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ISOLATION AND BIOLOGICAL CHARACTERIZATION OF THE BROAD HOST RANGE BACTERIOPHAGE ΨSA118 FOR THE BIOCONTROL OF *Staphylococcus aureus* IN FOOD SAFETY APPLICATIONS

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ABSTRACT

Staphylococcus aureus is a significant foodborne pathogen responsible for numerous outbreaks of food poisoning worldwide. Its increasing resistance to commonly used antibiotics, including methicillin, poses a critical challenge to food safety and public health, necessitating the development of alternative antimicrobial strategies. Bacteriophages (phages), as natural bacterial predators, offer a promising biocontrol approach due to their host specificity and ability to self-amplify at the site of infection. In this study, 25 bacteriophage isolates were screened for lytic activity against *S. aureus*, among which phage ΨSa118 was identified as the most promising candidate. Phage ΨSa118 exhibited a broad intraspecies host range, lysing all 9 tested *S. aureus* strains, while demonstrating strict species specificity. Transmission electron microscopy (TEM) revealed that ΨSa118 possesses an icosahedral head with a very short and non-contractile tail, classifying it within the Podoviridae family. Biological characterization showed that ΨSa118 achieved optimal replication at a multiplicity of infection (MOI) of 0.01, reaching a peak titer of 3.50×10^9 PFU/mL. The phage had a latent period of 20 minutes and a burst size of 73 PFU per infected cell, indicating efficient lytic potential. Environmental stability assays demonstrated that ΨSa118 remained viable across a wide range of conditions, including temperatures from 4 ^oC to 50 ^oC, pH levels from 2 to 12, and NaCl concentrations up to 10 M. Additionally, ΨSa118 maintained its viability following prolonged exposure to UV radiation (254 nm), further supporting its resilience. Taken together, these findings indicate phage ΨSa118 as a potent biocontrol agent against *S. aureus*, especially where conventional antimicrobials are limited by resistance or regulation.

Keywords: Bacteriophage, biocontrol, food safety, phage therapy, *Staphylococcus aureus.*

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INTRODUCTION

Foodborne illnesses remain a significant global public health concern, typically resulting from the consumption of food or beverages contaminated with pathogenic microorganisms or their toxins (Rahnama et al., 2022). Nutrient-rich food products such as meat, fish, milk, and dairy derivatives provide favorable environments for microbial growth and are common sources of contamination (Kali et al., 2021). If not effectively controlled, microbial contamination-especially bacterial-can lead to serious health consequences, economic losses, and widespread risks to community health (Zhou et al., 2018).

In Vietnam, foodborne illness remains a major public health concern, with thousands of cases reported annually. Improper handling, inadequate storage, and poor hygienic practices, particularly during the preparation and distribution of ready-to-eat foods, contribute significantly to the prevalence of *Staphylococcus aureus* in the food chain (Hue & Chris, 2021; Thanh et al., 2024). Although national food safety programs have improved in recent years, challenges persist in ensuring effective bacterial control at the production level. Among foodborne pathogens, *S. aureus* is a key causative agent. This Gram-positive coccus is capable of causing a wide range of infections in both humans and animals, including skin infections, respiratory tract infections, and notably, food poisoning through the production of heat-stable enterotoxins (Su et al., 2020). The overuse of antibiotics in both human medicine and animal husbandry has led to the increasing prevalence of antibiotic resistant bacteria, including methicillin-resistant *S. aureus* (MRSA). In 2017, the World Health Organization (WHO) listed MRSA as a high-priority pathogen for which new antibiotics are urgently needed. However, the development of novel antibiotics is time-consuming, costly, and their use in food may compromise sensory qualities or leave undesirable chemical residues (Nikolic et al., 2020; Ravindran et al., 2019). These limitations underscore the urgent need for alternative, safe, and effective bacterial control strategies in the food industry. One promising approach is the use of bacteriophages - viruses that specifically infect and lyse bacterial cells. In recent years, bacteriophages have gained renewed attention as natural biocontrol agents due to their high specificity, ability to self-replicate at the site of infection, and minimal impact on beneficial microbiota, particularly in applications related to food safety (Santos & Azeredo, 2019). Additional advantages include their relatively low production cost and environmentally friendly profile (Monteiro et al., 2018; Romero-Calle et al., 2023). The safety and efficacy of phage therapy have been validated in multiple animal models and human clinical contexts (Międzybrodzki, 2012; Takemura-Uchiyama et al., 2014).

