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RESEARCH ARTICLE

Staphylococcus aureus lytic bacteriophage: isolation and application evaluation

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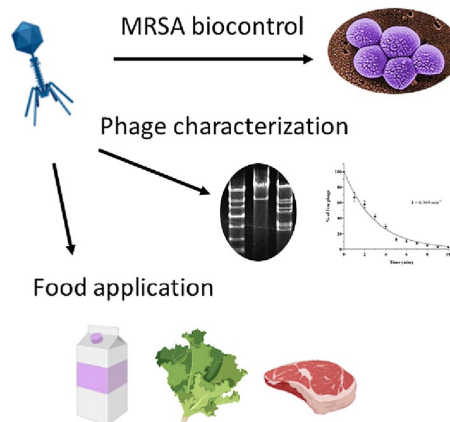
Abstract

Staphylococcus aureus is a significant pathogen associated with various illnesses, including food poisoning. The development of effective treatments is challenging, necessitating the exploration of novel antimicrobial options. Bacteriophages (phages) have emerged as promising candidates in this regard. In this study, a virulent phage called mSA4 was isolated and characterized. Furthermore, its efficacy in combating *S. aureus* biofilms and growth in various food products was evaluated. Phage mSA4 demonstrated a broad host range, targeting both *S. aureus* and methicillin-resistant *S. aureus* strains. Belonging to the *Myoviridae* family, it exhibited rapid adsorption (over 50% in 3 min), a short latent period (20 min), and a burst size of 97 phage particles per infected bacterial cell. Furthermore, phage mSA4 displayed stability across a wide range of pH values and temperatures, and effectively degraded established biofilms. Its performance was evaluated in chocolate milk, beef meat, and iceberg lettuce, resulting in significant reductions in bacterial counts (2.1 log CFU/mL, 2.8 log CFU/cm^2, and 3.2 log CFU/cm^2, respectively). These findings underscore the potential of phage mSA4 as a natural biocontrol agent against *S. aureus*.

Graphical abstract



Keywords *Staphylococcus aureus* · Biocontrol agents · Food poisoning · Phage · *Myoviridae* · Biofilm · Food safety

1 Introduction

Staphylococcal food poisoning is usually associated with the growth of *Staphylococcus aureus*, which produces heat-stable enterotoxins in protein rich foods like dairy products and meat (Mekhloufi et al. 2021). Symptoms such as abdominal pain, diarrhea, nausea and vomiting appear rapidly within a few hours after ingestion (Mekhloufi et al. 2021). Moreover, *S. aureus* can form biofilms, an organized multicellular community of bacteria, enclosed in extracellular polymeric substances, with the ability to withstand antibiotics and disinfectants (Duarte et al. 2021; Song et al. 2021). The emergence of methicillin-resistant

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S. aureus (MRSA), which is resistant to all available β-lactam antimicrobial drugs, makes the treatment of infections more challenging (Gharieb et al. 2020). Importantly, MRSA and vancomycin-resistant *S. aureus* (VRSA) were detected in ready-to-eat (RTE) meat, such as shawarma and burger (Saber et al. 2022). Hence, development of antibiotic alternatives, especially the use of bacterial viruses, so-called bacteriophages (phages), will be needed.

Recently, phage biocontrol has gained acceptance as green method in food safety since it selectively targets pathogenic bacteria without altering the food's natural microbiota (Moye et al. 2018). The use of virulent (lytic) phages is essential to prevent horizontal gene transfer and the possible spread of virulence factors among bacterial species (Garvey 2022). Also, phages can be used to disrupt bacterial biofilms. Several studies have shown the potential of phages to remove *S. aureus* biofilm (Alves et al. 2014; Song et al. 2021), and to control *S. aureus* in food (Chang et al. 2019; Gharieb et al. 2020).

