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RESEARCH

Isolation, genomic characterization, and biocontrol potential of a bacteriophage cocktail against *Staphylococcus aureus* in milk

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Abstract

The objectives of this study were to isolate, characterize, and evaluate the biocontrol potential of *Staphylococcus aureus*-targeting bacteriophages in milk. From wastewater and raw milk samples, a total of 19 lytic phages were isolated. Among them SAKAY1298 and SAKAY1306 phages were selected for detailed phenotypic and genomic analysis based on their lytic profiles, host range and growth kinetics. Both phages exhibited icosahedral capsids with short, non-contractile tails under transmission electron microscopy. The phages exhibited moderate acid tolerance by remaining stable at pH 4 and maintained thermal stability at temperatures up to 50 °C. They also maintained high titers over one year at+4 °C and -80 °C in tryptic soy broth and Tris-buffered saline (1×). Genome analyses revealed that both phages possess complete genomes (~17.5 kb), encoding 18 phage-related genes and lacking virulence and resistance determinants. Comparative and phylogenetic analyses confirmed assignment to the genus *Rosenblumvirus*, with 99% query coverage and~92% whole-genome nucleotide identity to *Staphylococcus* phage vB_SauR_SW21 (GenBank accession OR683639.1). Significant reductions in *S. aureus* counts were achieved following phage cocktail treatment, reaching up to 4.31 log CFU/mL in vitro at 37 °C and 1.44 log CFU/mL in UHT milk at 4 °C ($p<0.001$). Additionally, the phage cocktail fully inhibited the production of staphylococcal enterotoxin A in the in vitro assay. Altogether, the data supports the application of SA1298KAY and SA1306KAY phages have strong potential as effective biocontrol agents for reducing *S. aureus* in dairy applications.

Key points

SA1298KAY and SA1306KAY phages showed strong biocontrol against *S. aureus*.
Phage cocktail fully blocked SEA production in vitro assays.
Up to 1.44 log CFU/mL *S. aureus* reduction achieved in milk.

Keywords Biocontrol · Staphylococcal enterotoxin · UHT milk · TEM · Long-term storage · Whole-genome sequencing

Introduction

Staphylococcus aureus is one of the major causes of food poisoning due to its ability to produce heat-stable enterotoxins (Cieza et al. 2024). *S. aureus* is frequently present on the skin and mucous membranes of humans and animals, poses a significant transmission risk through both direct and indirect contact, especially to soft tissues (Raineri et al. 2022). Improper food handling remains a key contributor to foodborne infections, particularly in food industry. Over half of global outbreaks are linked to cross-contamination resulting from inadequate hygiene practices or unsanitary processing equipment (Lee et al. 2017). Its ability to persist in dry environments, such as air or dust, for extended

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periods further amplifies the risk of contamination during food processing and handling (Kozajda et al. 2019). The bacterium is capable of producing enterotoxins when it multiplies to high levels in food, particularly under conditions of inadequate refrigeration or hygiene. These enterotoxins are remarkably heat stable, persisting in foods even after cooking or pasteurization, and are a leading cause of rapid-onset foodborne illnesses worldwide (Henneike et al. 2012). Recent EU surveillance indicates that several member states, including Croatia, Germany, Greece, Italy, and Spain, have detected *S. aureus* in both food and animal samples. Overall, 5.4% of food samples and 10.8% of animal samples tested positive for *Staphylococcus* spp., with the highest prevalence observed in raw milk, processed foods, ready-to-eat products, meat products, and various cheese types (EFSA 2024).

Bacteriophages are viruses that specifically infect and replicate within bacterial cells, representing the most abundant and diverse biological entities on Earth (Hatfull 2015). Phages offer several advantages, including efficacy at low doses, ease of isolation, and low production costs. They can replicate efficiently even when host bacterial levels are low and exhibit high host specificity, thereby preserving beneficial microbiota (Garvey et al. 2022). Additionally, by reducing bacterial contamination, phages help extend the shelf life of food products while maintaining organoleptic qualities (Costa et al. 2023). To control bacterial contamination, phages can be effectively applied to a wide range of food matrices such as meat, dairy products, processed items, fruits, vegetables, as well as food packaging materials and processing environments (Cristobal-Cueto et al. 2021; Elois et al. 2023). Their host specificity and ability to disrupt bacterial populations support their use in food safety applications, yet it remains essential to continuously isolate new phages with broader lytic spectra and combine them into cocktails or with other interventions to counteract bacterial resistance and maximize effectiveness (Kazi & Annapure 2016).

In the present study, bacteriophages that infect *S. aureus* were isolated from wastewater and raw milk samples collected across Türkiye. Two phages were selected for further phenotypic and genotypic characterization, and their efficacy was evaluated as a cocktail against *S. aureus* both in vitro and in milk stored under refrigerated conditions. Additionally, the stability of *S. aureus* phages was investigated during long-term storage under different conditions.

Materials and method

Sampling

Sampling was carried out from January to December 2022. During this period, a total of 1,719 samples were collected from various facilities located in 24 cities across Türkiye (Fig. 1). These included samples from wastewater treatment plants, poultry and cattle/sheep slaughterhouses and raw milk. Sampling covered a wide range of matrices such

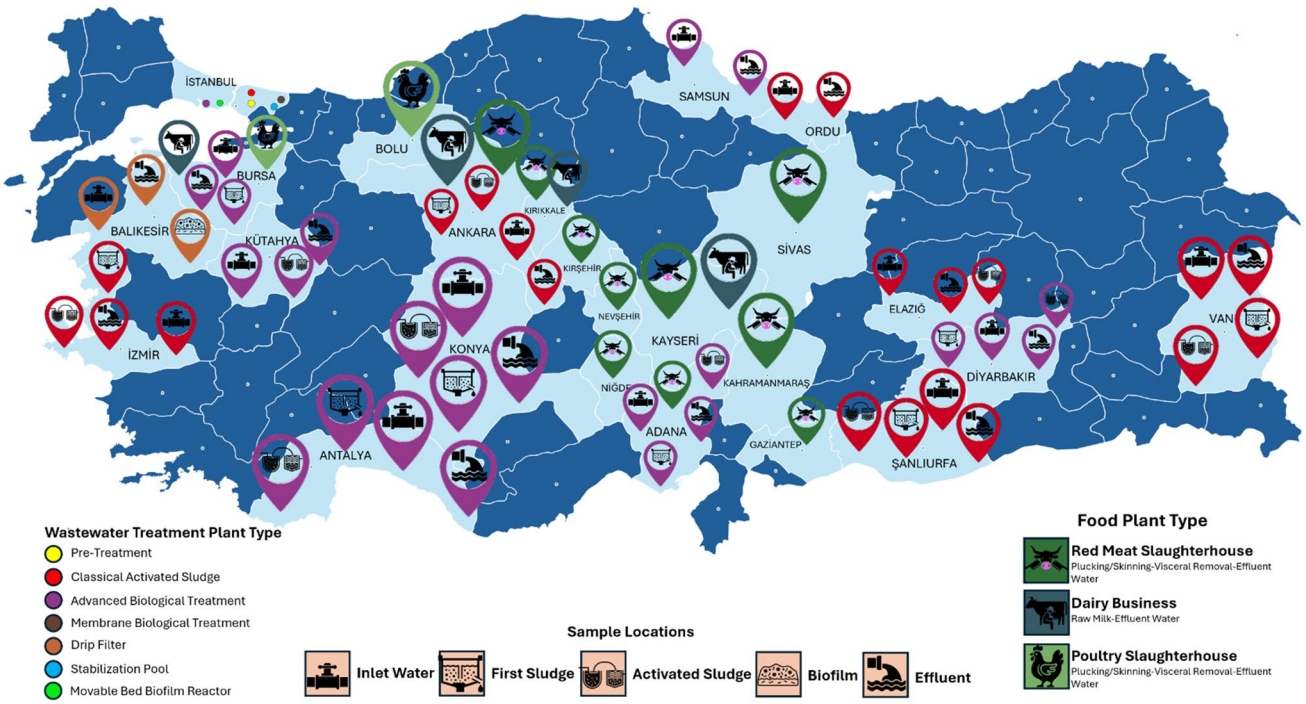


Fig. 1 Geographical distribution of sampling locations across Türkiye

as inlet water, activated sludge, wastewater from poultry and livestock processing, and effluents. Samples were kept under cold chain conditions and promptly transported to the laboratory for further analysis.