In this study, we screened and characterized the biological properties of the bacteriophage that exhibits strong lytic activity against *S. aureus*. This work aims to lay the foundation for the development of phage-based biocontrol formulations targeting *S. aureus* contamination in food products, contributing to sustainable food safety enhancement.

MATERIALS AND METHODS

Bacterial strains and bacteriophage isolates

A total of 25 bacteriophage isolates with lytic activity against *S. aureus* were previously isolated and stored in the Molecular Microbiology Laboratory (MML) - Institute of Biology, VAST. The test panel included nine *S. aureus* strains: six strains (771, 816, 2860, 3016, 3027, 3625), and the reference strain *S. aureus* ATCC 29213 from the Microbial strain collection of MML; two strains (VTCC70187 and VTCC70188) from the Vietnam National Microbial Resource Center - Institute of Microbiology and Biotechnology. For specificity testing, the *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, and *Bacillus cereus* ATCC 10876 strains were used as non-target controls.

Host range determination

The host range of each phage isolate was evaluated using the spot assay on double-layer agar (Glonti et al., 2022). Briefly, 100 μL of

indicator bacterial culture (10^8 CFU/mL) was mixed with 5 mL of molten Luria-Bertani (LB) soft agar (0.7% agar) and overlaid onto LB agar plates (2% agar). After solidification, 10 µL of purified phage suspension (10^9 PFU/mL) or SM buffer (200 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5) as a negative control was spotted onto the surface. Plates were incubated at 37 °C for 18–24 hours, and the presence of clear lytic zones was recorded as evidence of phage activity.

Transmission electron microscopy (TEM)

Phage morphology was visualized by TEM (Zhou et al., 2021). The purified phage (10^9 PFU/mL) was placed on carbon-coated copper grids, negatively stained with 2% uranyl acetate (pH 4.0) for 20 seconds, and air-dried. Images were captured using a JEM-1400 Flash TEM (JEOL, Japan). Phage classification was based on the taxonomy established by the International Committee on Taxonomy of Viruses (ICTV).

Optimal multiplicity of infection of the phage

The optimal MOI was determined according to the protocol described by Luo et al. (2021). The phage and *S. aureus* ATCC 29213 were mixed at MOIs of 0.001, 0.01, 0.1, 1, 10, and 100. Mixtures were incubated at 37 °C for 10 hours, centrifuged at 10,000 rpm for 5 minutes, and the supernatant was filtered through a 0.22 µm membrane. Phage titers were determined using the double-layer agar plaque assay. The titer of the original phage culture was calculated using the formula: phage titer (PFU/mL) = number of plaques × 10 × dilution factor. The MOI yielding the highest titer was considered optimal. All experiments were conducted in triplicate.

One-step growth curve assay

To determine the latent period and burst size, a one-step growth experiment was conducted as described by Luo et al. (2021). A mixture of 100 µL phage suspension (10^6 PFU/mL) and 100 µL *S. aureus* ATCC 29213 (10^8 CFU/mL) at a MOI of 0.01 was allowed to adsorb for 10 minutes at 37 °C. The mixture was then centrifuged at 10,000 rpm for 30 seconds at room temperature. The pellet was resuspended in 5 mL LB broth and incubated at 37 °C with shaking. Aliquots of 100 µL were collected at 10-min intervals post-infection for a total of 90 min. All experiments were conducted in triplicate. Phage titers were determined using the plaque assay, and the burst size was calculated as (final titer - initial titer)/initial infected cell count.