Some phage products such as EcoShield™, Listex™ P100, ShigaShield™ and SalmoFresh™ have been approved by the US Food and Drug Administration (FDA), and are used to control *Escherichia coli*, *Listeria monocytogenes*, *Shigella sonnei* and *Salmonella* Typhimurium in different food matrices, respectively (Lewis and Hill 2020). However, the number of lytic phages against *S. aureus* still insufficient. Therefore, the purpose of this study is to isolate a wild type lytic phage targeting *S. aureus*. The eradication of biofilm and biocontrol against food spiked with *S. aureus* will also be evaluated.

2 Material and methods

2.1 Bacterial cultures and phage isolation

Bacterial strains used in this study are listed in Table 1. *S. aureus* and other American Type Culture Collection (ATCC) strains were grown in tryptic soy broth (TSB; Merck, Darmstadt, Germany) medium with shaking at 180 rpm overnight. Mueller Hinton broth (MH; Merck, Darmstadt, Germany) medium was used to culture *Campylobacter jejuni* ATCC 33291. TSB supplemented with 0.25% (v/v) glucose (TSBg) was used for biofilm assays.

Phage mSA4 was isolated from water samples collected from retail seafood markets, using *S. aureus* ATCC 43300 as bacterial host strain. Briefly, 22.5 mL of water samples were mixed with 2.5 mL of 2X fresh TSB and 1% of the exponential-growth phase host strain. The mixture was centrifuged at 10,000×*g* for 10 min after overnight incubation at 37 °C and the collected supernatant was then filtered with a 0.22-µm filter (Sartorius, Göttingen, Germany). The presence of lytic phages in the filtrate was determined by a spot test (Tan et al. 2021). Phage propagation and purification were performed as previously described by Chang et al. (2019). The agar overlay method [tryptic soy agar (TSA; Merck, Darmstadt, Germany) and 0.6% (w/v) TSB soft top agar] was used to determine the phage titer (PFU/mL).

Table 1 Host range of phage mSA4

Bacterial strain	Infectivity	Source
Staphylococcus strains:		
S. aureus ATCC 43300	+	ATCC
S. aureus ATCC 33591	+	ATCC
MRSA1	±	Othman et al. (2018)
MRSA2	+	Othman et al. (2018)
MRSA3	+	Othman et al. (2018)
MRSA4	+	Othman et al. (2018)
MRSA5	+	Othman et al. (2018)
MRSA6	±	Othman et al. (2018)
MRSA7	+	Othman et al. (2018)
S. aureus ATCC 29213	+	ATCC
S. aureus ATCC 25923	+	ATCC
S. aureus ATCC 6538	+	ATCC
S. epidermidis ATCC 12228	–	ATCC
S. equorum ATCC 43958	–	ATCC
S. xylosus ATCC 29971	–	ATCC
Other Gram-positive bacteria:		
Enterococcus durans ATCC 6056	–	ATCC
Enterococcus faecalis ATCC 29212	–	ATCC
Bacillus cereus ATCC 13061	–	ATCC
Listeria monocytogenes ATCC 19115	–	ATCC
Gram-negative bacteria:		
Salmonella Enteritidis ATCC 13076	–	ATCC
Escherichia coli ATCC 25922	–	ATCC
Klebsiella pneumoniae ATCC 13883	–	ATCC
Campylobacter jejuni ATCC 33291	–	ATCC
Vibrio parahaemolyticus ATCC 17802	–	ATCC

+ clear plaque, ± turbid plaque, – no plaque

2.2 Host range analysis and electron microscopy

Phage host range was determined using spot test assays. Initially, a bacterial lawn of each indicator strain listed in Table 1 was prepared on an agar plate. Then, 10 µL of the purified phage lysate were spotted on the plate and dried at

room temperature for 15 min prior to overnight incubation at 37 °C. After incubation, plaque formation was observed and categorized as clear plaque (+), turbid plaque (±) or no plaque (–). Phage morphology was observed using transmission electron microscope (TEM, Philips HMG 400, Eindhoven, The Netherlands) after staining was carried out as previously described (Thung et al. 2019).