Bacteriophage isolation

Wastewater samples were filtered through filter paper, and then initially centrifuged (13,000 × g, 5 min). The supernatant, filtered through 0.22 µm Millipore filters subsequently. *S. aureus* ATCC 25923 used as the host strain. For phage enrichment, 4 mL of filtrate was mixed with 200 µL of log-phase host culture and 2 mL of Luria–Bertani (LB) broth in a sterile tube, followed by incubation at 37 °C overnight without agitation. The mixture was subsequently centrifuged at 10,000 rpm for 5 min, passed through a 0.22 µm filter, and preserved as phage stock. Phage presence was confirmed using the double-layer agar technique according to Cufaoglu and Ayaz (2019). Individual plaques were aseptically picked with a sterile pipette tip and transferred into fresh tubes containing log-phase host bacteria for further enrichment. This enrichment and centrifugation procedure was repeated three consecutive times, each round employing freshly grown host cultures to ensure plaque purification. Phage titers were initially estimated by spot assays on double-layer agar plates. For amplification, log-phase host bacteria were co-incubated with phage filtrate in Tryptic Soy Broth (TSB) at 37 °C overnight. The culture was then centrifuged (10,000 rpm, 5 min), filtered through a 0.22 µm membrane, and serially diluted. Aliquots (10 µL) from ten-fold dilution series were spotted onto LB soft agar overlays seeded with log-phase bacteria. Following overnight incubation at 37 °C, plaque-forming units per milliliter (PFU/mL) were calculated. In addition, phage stock titers were quantified using the double-layer agar method, and plaque characteristics such as morphology, clarity, and diameter were documented.

Host range determination and Efficiency of plating (EoP)

Host range determination was carried out against 30 *S. aureus* strains (Gencay et al. 2010). Additionally, to assess phage specificity and rule out potential cross-infectivity towards bacteria from other genera, 5 Gram-positive and 10 Gram-negative strains were included. The Gram-negative panel included various *Salmonella* Enteritidis, *Shigella dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*, *Yersinia enterocolitica* ATCC 23715, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *Pseudomonas aeruginosa* ATCC 15442, while the Gram-positive panel comprised *Enterococcus faecalis* ATCC 29212, *E. faecium* C68, *Bacillus cereus* NCTC 9946, *Clostridium perfringens* ATCC 13124, and *Listeria monocytogenes* ATCC 19111. All bacterial strains were cultured in tryptic soy broth (TSB) at 37 °C, except for *Clostridium perfringens*, which was cultured under anaerobic conditions. Host susceptibility was assessed using spot assays by applying 10 µL of phage lysates onto bacterial lawns prepared using the double-layer agar method. Lytic activity was evaluated based on plaque formation and clarity, following the same approach described for EoP determination (Ayaz et al. 2018).

EoP was assessed by spotting 10 µL aliquots of serially diluted phage lysates (10^6–10^8 PFU/mL) onto bacterial lawns of both the reference host strain and the tested isolates using the double-layer agar technique. Following overnight incubation, plaques were enumerated to determine phage titers. EoP values were calculated as the ratio of the mean phage titer (PFU/mL) obtained on each test strain to that on the original isolation host, which was designated as the reference (EoP = 1). Based on EoP results, infection efficiency was categorized as strong (0.5–1.0), medium (0.2–0.5), or weak (< 0.2), corresponding to plaque morphologies. The absence of visible plaques was interpreted as lack of infectivity (Park et al. 2011).

One-step growth curve

One-step growth curve analysis was performed using six selected phages (SA00.2 3–3, SA00.2 P-3, SA23.2 P-1, SAKay25923, SAKay1298, and SAKay1306), identified based on their host range, EoP values, and plaque characteristics (Table 2), on their host strain *S. aureus* ATCC 25923. The analysis was conducted by mixing mid-log phase *S. aureus* ATCC 25923 (~ 1×10^8 CFU/mL) with phage stock at a multiplicity of infection (MOI) of 0.1 in tryptic soy broth (TSB). The mixture was incubated for 5 min at room temperature to allow adsorption, and the adsorption efficiency (~ 90%) was determined in a parallel assay by measuring the reduction in free phage in the supernatant after centrifugation (5,000 × g, 5 min), (Banar et al. 2025). After adsorption, cells were pelleted (5,000 × g, 5 min), the supernatant was discarded, and the pellet was resuspended in 1 mL fresh TSB and incubated at 37 °C with gentle shaking. At defined time points (every 5 min for 60 min), 100 µL samples were taken from the suspension, immediately diluted in cold TSB, and centrifuged (5,000 × g, 5 min) to separate cells and free phages. The supernatant was then serially diluted and titrated by the double-layer agar method on the host to determine the concentration of extracellular phage particles (PFU/mL). Phage titers over time were used to construct one-step growth curves. The latent period was defined as the time between the end of adsorption and the first detectable increase in extracellular phage titer. Burst size was

calculated as the ratio between the maximum extracellular phage titer and the number of infected cells, where the latter was estimated from the initial bacterial concentration, the MOI (0.1), and the measured adsorption efficiency (~90%).

pH and heat stress

Phage suspensions were exposed to acidic conditions (pH 3.0 and 4.0, adjusted with lactic acid) and incubated at room temperature for 1 h. In parallel, thermal stability was tested by incubating the suspensions at 40 °C, 50 °C, and 60 °C for the same duration. Samples were taken at 0, 10, 30, and 60 min, and phage viability was assessed via the double-layer agar method. Changes in phage titers under each condition were recorded (Ayaz et al. 2018).

Transmission Electron Microscopy (TEM)

Phage morphology was examined using transmission electron microscopy (FEI/Tecnai G2 Spirit Biotwin) after negative staining of purified suspensions with 2% aqueous uranyl acetate on carbon-coated copper grids (Cufaoglu and Ayaz 2019).

Whole-genome sequencing and bioinformatic analysis

Two *Staphylococcus* phages SA1298KAY (SA1298KAY-MAS-S9) and SA1306KAY (SA1306KAY-MAS-S15) were subjected to whole genome sequencing for further genomic characterization. Genomic DNA was extracted using Phage DNA Isolation Spin Column Kit (Norgen Biotek, Canada) following standard laboratory procedures. Paired-end sequencing libraries were prepared and sequenced on the Illumina NextSeq platform with 2 × 150 bp read lengths. Raw sequence data were quality-checked using FastQC v0.11.9 (Andrews 2010), and combined summary reports were generated with MultiQC v1.12 (Ewels et al. 2016). Adapter trimming and removal of low-quality bases were performed using Trimmomatic v0.39 with sliding window and minimum length parameters (Bolger et al. 2014).