Thermal stability

Phage thermal stability was evaluated by incubating phage suspensions (10^8 PFU/mL) in LB broth at different temperatures (4, 20, 30, 37, 40, 50, 60, and 70 °C) for 1 hour. Aliquots were taken every 10 minutes, and phage titers were determined using the double-layer agar method as described by Luo et al. (2021).

pH stability

To assess pH stability, phage suspensions (10^8 PFU/mL) were added to LB broth adjusted to pH values ranging from 2.0 to 12.0 using 0.1 M HCl or 0.1 M NaOH. Samples were incubated at 37 °C for 1 hour. Phage titers were then determined by the double-layer plaque assay as described by Kim et al. (2018). All experiments were conducted in triplicate.

Salt tolerance

The effect of salt concentration on phage stability was tested by incubating *ΨSa118* (10^8 PFU/mL) in NaCl solutions at concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M. Samples were incubated at room temperature for 1 hour, and phage viability was assessed using the plaque assay according to Li et al. (2020). All experiments were conducted in triplicate.

UV resistance

UV resistance was evaluated following the method of Sui et al. (2021), with modifications. Phage suspensions (10^8 PFU/mL) were exposed to UV light (254 nm) under sterile conditions at a distance of 0.5 m from the UV source. Aliquots were taken every 10 minutes

for 1 hour, and phage titers were assessed using the plaque assay. All experiments were conducted in triplicate.

Statistical analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation (SD). Statistical differences between groups were analyzed using one-way ANOVA followed by post hoc tests, with significance accepted at $p \leq 0.05$. SPSS version 20 (IBM Corp., Armonk, NY, USA) was used for all analyses.

RESULTS

Host range determination of the phages

The host range of the 25 bacteriophage isolates was evaluated using spot assays against nine tested *S. aureus* strains. All phage candidates exhibited lytic activity against at least one of the tested *S. aureus* strains. Notably, phage ΨSa118 demonstrated the broadest host range, lysing all 9 tested *S. aureus* strains, including both reference and clinical isolates. In contrast, no lytic activity was observed against non-target bacteria such as *E. coli* ATCC 25922, *S. typhimurium* ATCC 14028, and *B. cereus* ATCC 10876, confirming high species-level specificity. The lysis zones produced by ΨSa118 were characterized by clear, sharply defined plaques, indicating efficient and uniform bacterial lysis (Fig. 1 & Table 1). These findings suggest that ΨSa118 possesses both broad intraspecies lytic capability and strict host specificity, making it a strong candidate for targeted biocontrol applications against *S. aureus*, and it was selected for further study.

Morphological characterization of phage ΨSa118

The plaque morphology of ΨSa118 on double-layer agar revealed clear, homogeneous lytic zones with an average diameter of 1.0 ± 0.1 mm (Fig. 2A). Transmission electron microscopy (TEM) showed that the phage exhibits an icosahedral head with an average diameter of 49.19 ± 2.0 nm and a short, non-contractile tail approximately 9.2 ± 2.3 nm in length (Fig. 2B). These structural features are characteristic of the Podoviridae family, whose members typically possess short tails and double-stranded DNA genomes.

Characterization of the phage infection

To determine the optimal multiplicity of infection (MOI), phage ΨSa118 was tested at various MOIs ranging from 0.001 to 100. The phage reached its highest titer (3.50×10^9 PFU/mL) at MOI = 0.01 after 10 hours of incubation at 37 ^{o}C, indicating that this MOI provides the most efficient phage amplification (Fig. 3A).