2.3 Phage DNA extraction and restriction enzyme analysis

The extraction of phage genomic DNA was conducted according to the method of Sambrook and Russell (2001). NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used to determine the quality and concentration of purified phage DNA. For restriction enzyme analysis, 10 µg of genomic DNA was digested with 3 different restriction enzymes (*Eco*RI, *Sal*I and *Bam*HI; New England Biolabs, Ipswich, USA) according to the conditions recommended by the manufacturer. The digested DNA was subjected to electrophoresis and visualized with Gel Documentation System (SynGene, Frederick, USA).

2.4 One-step growth and adsorption assay

Phage one-step growth was performed as previously described (Kornienko et al. 2020) with some modifications. Briefly, a multiplicity of infection (MOI) of 0.001 was prepared by adding phage suspension (10^5 PFU/mL) to the exponential-growth phase of *S. aureus* ATCC 43300 (10^8 CFU/mL), followed by incubation at room temperature for 10 min. The mixture was then centrifuged at $8{,}000 \times g$ for 5 min. Thereafter, the pellet was resuspended in fresh TSB and incubated at 37 °C with shaking. Samples (100 µL) were taken every 5 min over a period of 1 h and subjected to phage titration by agar overlay method. For adsorption assays, a phage inoculum with MOI of 0.01 was used for infection. The percentage of free phage was determined according to the rate equation as described (Alves et al. 2014). 3 independent experiments were conducted.

2.5 Stability analysis

Phage stability was evaluated under different pH conditions (ranging from pH 3 to 12, at 37 °C) and at various temperatures (4 °C, 25 °C, 37 °C, and 65 °C) as previously described (Shimamori et al. 2021), using *S. aureus* ATCC 43300 as bacterial host strain. An agar overlay assay was performed after 1 h incubation of phage suspension (10^8 PFU/mL).

2.6 Biofilm formation and phage treatment on 96-well microplate

The host strain *S. aureus* ATCC 25923 was used for biofilm formation as described previously with some modifications (Gharieb et al. 2020). An overnight culture was diluted 1:100 (v/v) (2 µL of bacterial suspension to 198 µL of TSBg) in each well of a 96-well polystyrene microtiter plate and incubated at 37 °C for 24 h. A set of wells with broth-only served as negative control. All wells were then washed twice with phosphate-buffered saline (PBS) prior to phage treatment (200 µL of 10^9–10^{11} PFU/mL) for 24 h at 37 °C. After incubation, all wells were washed twice with PBS and stained with 0.1% crystal violet for 15 min. After washing with PBS, 33% (v/v) acetic acid was added to each well and the absorbance was measured at 570 nm using a Microplate Reader (Tecan, Männedorf, Switzerland).

2.7 Confocal laser scanning microscopy

The effect of phage on *S. aureus* ATCC 25923 biofilms was also observed using confocal laser scanning microscopy (CLSM, SP8 Leica Microsystems, Wetzlar, Germany) as described previously (Duarte et al. 2021). *S. aureus* biofilms were grown in 2-well µ-slides with glass bottom (ibidi, Martinsried, Germany). After growth, biofilms were rinsed with PBS, followed by phage treatment with 10^{11} PFU/mL. Then, wells were washed twice with PBS and stained with Live/Dead BacLight kit (Invitrogen, Carlsbad, USA) prior to microscopic examination.

2.8 Phage treatment in retail food

Food samples of pasteurized chocolate milk, beef meat and iceberg lettuce were purchased from local market and kept at 4 °C before use. The host strain *S. aureus* ATCC 43300 (10^5 CFU/mL) was used to artificially contaminate the food samples, and different MOI values (100 and 1000) (10^7 and 10^8 PFU/mL) of phage suspensions were prepared for treatment.