High-quality reads were assembled de novo using SPAdes v3.15.5 with the "–metaviral" flag, which is optimized for viral and extrachromosomal DNA assembly (Antipov et al. 2020). The assembly process utilized 12 threads and the default k-mer selection. Contigs were filtered based on length (500) and sequencing coverage (10x), and viral contigs were assessed using CheckV v1.0.1 to estimate genome completeness and detect potential host contamination (Nayfach et al. 2021). Taxonomic classification of viral contigs was carried out with Kraken2 using the RefSeq standard database (Wood et al. 2019). Gene prediction and functional annotation were performed using Prokka v1.14.6 with the "–kingdom Viruses" parameter (Seemann 2014) and PHASTER (Arndt et al. 2016). Protein-coding sequences were compared to the NCBI non-redundant protein database using BLASTp to identify homologous viral proteins. To evaluate phage lifestyle, annotated genomes were examined for the presence of integrase, Cro-like repressor, holin, endolysin, and attachment site sequences (*attL, attR*) to differentiate between temperate and lytic replication strategies. Antimicrobial resistance (AMR) genes were screened using Abricate (Seemann 2020) against the ResFinder and CARD databases. The complete phage genomes obtained from both isolates were deposited in NCBI GenBank and assigned accession numbers. The genome visualized with the Proksee online tool (Grant et al. 2023). The comparative circular genome map was generated using the annotated phage genomes of SA1298KAY and SA1306KAY together with the closely related reference phage *Staphylococcus* phage vB_SauR_SW21 (OR683639.1). Coding sequences were displayed according to genomic location, strand orientation, and predicted product annotation to facilitate visualization of structural, lysis-associated, and packaging-related proteins.

To predict bacteriophage lifestyles, we utilized Bacphlip (Hockenberry & Wilke 2021), which employs Hidden Markov Models (HMMs) to classify phages as temperate or virulent based on genomic features. Each phage genome was analysed using Bacphlip's pre-built HMM profiles through the hmmsearch tool from the HMMER suite (v3.4). Predictions were evaluated by Bacphlip to provide probabilistic classifications. To investigate the taxonomic relationships and clustering patterns of the recovered phage genomes, we performed a viral clustering analysis using vConTACT2. This tool applies a gene-sharing network approach to group viral genomes based on shared protein clusters and their connectivity within reference datasets. Using the ProkaryoticViralRefSeq database and MCL clustering, the phage genomes were analyzed alongside known reference phages (Jang et al. 2019).

Phylogenetics analysis

For whole-genome phylogeny, complete genome sequences of the phages SA1298KAY (SA1298KAY-MAS-S9) and SA1306KAY (SA1306KAY-MAS-S15), together with reference *Staphylococcus* phages, were aligned using MAFFT v7.505 (Katoh & Standley 2013) with default parameters. Maximum-likelihood (ML) phylogenetic trees were constructed in IQ-TREE v2.3.5 (Minh et al. 2020), applying the LG + G4 amino acid substitution model selected by ModelFinder. Branch support was estimated with 1000 ultrafast bootstrap replicates, and bootstrap values ≥ 50 were indicated

at the nodes. For protein-level phylogeny, the major capsid protein (MCP) sequences were predicted from annotated genomes using Prokka. Homologous MCP sequences from reference phages were retrieved from GenBank and aligned using MAFFT. A maximum-likelihood tree was generated in IQ-TREE under the JTT+G4 model with 1000 bootstrap replicates. Trees were visualized and edited using MEGA X (Kumar et al. 2018). To further assess intergenomic relatedness at the species level, pairwise genome similarity analysis was performed using VIRIDIC with complete *Rosenblumvirus* genomes ($n=20$) retrieved from GenBank. Reference genomes were selected based on BLAST similarity and taxonomic relevance. The resulting intergenomic similarity matrix was visualized as a heatmap and used to support the taxonomic interpretation of SA1298KAY and SA1306KAY.

Optimization of storage conditions for phages

To evaluate long-term stability, bacteriophage suspensions were stored for one year in four different media [TSB (tryptic soy broth), SM buffer (100 mM NaCl, 50 mM Tris–HCl, 8 mM $MgSO_4$, 0.01% gelatin, pH 7.5), physiological saline (PS, 0.09%), and Tris-buffered saline (TBS 1×)] at+4 °C, −20 °C, and −80 °C. Glycerol (20%, v/v) was added to samples stored at −20 °C and −80 °C. Phage stability was assessed a three-month period through serial dilution and plaque assays, and titer changes were recorded to determine the most effective preservation conditions.

In vitro lytic activity of the phage cocktail

The efficacy of the phage cocktail was evaluated against the *S. aureus* D4508 strain, which was previously identified as a carrier of the *sea* and *seh* staphylococcal toxin genes (Gencay et al. 2010). The test was conducted at two different bacterial concentrations. Briefly, overnight cultures in TSB (37 °C) were diluted to 10^2 and 10^4 CFU/mL and infected with the phage cocktail (SA1298KAY and SA1306KAY) at 10^8 PFU/mL and divided into control (C) and phage-treated (P) groups. Cultures were incubated at 37 °C, and samples were collected at 0, 30, 60, 90 min, and 3, 6, and 24 h. Bacterial loads were determined by plating serial dilutions on Baird-Parker agar. Simultaneously, phage titers were measured using the double-layer agar method. Additionally, staphylococcal enterotoxins (SEs) were determined with a toxin detection kit (Oxoid TD0900A, SET-RPLA Staphylococcal toxins A, B, C, D) during the incubation period.

S. aureus biocontrol in milk

The biocontrol potential of the phage cocktail against *S. aureus* D4508 was investigated in milk under refrigerated storage conditions to mimic post-opening household use. For this purpose, UHT milk was used and the samples were artificially contaminated with *S. aureus* at two levels (final concentrations of 10^2 and 10^4 CFU/mL), representing relatively low and higher contamination scenarios. The samples were subsequently assigned to control (C) and phage-treated (P) groups same as in vitro test. The P group samples were treated with 1 mL of phage cocktail (10^8 PFU/mL) and the C group received 1 mL of sterile distilled water. Following a 10-min gentle shaking step, all samples were stored at 4 °C. Bacterial loads, bacteriophage counts, and staphylococcal enterotoxins (SEs) were determined at 0, 1, 3, 6, and 24 h using Baird-Parker agar, double-layered LB agar and toxin detection kit, respectively.

Statistical analysis

All experiments were carried out in triplicate as independent biological replicates, and data were presented as mean log CFU/mL ± standard error. Changes in bacterial counts in phage-treated samples (P group) were compared with those in the untreated control group (C group). Statistical analyses were performed using one-way ANOVA to determine significant differences between groups. When significant differences were detected, pairwise comparisons between control and phage-treated groups were performed using unpaired Student's t-test. Data distribution was summarized through Least Squares Means (LSM) and their corresponding standard errors. All statistical evaluations were conducted using IBM SPSS Statistics version 27, and results with $p<0.001$ were considered statistically significant.

Results

Phage isolation

In the study, a total of 19 lytic clear plaque forming bacteriophages were isolated from wastewater and raw milk samples using *S. aureus* ATCC 25923 as the host strain. Given the large number of samples ($n=1719$), a single host strain was selected during the initial isolation step as an enrichment strategy to facilitate efficient phage recovery. Based on their sources, the majority of isolates were recovered from PSW (8 isolates, 42.1%), followed by CSW (6 isolates, 31.6%) and WTP (4 isolates, 21.1%), while only one phage (5.2%) was obtained from raw milk. In terms of seasonal distribution, most phages were isolated in spring (12 isolates), followed by summer (6 isolates), whereas only a single phage was recovered in autumn which was from raw milk. Phage isolation was most frequent from outlet water samples (8/19, 42.1%) from wastewater treatment

plants. The isolated *S. aureus* bacteriophages, categorized by source, location, sample type, and season are presented in Table 1.