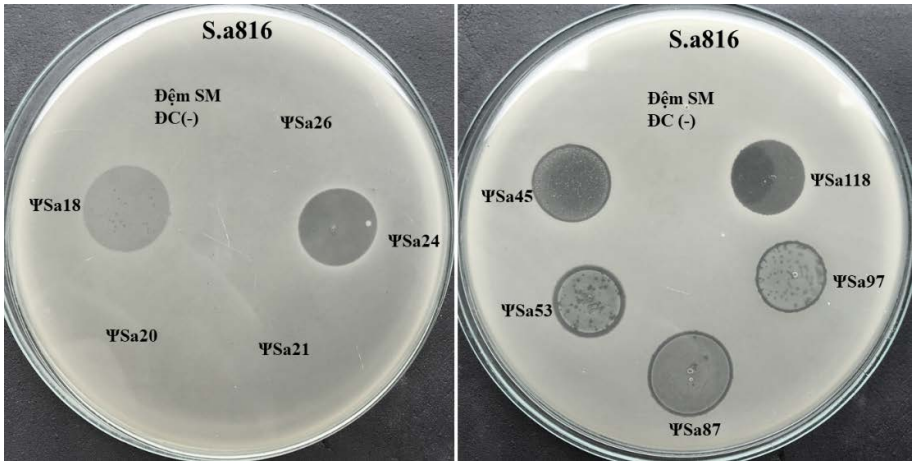

Figure 1. Lytic activity of several tested bacteriophages using *Staphylococcus aureus* strain 816 as the host bacterium, determined by the double-layer agar method. SM buffer was used as the negative control

Table 1. Results of host range determination of the bacteriophages

Phage	*Staphylococcus aureus*									*E.coli* ATCC 25922	*S.a* ATCC 14028	*B.c* ATCC 10876
	S.a771	*S.a816*	*S.a2860*	*S.a3016*	*S.a3027*	*S.a3625*	*S.a* ATCC 25923	S.a VTCC 70187	S.a VTCC 70188			
ΨSa14	-	+	-	-	-	+	-	-	-	-	-	-
ΨSa15	-	+	+	-	-	-	-	+	-	-	-	-
ΨSa17	-	+	-	-	+	-	-	-	+	-	-	-
ΨSa18	+	+	-	+	-	-	-	+	-	-	-	-
ΨSa20	-	-	-	-	+	-	+	-	-	-	-	-
ΨSa21	-	-	+	-	-	+	-	-	+	-	-	-
ΨSa24	-	+	-	+	-	-	-	+	-	-	-	-
ΨSa26	+	-	-	-	-	-	-	-	+	-	-	-
ΨSa28	-	+	-	-	-	+	-	-	-	-	-	-
ΨSa31	-	-	-	-	-	-	+	-	-	-	-	-
ΨSa45	+	+	-	-	-	-	-	-	+	-	-	-
ΨSa53	-	+	-	+	-	-	+	-	-	-	-	-
ΨSa87	+	+	+	+	-	+	+	+	+	-	-	-
ΨSa97	+	+	-	+	+	-	-	-	+	-	-	-
ΨSa118	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**-**	**-**
ΨSa134	+	+	+	-	+	+	-	+	+	-	-	-
ΨSa145	-	-	+	-	-	-	-	+	-	-	-	-
ΨSa201	-	+	-	-	+	-	-	-	-	-	-	-
ΨSa215	+	-	-	-	-	+	-	+	-	-	-	-
ΨSa255	-	-	-	-	-	+	+	-	+	-	-	-
ΨSa301	-	+	-	+	-	-	-	+	-	-	-	-
ΨSa331	+	-	+	-	-	-	-	-	-	-	-	-
ΨSa442	-	+	-	-	-	+	-	+	-	-	-	-
ΨSa455	-	-	+	-	+	-	-	-	-	-	-	-
ΨSa467	+	-	-	-	-	-	+	-	-	-	-	-

Note: (+) indicates clear plaques; (–) indicates no plaques.