Chocolate milk samples were inoculated with bacterial cultures and pre-incubated at 4 °C for 1 h prior to the phage treatment (Chang et al. 2019). For beef meat, samples were aseptically cut into pieces (approximately 10 cm^2) followed by UV-irradiation for 30 min, and selected samples (3 pieces) were tested for natural *Staphylococcus* contamination by plate counting on Baird-Parker agar (BPA; Oxoid, Hamphire, UK). The bacterial suspension was spotted onto the surface of meat samples followed by pre-incubation at 4 °C for 1 h. Afterwards, meat samples were inoculated with phage suspension and incubated at 4 °C. For iceberg lettuce, samples (inner leaves) were initially sliced in pieces (approximately 10 cm^2), rinsed with

sterile water, and UV-sanitized for 30 min. The bacterial suspension was then added onto the samples and allowed to dry in the biosafety cabinet for 1 h. Subsequently, the phage suspension was pipetted onto the surface of the samples and incubated at 4 °C. All treated samples (beef meat and iceberg lettuce) were subjected to homogenization and centrifugation prior to enumeration. The viable cells were enumerated at different time points (0, 2, 4, 6 and 24 h) by plating on BPA, and a phage-free control was prepared by using SM buffer (8 mM $MgSO_4 \cdot 7H_2O$, 100 mM NaCl, 50 mM Tris–HCl and 0.01% gelatine, pH 7.5). 3 independent experiments were conducted. One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests were used to analyze the *S. aureus* counts for each time point.

3 Results

Bacteriophage mSA4 was isolated from water samples collected from retail seafood markets located in Serdang, Selangor. Phage mSA4 was able to form plaques and propagate in liquid culture at 37 °C. Morphological analysis of mSA4 revealed a phage with an approximately 190 nm contractile tail and a 90 nm icosahedral head, which can be clearly placed in the order *Caudovirales*, family *Myoviridae* (Fig. 1a). As shown in Table 1, phage mSA4 was able to infect *S. aureus* strains, forming clear or turbid plaques. No infectivity was observed for other Gram-positive and Gram-negative bacteria, suggesting that it has a high host specificity to *S. aureus*.

Genomic DNA extracted from phage mSA4 was successfully digested by *Eco*RI, *Sal*I and *Bam*HI (Fig. 1b). A one-step growth curve and the adsorption assay were further performed to characterize the infection cycle of phage mSA4. Phage mSA4 showed a short latent period of around 20 min and the burst size was approximately 97 phage particles per infected bacterial cell (Fig. 1c). For the adsorption efficiency, more than 50% of the phage particles were adsorbed to the bacteria within 3 min (Fig. 1d). Additionally, phage mSA4 was stable between pH 4 and 11 (Fig. 1e). The viability of phage mSA4 decreased significantly after heating at 65 °C for 1 h (Fig. 1f).

To determine the efficacy of phage mSA4 in reducing *S. aureus* biofilms, we treated the established 24 h biofilms with different phage titers ranging from 10^9 to 10^{11} PFU/mL. Treatment with 10^{11} PFU/mL significantly reduced the biofilm biomass compared to the untreated control (Fig. 2). An approximately 50% of the reduction in biomass was observed when applying 10^9 PFU/mL of phage mSA4, demonstrating that this titer was less effective for biofilm removal. In addition, CLSM images revealed that phage mSA4 (10^{11} PFU/mL) reduced the viability of the residual cells (Fig. 3), suggesting that it might be a potential biofilm agent.

The effectiveness of phage mSA4 against *S. aureus* contamination on food products was evaluated at an MOI of 100 and 1000. Reductions of viable cells were observed in all food samples after just 2 h of phage treatment ($p < 0.05$) (Fig. 4). For chocolate milk samples (Fig. 4a), after 24 h of treatment an approximately 1.9 log CFU/mL and 2.1 log CFU/mL reduction of bacteria was achieved when applying a MOI of 100 and 1000, respectively. Meanwhile, treatment with a MOI of 1000 for 24 h in beef meat and iceberg lettuce samples led to decreases of viable cell counts by 2.8 log CFU/cm^2 and 3.2 log CFU/cm^2, respectively (Fig. 4b, c).

