2, STP-3, and STP-4 against *Salmonella* spp. Owing to the apparent plaques that these phages produced in the agar overlay experiment, they were regarded as lytic bacteriophages. The phages discovered in this study potentially lyse strains belonging to a single genus without harming strains belonging to other species. This trait may be helpful when beneficial bacteria are present or in oral tests that reveal additional gut microbiota. Because the location of the cell receptor depends on the phage and host, exclusively infecting strains with the same receptor as the host at the time of isolation is typically possible (Hyman, 2019). Receptors are present in cell walls, flagella, pili, capsules, or bacterial cell surface proteins, which are inaccessible or noncomplementary to receptor-binding proteins; hence, phages become incapable of binding to host cells (de Jonge et al., 2019).

EF-TEM was used in the current investigation to conduct morphological characterization, revealing that the *Caudoviricetes* class exhibited the presence of all 4 phages. These findings are consistent with those of recent studies (Bardina et al., 2012; Duc et al., 2018; Kim et al., 2020) that discovered that *Salmonella*-specific phages were members of the *Caudovirales* order. Based on the morphology of their tails, the 4 families of tailed bacteriophages (*Caudovirales*) were subdivided. *Myoviridae* have long, rigid, contractile tails that range in length from 80 to 485 nm, and their typical head diameter is 85 nm. The *Siphoviridae* family possesses a moderate head diameter of 55 nm and noncontractile, long, and flexible tail. The typical head diameter of *Podoviridae* is 58 nm, and its noncontractile, short tail is <40 nm long (Harada et al., 2018; Dion et al., 2020).

Phages potentially increase the mortality of targeted bacteria and exposure to bacteria through self-replication in host bacterial cells. In studies by Kwon et al. (2008) and Huang et al. (2018), the burst size of phage LPST10 measured using the first-stage growth curve was approximately 100 PFU/infected cell, exhibiting similarity to phage SG-JL2, a T7-like lytic bacteriophage. In our study, the burst size of STP-1 was approximately 31 virons/infected cell, and that of STP-2, STP-3, and STP-4 was approximately 200 virons/infected cell. Furthermore, Huang et al. (2018) reported a short latent time of 10 min, whereas in this study, all 4 bacteriophage types had a latent time that exceeded 30 min. On comparing the 1-step growth and burst size yielded in this study with those reported in our previous study (Kim et al., 2020), 1 phage (CAU-SEP-1) had a similar latent time; however, most phages had a latent time of approximately 10 min, and a difference in burst size was also noted. Two phages (CAU-SEP-2 and CAU-SEP-4) survived at pH 3, which is a strong acidic condition, demonstrating similarity to the pH stability of STP-3 in this study. In contrast to the present study, which revealed stability at temperatures >70°C, Kim et al. (2020) reported stability for 1 h at temperatures <65°C (Kim et al., 2020). Therefore, the phages isolated in this study can be applied to various foods owing to their broad pH stability (pH 3−13) and thermal stability (up to 70°C).

According to earlier research, developing bacteria-insensitive mutants (**BIMs**) during overnight

incubation potentially leads to phage resistance and subsequently reduces the phage's long-term efficiency (O'flynn et al., 2006). This study's growth inhibition experiment results revealed that all phages, except MOI 10, demonstrated a regrowth phenomenon for 24 h. Two or more combinations of phage cocktails with a vast host range can partially reduce the likelihood of BIM formation due to the various receptors involved in the interaction between phage and bacterial receptors.

As a result of supporting this, in our study, the host bacteria continued to decline when the phage was cocktailed and applied to food (chicken breast). Artawinata et al. (2023) reported a reduction of up to 96.06% by phage ETEC-S3 on chicken meat samples after incubation at 28°C. The reason for the more significant reduction than that in this study, which involved storage at 4°C, is the possible promotion of death as bacteria grow better at high temperatures, and phage self-replication occurs more rapidly. Our in-food results indicate that *S.* Typhimurium counts in chicken breast were significantly reduced ($P < 0.05$) after 7 d. In contrast, the study by Kim et al. (2020), which involved storage at 4°C for 7 d, suggests that considering a 1.5-log reduction in MOI > 10^4, this study's results, in which a decrease in MOI 1,000 of 1.2 logs ($P < 0.05$) after 7 d occurred, exhibited a more significant reduction rate.

Notwithstanding, this study is limited in that it did not include genomic analysis of isolated bacteriophages; nonetheless, genomic analysis of the 4 isolated phages will be performed in future studies. We also intend to investigate the relationship between *S.* Typhimurium host and bacteriophages.

CONCLUSIONS

This study successfully demonstrated the effectiveness of 4 *Salmonella*-specific bacteriophages (STP-1, STP-2, STP-3, and STP-4) as biocontrol agents against MDR *S.* Typhimurium in vitro and in chicken breast meat. Phage morphology suggests that the phage belongs to the Caudoviricetes class. The host range results, stability under various temperatures and pH, adsorption rate, 1-step growth curve, and inhibition effect of *S.* Typhimurium in vitro indicate that these phages are promising biocontrol agents. In addition, the 4-phage cocktail treatment of chicken breast was considerably effective against *S.* Typhimurium stored at 4°C. The results of this study clearly indicate that the combination of 4 isolated bacteriophages, when used as a cocktail, exhibits a notably superior effectiveness in reducing S. Typhimurium levels in chicken breast. These findings highlight the potential of utilizing these isolated phages as valuable biocontrol agents against MDR S. Typhimurium within the realm of the food industry, with particular relevance to the advancement of poultry product development. However, further study on their genomic characterization and suitability for use in the food industry is warranted.

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DISCLOSURES

There is no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.103073.

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