Plaque morphology, lytic spectrum and growth characteristics

Plaque morphology was examined and recorded following the purification of bacteriophage stocks on double-layered agar plates. The lytic spectrum of the isolated phages was determined using the spot assay method to assess host susceptibility. Plaque characterization, lytic spectrum and EoP of the 19 phages are given in Table 2. None of the phages exhibited lytic activity against the other bacterial species tested.

Among 19 phages, six phages (SA00.2 3–3, SA00.2 P-3, SA23.2 P-1, SAKay25923, SAKay1298, and SAKay1306) exhibiting the broadest and partially distinct host range profiles, highest EoP values, and clear plaque morphology were selected for one-step growth analysis (Table 2). Their latent periods ranged between 20–40 min and burst sizes between ~ 10–60 PFU/cell. Among them, SAKay1298 and SAKay1306 were selected for further characterization due to their relatively shorter latent periods and more consistent burst size production compared with the other candidates. Specifically, the burst size of SA1298KAY was 21.66 PFU/cell, with a latent period of 40 min. SA1306KAY exhibited a latent period of 30 min and a burst size of 30.06 PFU/cell. These values correspond to final titers of ~ 2.17×10^8 and ~ 3.01×10^8 PFU/mL, respectively, in agreement with the growth curves shown in Fig. 2.

Table 1 List of isolated *S. aureus* bacteriophages according to source, location, sample type, and season

Phage code	Source	Province/Location	Sample type/Code	Season
SA00.2 3–1	WTP	Kırıkkale	Inlet water/2.3–1	Spring
SA00.2 3–2	WTP	Kırıkkale	Primary sludge/2 3–2	
SA00.2 3–3	WTP	Kırıkkale	Activated sludge/2 3–3	
SA00.2 3–4	WTP	Kırıkkale	Outlet water/2 3–4	
SA00.2 P-1	PSW	Bursa	Defeathering/2 P-1	
SA00.2 P-2	PSW	Bursa	Evisceration/2 P-2	
SA00.2 P-3	PSW	Bursa	Chilling/2 P-3	
SA00.2 P-4	PSW	Bursa	Outlet water/2 P-4	
SA23.2 P-1	PSW	Bolu	Defeathering/2 P-1	
SA23.2 P-2	PSW	Bolu	Evisceration/2 P-2	
SA23.2 P-3	PSW	Bolu	Chilling/2 P-3	
SA23.2 P-4	PSW	Bolu	Outlet water/2 P-4	
SA Kay69	CSW	Nevşehir	Outlet water/MN1	Summer
SA Kay92	CSW	Adana	Outlet water/YA1	
SA Kay102	CSW	Niğde	Outlet water/MN1	
SA Kay25923	CSW	Merkez-Kayseri	Outlet water/MK1	
SA Kay1298	CSW	Melikgazi-Kayseri	Outlet water/MK2	
SA Kay1306	CSW	Kocasinan-Kayseri	Outlet water/KK1	
SA.5 P	DP	Ankara	Raw milk/5 P-1	Autumn

WTP Wastewater Treatment Plant; *PSW* Poultry Slaughterhouse Wastewater; *CSW* Cattle/Sheep Slaughterhouse Wastewater; *DP* Dairy Plant

pH and thermal stabilities

The acid tolerance of phages SA1306KAY and SA1298KAY was assessed starting from an initial concentration of 8 log PFU/mL. At pH 3, both phages maintained low viability (2 log PFU/mL) after 10 min, and became undetectable at 30 and 60 min. In contrast, at pH 4, they showed consistent stability, maintaining the titer of 5 log PFU/mL across all time points (10, 30, and 60 min).

For thermal tolerance, at 40 °C and 50 °C both SA1298KAY and SA1306KAY phages remained completely stable, retaining their initial titers of 8 log PFU/mL throughout the 60-min incubation period. However, at 60 °C, phage titers dropped to 5 log PFU/mL at 10 min and further to 4 log PFU/mL at 30 and 60 min, respectively. Overall, both phages showed moderate acid tolerance and remained stable up to 50 °C.

Phage stability under various storage conditions

SA1306KAY and SA1298KAY remained stable in all tested media (TSB, SM buffer, PS, and TBS) at + 4 °C showing no detectable reduction in titer (9 log PFU/mL) throughout the 12-month period. At −20 °C, titers in all media also remained constant for up to 6 months. However, a slight decrease (to 8 log PFU/mL) was observed in SM buffer and PS by the 9th and 12th months. Similarly, at −80 °C, phage suspensions in TSB and TBS remained stable throughout the study, while minor reductions (to 8 log PFU/ml) were noted in SM buffer and PS beginning from the 6th

Table 2 Plaque characterization and lytic spectrum of *S. aureus*-specific bacteriophages

Phage Codes	ATCC 25923	RIMD 31092*	D4508 *	NCTC 9393	ATCC 19095	A900 322a*	ATCC 29213	ATCC 14458	ATCC 6538	AB 80002*	*S. aureus* isolates (n:20)
SA00.2 3-1	++	-	++	-	+	-	+	+	+	+	+
SA00.2 3-2	+	-	+	-	+	+	+	-	+	+	+
SA00.2 3-3	++	+	++	-	+	-	++	-	+	+	+
SA00.2 3-4	+	-	+	+	+	-	+	+	+	+	+
SA00.2 P-1	+	-	+	-	-	-	-	-	-	+	-
SA00.2 P-2	+	-	+	-	-	-	-	-	-	-	-
SA00.2 P-3	++	-	+	-	-	+	-	-	-	-	+
SA00.2 P-4	+	-	+	-	-	-	-	-	-	-	-
SA23.2 P-1	++	-	+	-	+	-	-	-	+	-	+
SA23.2 P-2	++	+	+	-	-	-	-	-	-	+	+
SA23.2 P-3	+	-	+	-	-	-	-	-	-	-	-
SA23.2 P-4	+	-	+	-	-	-	-	-	-	-	-
SAKay69	+	-	-	-	-	-	-	-	-	-	-
SAKay92	+	-	-	-	-	-	-	+	-	-	-
SAKay102	+	-	-	-	+	-	-	-	-	-	-
SAKay25923	++	+	-	+	+	-	++	+	+	+	+
SAKay1298**	++	+	++	+	+	+	++	+	++	+	+
SAKay1306**	++	+	++	+	++	++	++	+	+	+	+
SA.5 P	+	-	+	-	-	-	-	-	-	-	-

++: Strong lytic effect with clear zone with 1–2 mm diameter;+: Weak plaque formation -: No plaque formation. ATCC: American Type Culture Collection. RMID: Research Institute for Microbial Diseases. NCTC: National Collection of Type Cultures. * Reference strains of *S. aureus* D4508 (*sea*, *seh*), RIMD31092 (*seb*, *sec, egc*), AB 80002 (egc [seg, sei, SEM, sen, seo]) and A900322a (*seg, sei*) were kindly provided by G. Blaiotta, Dipartimento di Scienza degli Alimenti, Sezione di Microbiologia Agraria, Alimentare, Ambientale e di Igiene, Stazione di Microbiologia Industriale, Universita degli Studi di Napoli "Federico II", Via Universita, Portici, Italy. **Indicates the phages selected for further characterization and biocontrol assays. Gray lines indicate the six phages selected for one-step growth assay.++: Clear plaques (1–2 mm diameter); EoP between 0.5 and 0.2+: Weak plaque formation; EoP below 0.2 –: No plaque formation