4 Discussion

A lytic *S. aureus*-specific phage described in this work was isolated from water samples. Previously, *S. aureus* phages have been isolated from different sources such as sewage (Alves et al. 2014), wastewater (Gharieb et al. 2020), goat feces (Chang et al. 2019), and environmental swabs from swine production (Korn et al. 2021). Accordingly, *Myoviridae* phages, which bind either the wall teichoic acid (WTA) ribitol-phosphate backbone or *β*-O-*N*-acetylglucosamine (GlcNAc) decorated WTA, have previously been reviewed as virulent among the isolated *S. aureus* phages (Moller et al. 2021). Phage mSA4 presented a narrow host range against *S. aureus* strains. In accordance with this finding, phage vB_SauM_SDQ (Song et al. 2021) and phage SaGU1 (Shimamori et al. 2021) have been shown as narrow spectrum disinfectant against *S. aureus*. On the contrary, *S. aureus* phages with broad host range have been reported. For example, a lytic *S. aureus*-specific phage, vB_SauM-515A1, was able to lyse *S. epidermidis* (Kornienko et al. 2020). A Twort-like phage, vB_SauM-fRuSau02, showed different rates of infection on several coagulase-negative *Staphylococcus* isolates, including *S. epidermidis*, *S. haemolyticus*, *S. intermedius*, *S. lugdunensis*, *S. pseudointer*, and *S. saprophyticus* (Leskinen et al. 2017).

In this study, phage mSA4 encapsulates a double stranded DNA which was susceptible to restriction endonuclease digestion. A previous study showed that phages with DNA modifications might only be digested with certain enzymes such as *Taq*I and *Msp*I (Korn et al. 2021). On the other hand, phages with short latent periods and high burst sizes would be preferred for usage as antimicrobial agent. The life cycle of phage mSA4 was comparable to those of other *S. aureus* phages like DRA88 (latent periods of 25 min; burst size of 76 PFU/cell) (Alves et al. 2014) and vB_SauM_ME18 (latent periods of 15 min; burst size of 114 PFU/cell) (Gharieb et al. 2020). Moreover, the high stability of phage mSA4 suggests that it could be used as a biocontrol agent either during pre- or post-harvest processing and storage of food products.