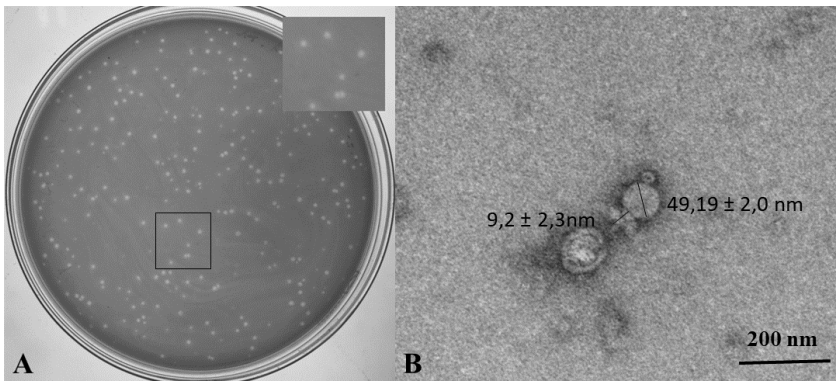

Figure 2. (A) Plaque of bacteriophage ΨSa118 on double-layer agar. A magnified image of the plaque is shown in the inset box; (B) TEM image of bacteriophage ΨSa118

The one-step growth curve, conducted at the optimal MOI, showed a latent period of approximately 20 minutes, followed by a rise period during which phage titers rapidly increased, peaking between 50 and 60 minutes post-infection. The calculated burst size was 73 PFU per infected cell (Fig. 3B), confirming robust replication and lytic capacity.

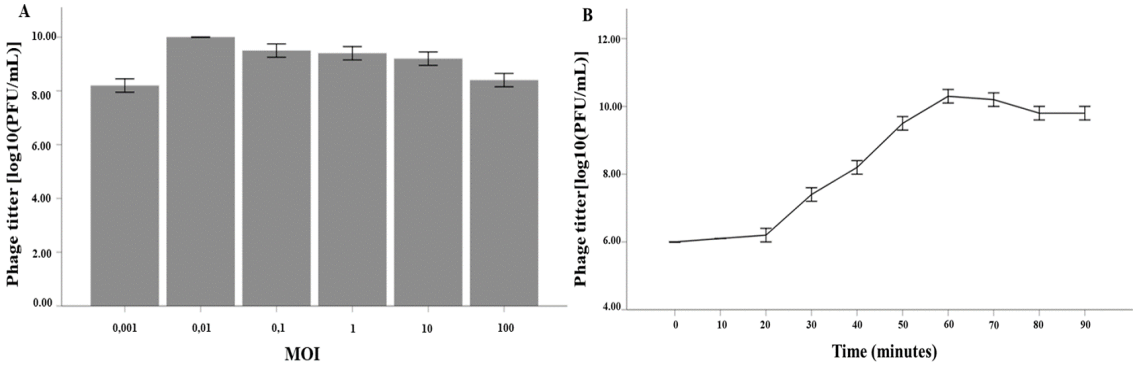

Figure 3. (A) Variation in the concentration of bacteriophage ΨSa118 at different MOIs; (B) One-step growth curve of bacteriophage ΨSa118 at an MOI of 0.01

Stability of ΨSa118 under varying pH and temperature conditions

The stability of ΨSa118 was assessed across a broad range of pH (2–12) and temperature conditions (4–70 ^{o}C). The phage maintained high viability between pH 6.0 and 10.0, with titers ranging from 7.96 to 8.00 $\log_{10}$ PFU/mL. Slight reductions in titer were observed at pH 4, 5, and 11, while significant reductions occurred at pH 2, 3, and 12 (1.55–1.72 $\log_{10}$ PFU/mL), indicating susceptibility to extreme acidic or alkaline environments (Fig. 4A).

Thermal stability assays revealed that ΨSa118 remained stable from 4 to 40 ^{o}C, with titers maintained between 7.86 and 8.00 $\log_{10}$ PFU/mL. A statistically significant decline in phage viability was observed at temperatures above 50 ^{o}C, with reductions of 2.56, 4.65, and 5.57 $\log_{10}$ PFU/mL at 50, 60, and 70 ^{o}C, respectively (Fig. 4B). These results demonstrate the phage's strong resilience to temperature fluctuations typically encountered in food production environments.