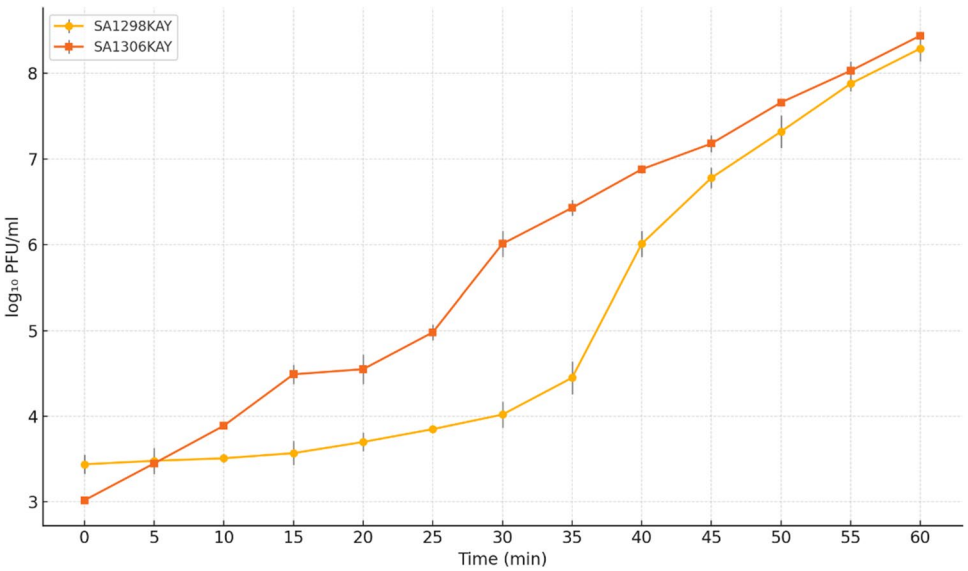


Fig. 2 One-step growth curves of SA1298KAY and SA1306KAY phages. Free extracellular phage titers in the supernatant (log_{10} PFU/mL) were measured at 5-min intervals post-infection (MOI = 0.1). Latent periods were 40 min for SA1298KAY and 30 min for SA1306KAY. Final PFU values (~2.5×10^8 PFU/mL) correspond to the reported burst sizes (21.66–30.06 PFU/cell), based on an estimated 10^7 infected cells/mL

month. Overall, TSB and TBS at all temperatures, particularly at + 4 °C and −80 °C, showed the most consistent performance in preserving phages over one year. Given that changes in phage titers were minimal under most tested conditions, and that multiple media and temperature combinations were evaluated, the stability data are presented in text form only to avoid overly complex and difficult-to-interpret figures.

TEM

TEM revealed that SA1306KAY and SA1298KAY phages exhibit icosahedral capsids with short, non-contractile tails as shown in Fig. 3.

Phage genome analysis

The initial metaviralSPAdes assemblies generated multiple contigs for each sample. However, downstream analyses were restricted to high-coverage (>1000x) contigs consistent with complete phage genomes. The remaining contigs exhibited substantially lower coverage and lacked clear association with the phage genomes of interest, and were therefore excluded from further analysis. Two high-coverage (>1000x) phage genome contigs corresponding to SA1298KAY (SA-1298-KAY-MAS-S9) and SA1306KAY (SA-1306-KAY-MAS-S15) were selected for downstream analysis. These contigs were approximately 17.5 kb in length and showed high sequencing depth (1310× and 1391×, respectively). CheckV classified both as complete/high-quality phage genomes, with no evidence of proviral or host-associated contamination. The high sequencing depth and consistent functional annotation further support that these contigs represent near-complete phage genomes. Each genome encoded 18 phage-related coding sequences and was therefore retained for functional, comparative, and phylogenetic analyses. No AMR genes, tRNA genes, or CRISPR elements were identified. BLAST analysis showed that both SA1298KAY and SA1306KAY shared 99% query coverage and approximately 92% nucleotide identity with Staphylococcus phage vB_SauR_SW21 (GenBank accession OR683639.1), supporting their close relationship to previously reported members of the genus *Rosenblumvirus*, which typically exhibit genome sizes of approximately 17–18 kb and similar modular structures. The comparative genome map (Fig. 4) further illustrates the conserved organization of both phages relative to the closely related reference phage OR683639.1. In addition to structural modules, the map highlights lysis-associated proteins, including holin and amidase, and packaging/replication-associated proteins such as the putative encapsidation protein and DNA polymerase. Taxonomically, the isolates were assigned to the class *Caudoviricetes* and showed close relatedness to *Staphylococcus* phages associated with the genus *Rosenblumvirus*, supported by vConTACT2 clustering and BLAST-based similarity. Bacphlip lifestyle analysis predicted a lytic lifestyle with a high confidence score of 98.75% and 89.75%, and the absence of integrase genes or *att* sites further supports their strictly virulent nature. Genomic annotation identified protein-coding regions associated with structural, lysis, and replication/packaging functions. Structural components included a major capsid/scaffold protein (807–2033 nt), a putative collar/head–tail connector protein (2049–3032 nt), a lower collar protein (3025–3780 nt), a putative structural protein (3794–5737 nt), and tail fiber-associated proteins (6561–7490 nt and 7547–9310 nt). Lysis-related proteins included an N-acetylmuramoyl-L-alanine amidase (5749–6498 nt) and a holin (9312–9731 nt). Replication- and packaging-associated proteins included a DNA polymerase (11260–13545 nt) and a putative encapsidation protein (13560–14807 nt). These features support a conserved modular genome organization consistent with lytic *Staphylococcus* phages. The phage genomes detected and characterized in this study were submitted to NCBI GenBank under the accession numbers PX278216 (SA1298KAY-MASS9) and PX376716 (SA1306KAY-MASS15).

Phylogenetics analysis

Whole-genome-based phylogenetic analysis (Fig. 5) revealed that SA1306KAY and SA1298KAY form a distinct sub-branch, supported by a moderate bootstrap value (79), indicating their close evolutionary relationship. This observation aligns with the ~92% genomic identity found