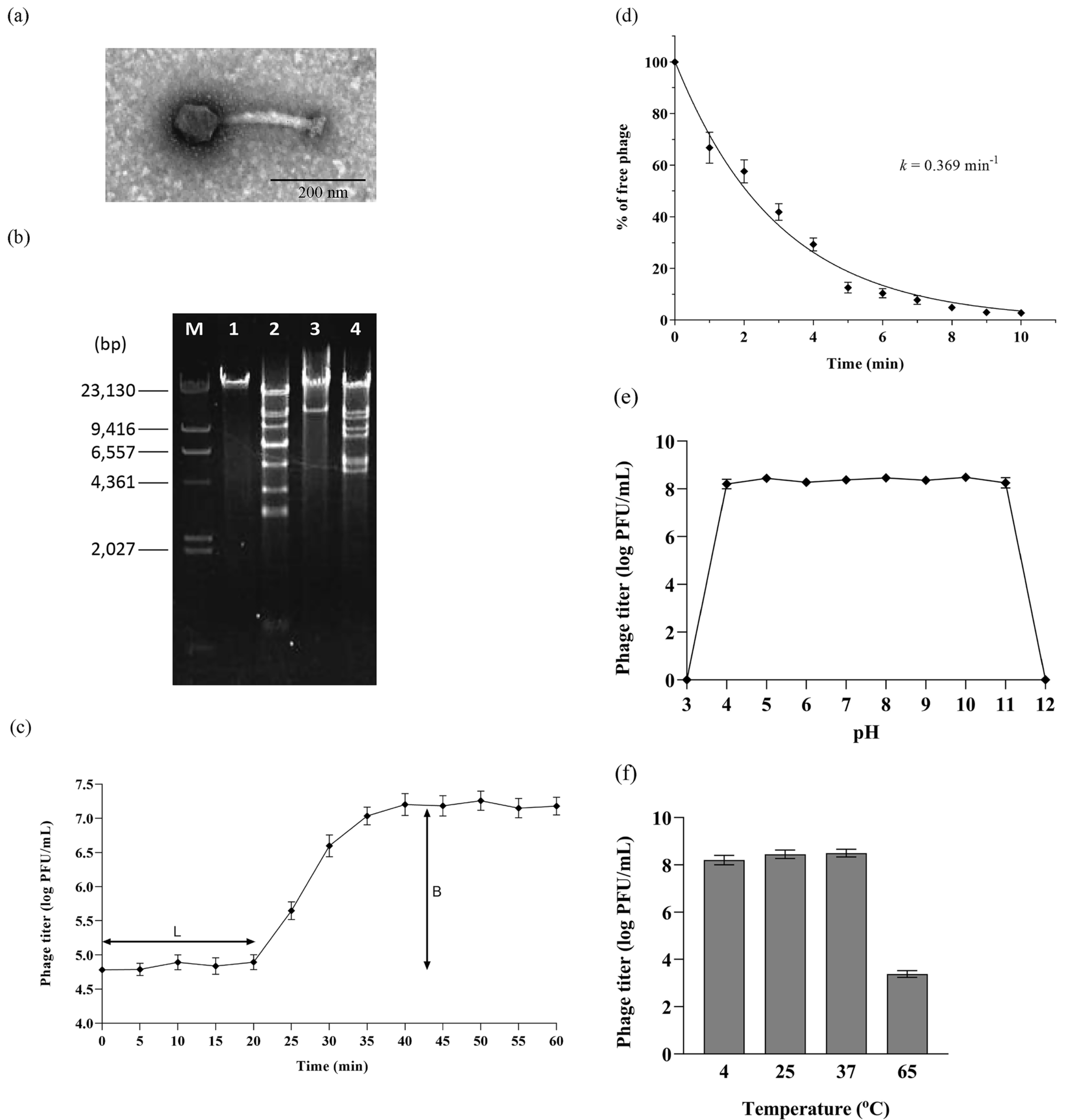

Fig. 1 Characteristics of phage mSA4. (**a**) Electron micrograph of *Myoviridae* phage mSA4. The bar represents 200 nm. **b** Restriction digestion patterns of mSA4. Lane M: Lamda DNA-*Hind*III marker (New England Biolabs, #N3012); Lane 1: undigested mSA4; Lane 2: mSA4 digested with *Eco*RI; Lane 3: mSA4 digested with *Sal*I, and Lane 4: mSA4 digested with *Bam*HI. **c** One-step growth curve of mSA4 in TSB broth at 37 °C. L, latent period; B, burst size. **d** Adsorption assays of mSA4 on *S. aureus* ATCC 43300. **e** Stability of mSA4 under different pH values. **f** Stability of mSA4 at various temperatures. 3 independent experiments were combined and data are presented as means with standard deviation

It is well known that biofilm-forming *S. aureus* strains are more resistant to antibiotics. Hence, an increasing number of studies in recent years have demonstrated that phages can be used for biofilm removal. For instance, Song et al. (2021) demonstrated that a *S. aureus*-specific phage could significantly reduce the biofilms formed on polystyrene microtiter plate, milk, and mammary-gland tissue. Interestingly, a combination of phage and phage-derived

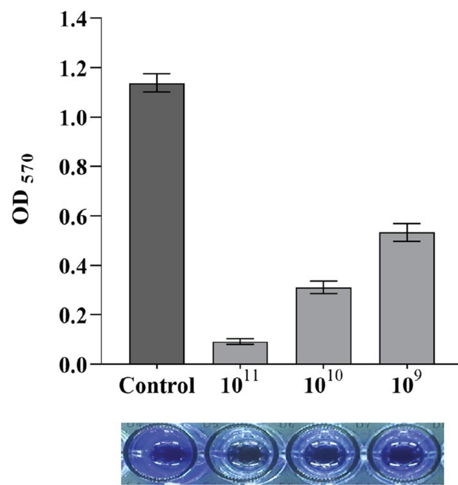

Fig. 2 Effect of phage mSA4 on *S. aureus* ATCC 25923 24 h-old biofilms in 96-well microplates. Phage titers of 10^{11}, 10^{10} and 10^9 were used for 24 h post-infection and the absorbance was measured at 570 nm. Data are the mean ± standard deviation of 3 independent experiments

lytic protein has been described as alternative for biofilm elimination, where the biofilm might be loosened by the lytic protein and making cells more accessible to phage infection (Duarte et al. 2021).