Effect of salt concentration and UV exposure on ΨSa118 stability

To assess salt tolerance, ΨSa118 was incubated in NaCl solutions ranging from 0.5 M to 10 M. Phage viability was stable at

concentrations up to 4 M, with no statistically significant differences in titer. However, a moderate yet significant reduction was observed at 5 M and 10 M NaCl, with decreases of 0.25 and 0.73 $\log_{10}$ PFU/mL, respectively (Fig. 5A). These findings highlight the phage's suitability for application in high-salt environments such as fermented or processed food products.

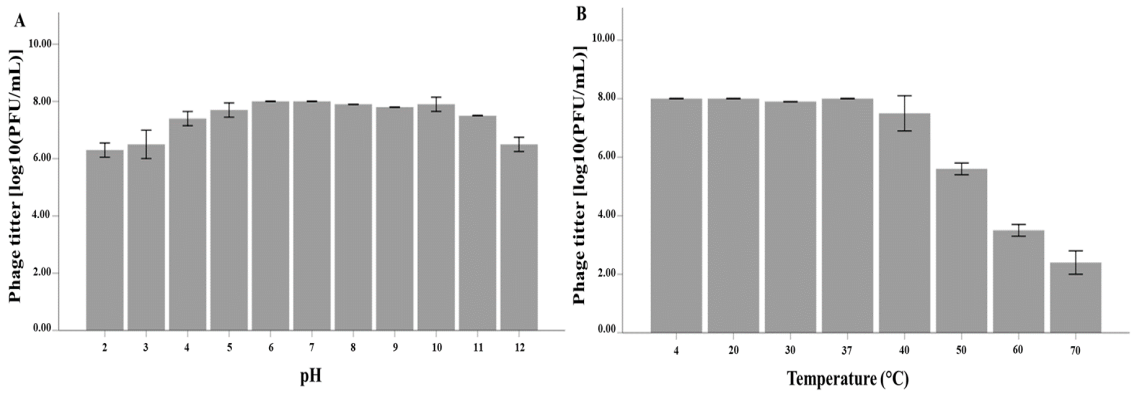

Figure 4. (A) Stability of bacteriophage ΨSa118 under different pH conditions (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12) after 1 hour of incubation; (B) Stability of bacteriophage ΨSa118 at different temperatures (4, 20, 30, 37, 40, 50, 60, and 70 ^{o}C) after 1 hour of incubation

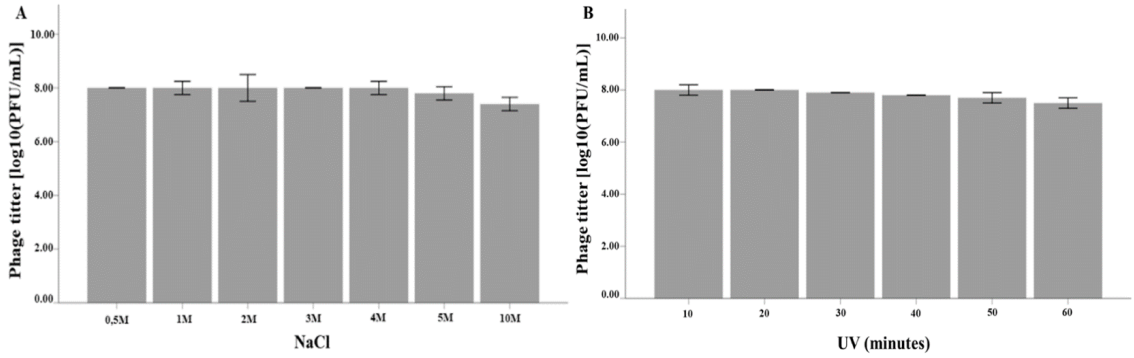

Figure 5. (A) Stability of bacteriophage ΨSa118 at different salt concentrations; (B) Stability test of bacteriophage ΨSa118 under UV exposure at different time points

Furthermore, ΨSa118 exhibited excellent resistance to ultraviolet (UV) radiation. Following 60 minutes of exposure to 254 nm UV light, no significant decrease in phage titer was observed (Fig. 5B). This property enhances the applicability of ΨSa118 in sanitation practices where UV treatment is commonly used, such as surface disinfection in food processing facilities.