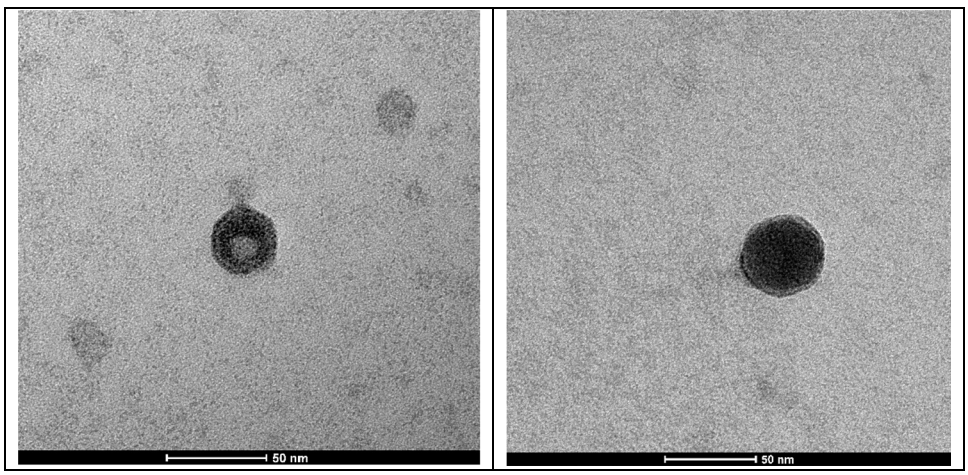


Fig. 3 TEM images of SA1306Kay (left) and SA1298Kay (right) phages

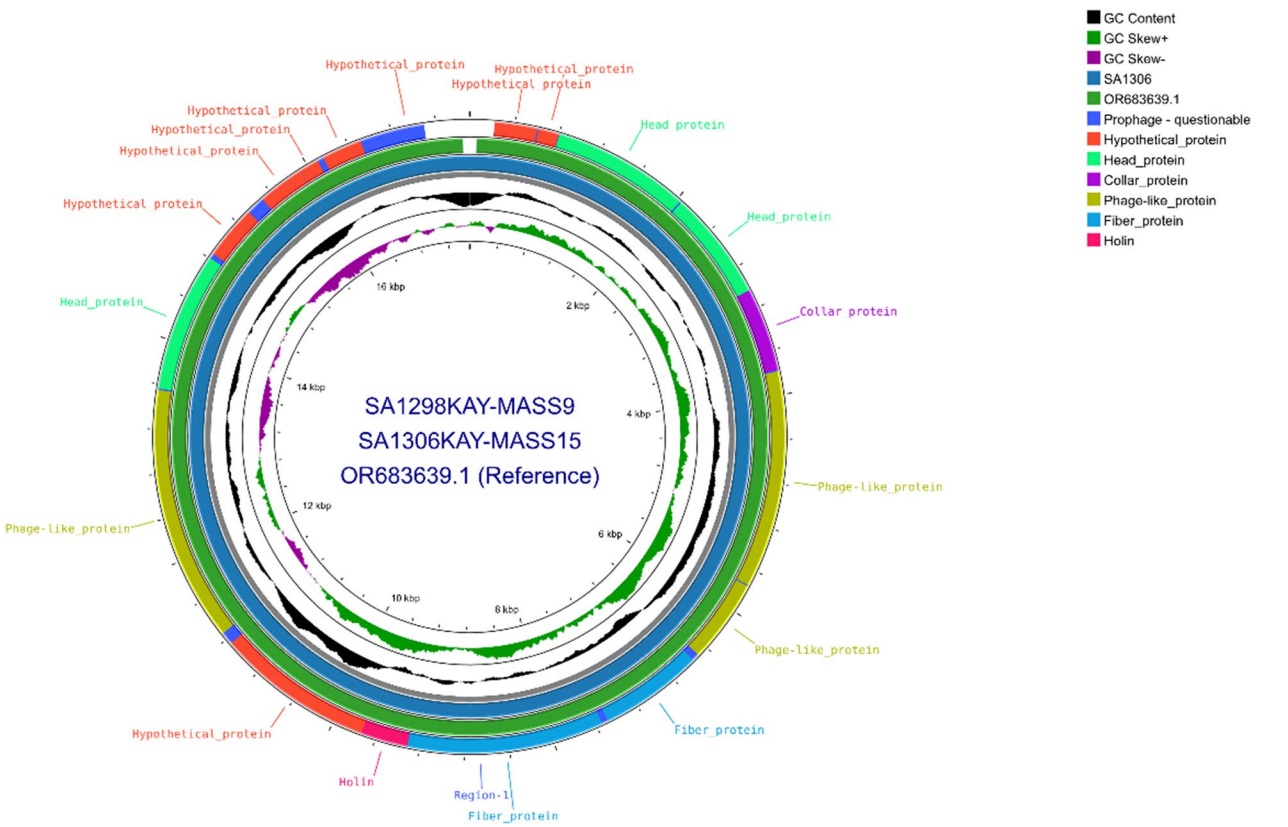


Fig. 4 Comparative circular genome map of SA1298KAY, SA1306KAY, and the closely related reference phage *Staphylococcus* phage vB_SauR_SW21 (OR683639.1). Coding sequences are displayed according to genomic position and predicted product annotation. Representative structural proteins, including head, collar, and fiber/tail-associated proteins, are indicated together with lysis-associated and packaging/replication-associated proteins, as indicated by the color-coded functional categories in the genome map. Inner tracks represent GC content and GC skew. The overall genome organization shows a conserved modular architecture among the two study phages and the reference *Rosenblumvirus* genome

in comparative analyses, suggesting that both isolates are closely related variants within the genus *Rosenblumvirus*. Further phylogenetic analysis based on major capsid protein sequences (Fig. 6) confirmed this relationship, placing both phages within the *Rosenblumvirus* clade with strong bootstrap support, reinforcing their taxonomic assignment. To further evaluate genome-wide relatedness within the genus *Rosenblumvirus*, a VIRIDIC-based intergenomic similarity analysis was performed using complete reference genomes ($n=20$) selected from BLAST-supported matches and publicly available *Rosenblumvirus* sequences. The heatmap matrix showed that SA1298KAY and SA1306KAY cluster are the most closely recognized with *Rosenblumvirus* phages, supporting their taxonomic placement within this genus (Supplementary Fig. 1).

In vitro effect of phage cocktail on *S. aureus* growth and enterotoxin production

The biocontrol efficacy of the phage cocktail (SA1306KAY and SA1298KAY) at 37 °C in tryptic soy broth was evaluated at two initial inoculum levels of *S. aureus* D4508; 2 log CFU/mL (C2 and P2) and 4 log CFU/mL (C4 and P4) (Fig. 7). The phage cocktail (8 log PFU/mL) effectively reduced bacterial counts by 0.71–4.31 log CFU/mL ($p<0.001$). The highest reduction was observed at 6 h in the P2 group, where *S. aureus* dropped below the detection limit, corresponding to at least a 4.31 log CFU/mL decrease. In the P4 group, the maximum reduction was 4.06 log CFU/mL at the same time point. Phage activity was more sustained at higher inoculum levels, while regrowth was noted in the P2 group after 6 h. Overall, significant reductions were achieved at both concentrations ($p<0.001$). Exact CFU/mL values and standard deviations for each time point are provided in Supplementary Table 1.

Additionally, the effect of the phage cocktail on staphylococcal enterotoxin production was also evaluated. SEA was detected only in the control groups, while no toxin production was observed in the phage-treated groups throughout the incubation. Specifically, in the C4 group, weak toxin expression (SEA^{+}) was first observed at 6 h, progressing to strong positivity (SEA^{+++}) in both C2 and C4 at 24 and 48 h.