Phage application in food considering phage concentration, properties of the target matrix, and application method such as dipping, spraying, microencapsulation, and forming cocktail of phages have been reported (Lewis and Hill 2020). For phage mSA4, we observed that a MOI of 100 was less effective to inhibit the growth of *S. aureus* in chocolate milk. In contrast, a virulent mutant phage SA13m with MOI of 100 was able to completely lyse the bacterial cells in milk samples after overnight incubation at refrigerator temperature (Chang et al. 2019). Moreover, Gharieb et al. (2020) reported that phages with low MOI of 10 completely eradicated *S. aureus* from milk after 6 h incubation. With that in mind, different isolated phages might have their unique features such as genetic constitution and the presence of multiple copies of receptor binding complexes among phages. Moreover, further findings on phage mSA4 encoded peptidoglycan enzyme (i.e. endolysin) could be used as alternative antimicrobials in the practical application.

5 Conclusion

In summary, the isolated phage mSA4 was morphologically characterized as myovirus with a short latent period and large burst size. The phage can specifically infect *S. aureus* and reduce the formation of biofilm. In addition, it effectively eradicated *S. aureus* in foods, making it a good candidate as biocontrol agent. Further complete genomic analysis and genome structure studies are currently under way to verify the genomic safety.

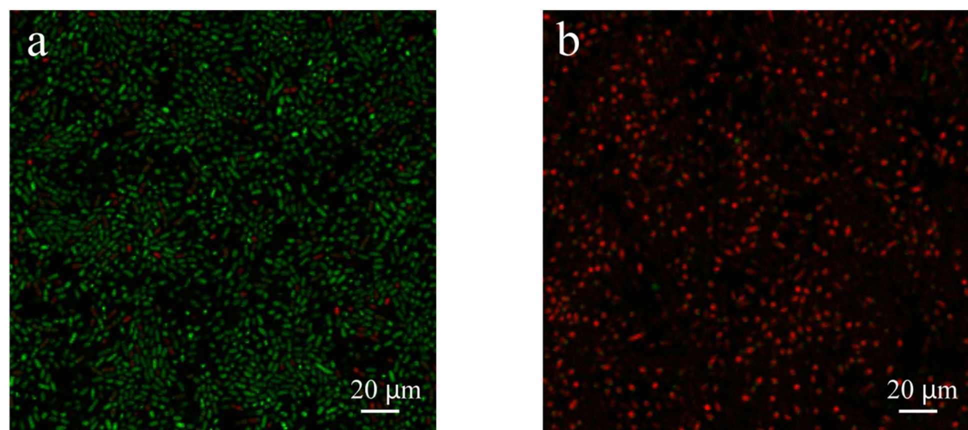

Fig. 3 Confocal laser scanning microscopy (CLSM) of *S. aureus* ATCC 25923 biofilms before and after phage treatment. **a** Control. **b** Lytic action of phage mSA4 at 10^{11} PFU/mL. Cells with green stain are alive, whereas those with red stain are dead. Scale bars represent 20 µm