DISCUSSION

The emergence of antibiotic-resistant *S. aureus* strains in food products presents a significant challenge to food safety and public health. Among these, methicillin-resistant *S. aureus* (MRSA), commonly referred to as a "superbug," is of particular concern due to its resistance to multiple antibiotics. Bacteriophages, viruses that specifically infect and lyse bacteria, have re-emerged as promising natural antimicrobial agents with high host specificity, low toxicity, and environmental safety, making them ideal candidates for biocontrol in food systems.

In this study, we screened 25 phage isolates and identified ΨSa118 as the most promising

candidate based on its broad host range and species-specific activity. Phage ΨSa118 was capable of lysing all 9 tested *S. aureus* strains while exhibiting no lytic activity against non-*Staphylococcus* species, such as *E. coli*, *S. typhimurium*, and *B. cereus*. This high level of specificity minimises the risk of disturbing beneficial microbiota, a key consideration for biocontrol applications. Similar broad-spectrum activity against *S. aureus* has been reported for other phages such as PSA2 and SapYZU11, which were able to lyse 6 and 54 *S. aureus* strains, respectively (Son & Minh, 2024; Hua et al., 2023).

Transmission electron microscopy (TEM) revealed that ΨSa118 has an icosahedral head and short, non-contractile tail, classifying it within the Podoviridae family, in accordance with the International Committee on Taxonomy of Viruses (ICTV). This morphology is frequently associated with high infectivity and stability. Previous studies have suggested that short-tailed Podoviridae phages may exhibit greater resistance to environmental stressors and antibacterial agents, further supporting their utility in food safety applications (Vandamme, 2019).

The optimal multiplicity of infection (MOI) for ΨSa118 was determined to be 0.01, consistent with previously reported values for other anti - *S. aureus* phages such as MikSA913 (MOI = 0.001) and SapYZU series phages (MOI = 0.01) (Hua et al., 2023; Çotak, 2019). At this MOI, ΨSa118 achieved a high viral titer (3.50×10^9 PFU/mL) and demonstrated effective replication dynamics, with a latent period of 20 minutes and a burst size of 73 PFU/infected cell. While this burst size is moderate compared to other phages like ME126 (140 PFU/cell) (Gharieb et al., 2020), it is sufficient to support sustained replication without overwhelming or depleting the bacterial host population too rapidly, thereby potentially reducing the risk of phage resistance development.

Environmental stability is a critical feature for phages intended for real-world applications. ΨSa118 remained stable across a wide pH range (2–12) and temperature range (4–50 ^oC). Although extreme pH (2, 3, 12) and high temperature (≥ 60 ^oC) significantly reduced phage viability, ΨSa118 demonstrated superior stability under typical food processing and storage conditions. These results compare favorably to previously reported phages such as JD419, whose pH and thermal tolerance were more limited (Feng et al., 2021).

Moreover, ΨSa118 maintained its activity under high salinity conditions, tolerating NaCl concentrations up to 10 M, which is relevant for salted or fermented food products. Notably, the phage also exhibited high UV resistance, showing no significant reduction in titer following UV-C (254 nm) exposure over 60 minutes. This attribute is particularly advantageous for applications in food processing environments where UV irradiation is routinely used for surface decontamination.

Collectively, these findings position phage ΨSa118 as a robust, versatile candidate for biocontrol of *S. aureus* in the food industry. Its biological properties, species-specific lytic activity, efficient replication, and environmental resilience support its potential as a safe and effective alternative to antibiotics in food safety applications. Further *in vivo* studies and formulation development are warranted to validate its performance under industrial conditions and assess its regulatory feasibility.

CONCLUSION

Phage ΨSa118 exhibits strong lytic activity, high environmental stability, and strict species specificity against *S. aureus*. These attributes highlight its potential as a biocontrol agent in food safety applications. Future work will focus on formulation development and field evaluations.

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