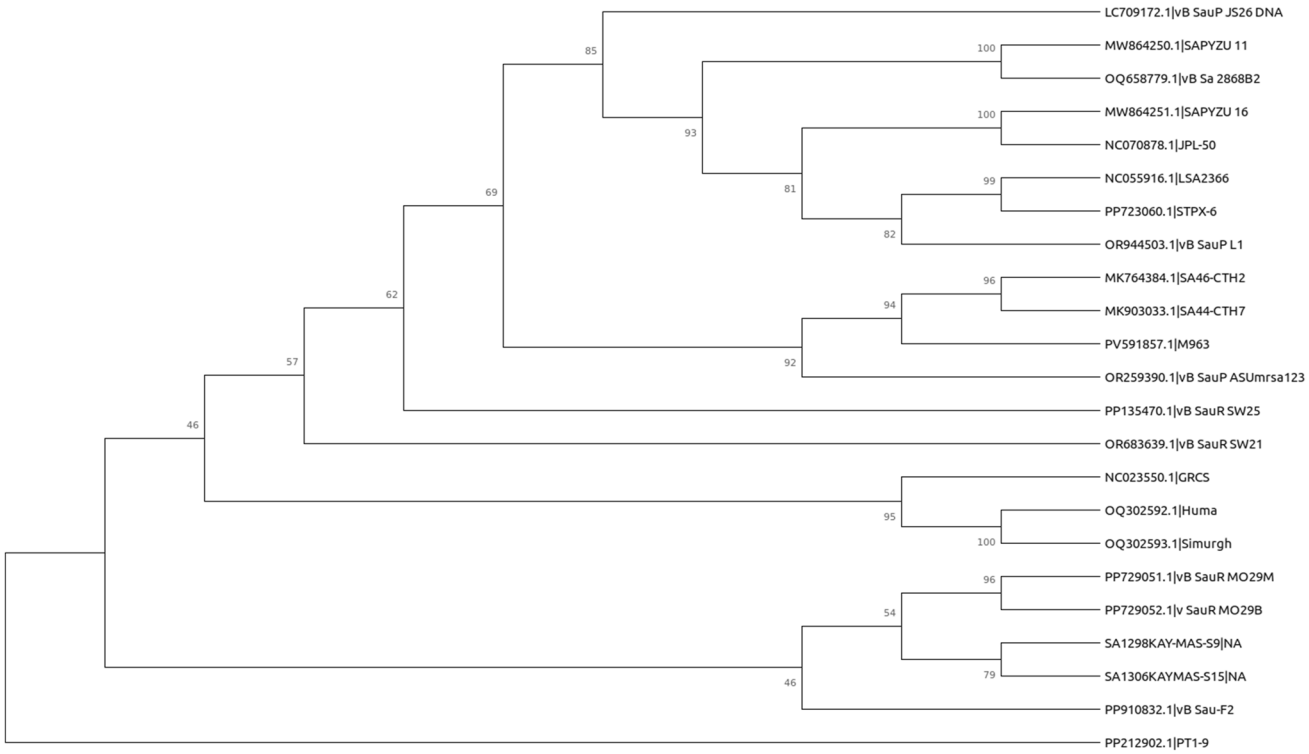


Fig. 5 Maximum-likelihood phylogenetic tree of *Staphylococcus* phages based on whole-genome alignments. Bootstrap values ≥ 50 are shown at the nodes. SA1298KAY-MAS-S9 (SA1298KAY) and SA1306KAY-MAS-S15 (SA1306KAY) clustering together within the *Rosenblumvirus* genus and forming a distinct sub-branch separated from other reference phages

In contrast, no SEA production was observed in the phage-treated groups (P2 and P4) throughout the 48-h incubation. The degree of SEA positivity (e.g., SEA^{+} to SEA^{+++}) was determined visually based on the manufacturer's guidelines for agglutination intensity using the RPLA kit. The results indicate that the phage cocktail not only reduced *S. aureus* populations but also effectively suppressed SEA expression under the tested conditions.

Biocontrol of *S. aureus* growth and enterotoxin production in milk

In the UHT milk model, the phage cocktail (8 log PFU/mL) showed moderate lytic activity against *S. aureus* D4508 at both inoculum levels (Fig. 7). In the P2 group (2 log CFU/mL), the maximum reduction was 0.94 log CFU/mL at 6 h, while in the P4 group (4 log CFU/mL), the highest reduction was 1.44 log CFU/mL at 3 h. Compared to the TSB medium, the phage exhibited reduced efficacy in milk, which can be attributed to the lower incubation temperature (4 °C). This temperature likely slowed down bacterial metabolism and phage replication dynamics, limiting the overall lytic effect. Nonetheless, the phage retained measurable activity even under refrigeration conditions, achieving statistically significant reductions ($p < 0.001$) and indicating its potential for application in cold-stored foods. Exact CFU/mL values and standard deviations for each time point are provided in Supplementary Table 1. Enterotoxin production was not detected in either the control or phage-treated groups, likely due to the inhibitory effect of storage at 4 °C.

Discussion

In recent years, studies on bacteriophages as alternative antimicrobials, especially against multidrug-resistant pathogens, have increased significantly. In this current study, a large number of samples collected over a year from food enterprises and municipal wastewater treatment plants across different regions of Türkiye. With detailed phenotypic, morphological and genetic characterizations, two phages (SA1306KAY and SA1298KAY) were applied in milk food model and significant bacterial reductions were recorded in both in vitro and milk food model assays.

A critical strength of phage biocontrol lies in the ecological versatility of these viruses, which can be recovered from a wide spectrum of environments wherever susceptible hosts are present. Numerous studies have reported the successful isolation of *S. aureus*-targeting phages from sources such as raw milk, seafood market water, animal processing facilities, and municipal wastewater (García et al. 2009; Synnott et al. 2009; Yoon et al. 2013; Son et al. 2024; Titze

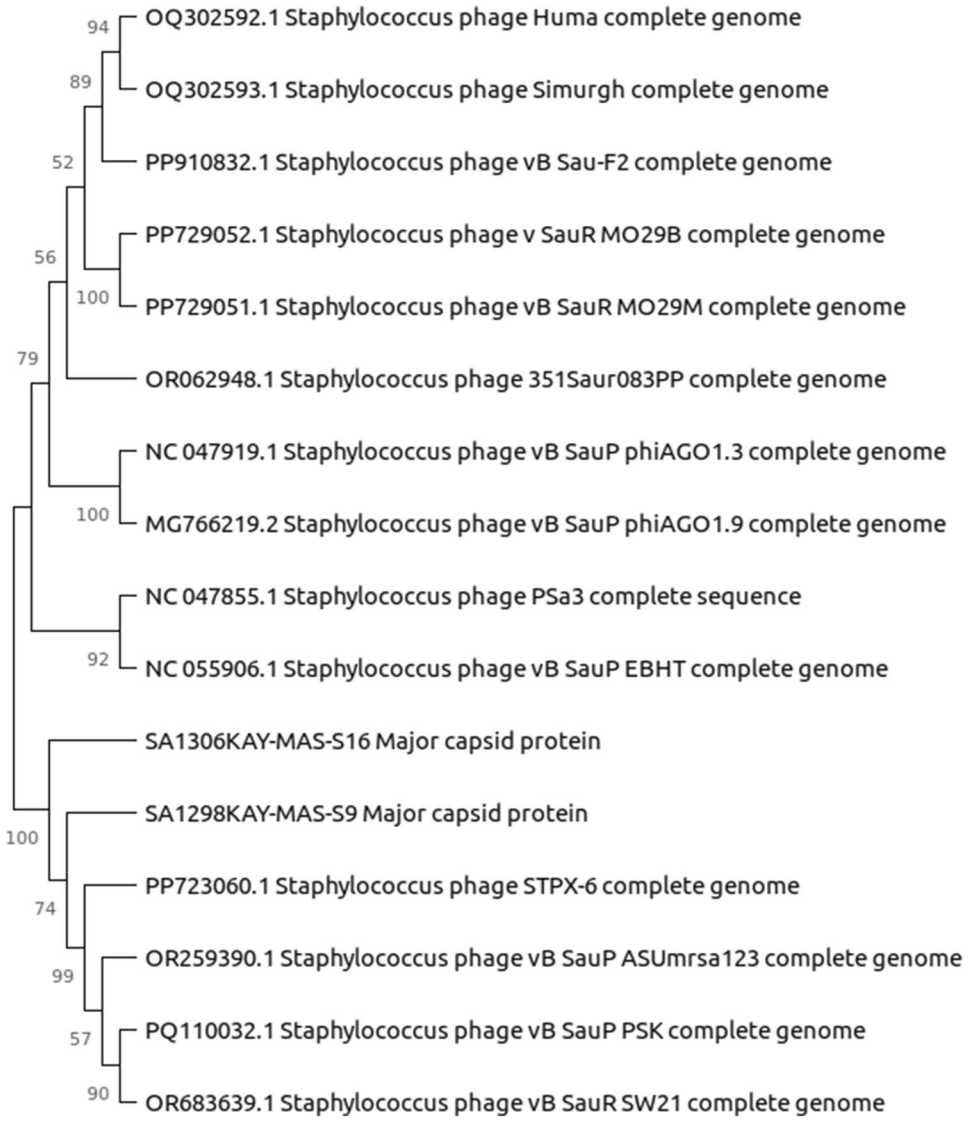


Fig. 6 Phylogenetic tree based on the major capsid protein sequences of *Staphylococcus* phages. The newly characterized phages SA1298KAY-MAS-S9 and SA1306KAY-MAS-S15 cluster within the *Rosenblumvirus* clade with high bootstrap support, confirming their close evolutionary relationship to reference *Rosenblumvirus* members. Bootstrap values are shown at the nodes