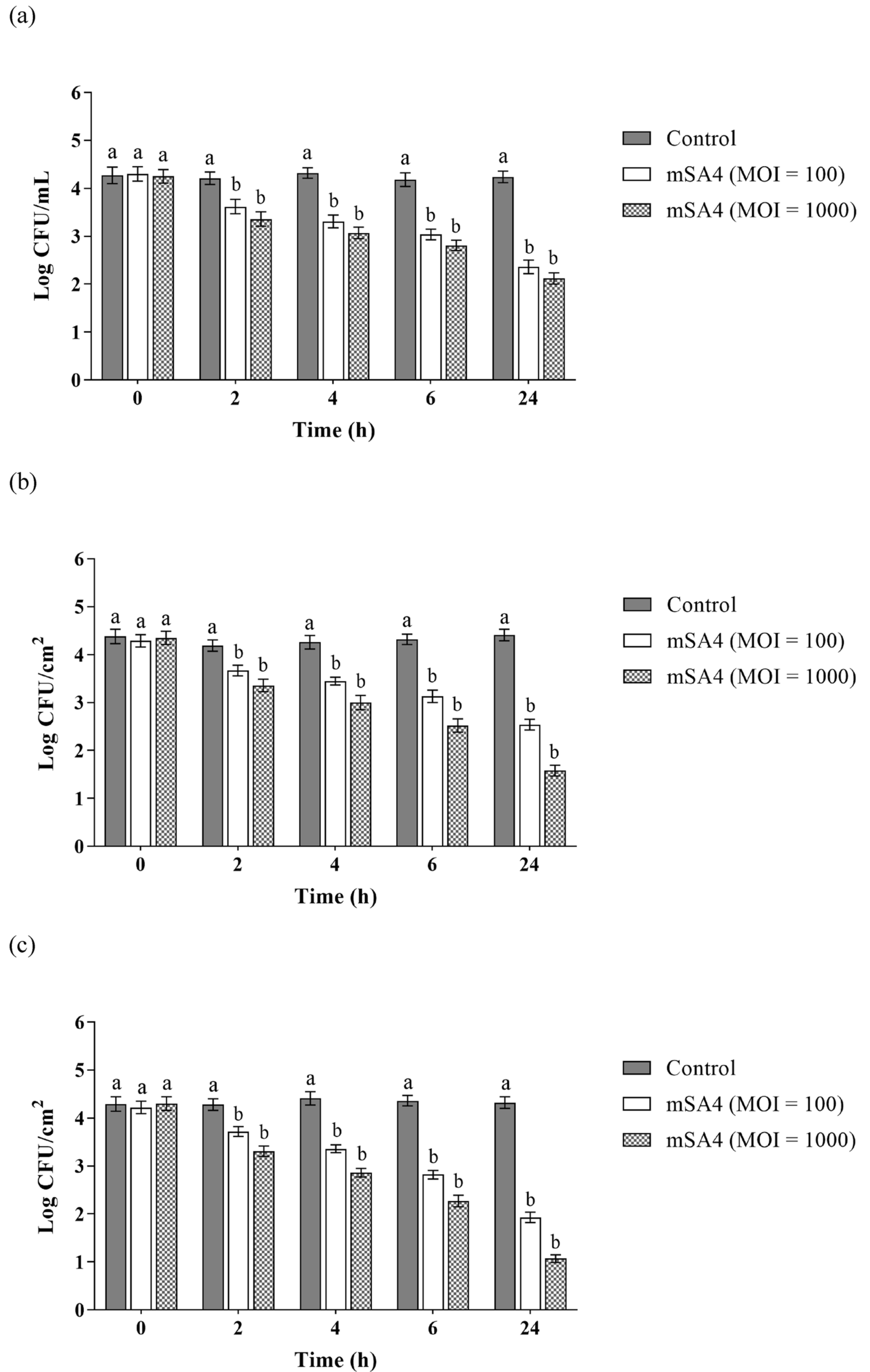

Fig. 4 Effectiveness of phage mSA4 in reducing *S. aureus* ATCC 43300 on **a** chocolate milk, **b** beef meat and **c** iceberg lettuce at 4 °C. All bars shown are the mean of 3 independent experiments and the error bars are the standard deviation. The lowercase letters indicate significant differences ($p < 0.05$) between test groups

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Author contributions FL and DL conducted the biological experiments. NM and EN performed the statistical analysis and wrote the draft. TYT conceptualized the idea and performed the final review of the manuscript.

Declarations

Conflict of interest All authors declare no conflicts of interest.

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