et al. 2020; Lambuk et al. 2024; Gungor et al. 2024). Building on this knowledge, our findings expand the landscape for phage hunting by demonstrating successful isolation of lytic *S. aureus* phages not only from food industry sources but also from slaughterhouse effluents and municipal wastewater. This broader approach increases the likelihood of discovering phages with unique host ranges or lytic capacities, critical for designing effective phage applications in foods.

To assess the bactericidal potential and infection dynamics of the isolated phages, we conducted one-step growth curve analyses. SA1298KAY and SA1306KAY exhibited latent periods of 40 and 30 min, with burst sizes of 21.66 and 30.06 PFU/cell, respectively. These values are consistent with previously reported ranges for lytic *S. aureus* phages. For example, Abdurrahman et al. (2021) described phages Trsa205, Trsa207, and Trsa220/222 with latent periods of 20–25 min and burst sizes ranging from 20 to 45 PFU/cell. Similarly, Feng et al. (2021) reported that phage JD419 had a latent period of 40 min and a burst size of 48 PFU/cell, closely aligning with *SA1298KAY*. Xiao et al. (2024) further reported that phage JPL-50 exhibited a burst size of 25 PFU/cell and a latent period of 20 min, falling within a similar range. In contrast, higher burst sizes were observed for CSA13 (230 PFU/cell, 20-min latent period) as reported by Cha et al. (2019). Taken together, the lytic parameters of SA1298KAY and SA1306KAY fall within the expected kinetic range for *S. aureus*-specific phages and support their potential as biocontrol agents.

Thermal and pH stability tests demonstrated that the selected phages remained viable at 40 °C–60 °C for up to 60 min. However, they were rapidly inactivated at pH 3 within 30 min. These findings align with those of Pradeep et al. (2022), who reported that phages isolated from wastewater lost viability below 21 °C and above 45 °C, and showed optimal survival between 26 °C and 45 °C at pH 7. Outside the pH 6–8 range, infectivity was significantly

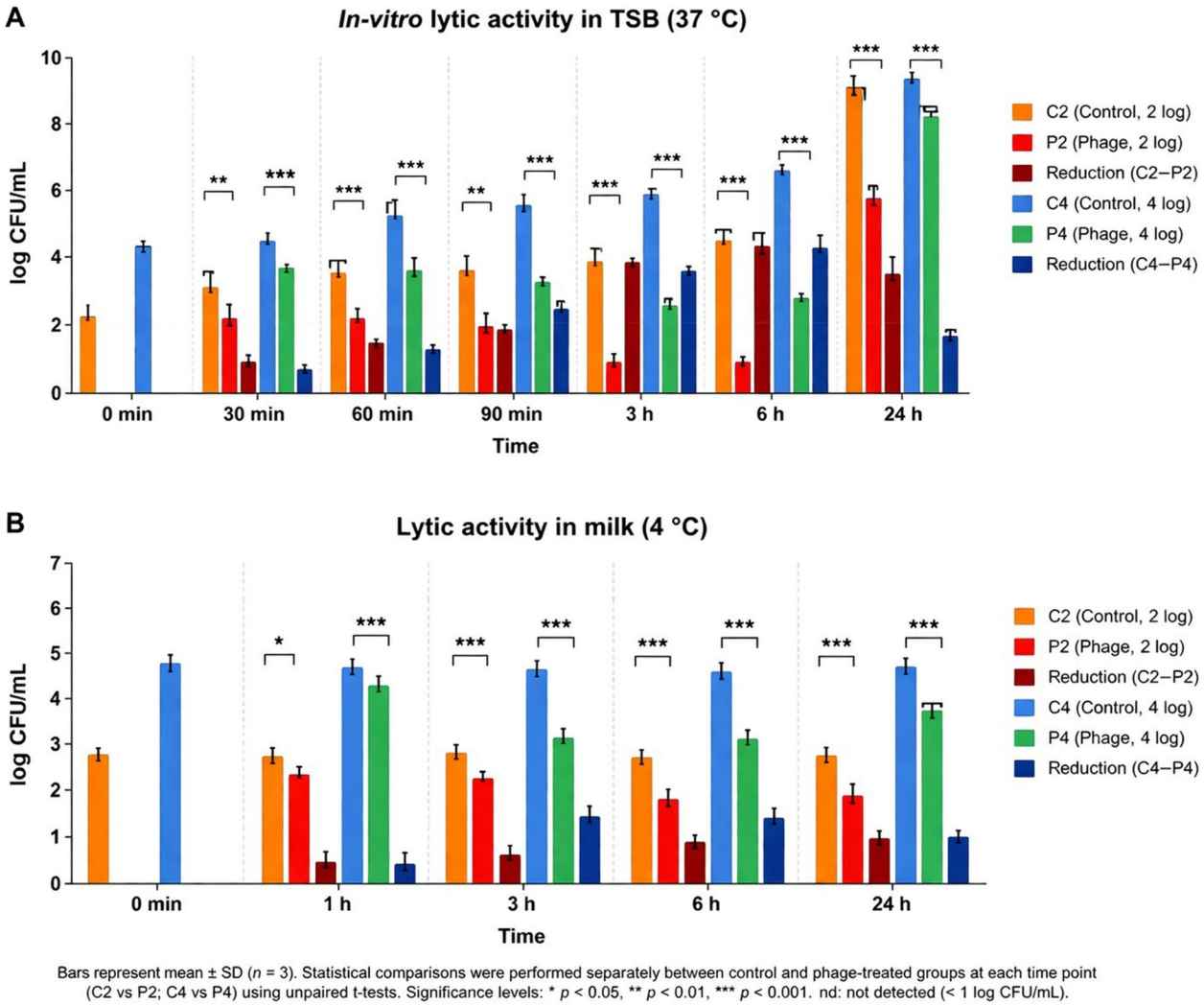


Fig. 7 Lytic efficacy of the bacteriophage cocktail (SA1306KAY and SA1298KAY) against *S. aureus* D4508 in tryptic soy broth (TSB) (**A**) at 37 °C and in milk during storage at 4 °C (**B**). C2: Control group with 2 log CFU/mL bacteria; C4: Control group with 4 log CFU/mL bacteria; P2: Phage added group with 2 log CFU/mL bacteria; P4: Phage added group with 4 log CFU/mL bacteria. Phage cocktail titer: 8 log PFU/ml. Bars represent mean log CFU/mL ± standard deviation ($n = 3$). At 3 h and 6 h in TSB (Panel **A**), bars for phage-treated groups are not shown due to undetectable bacterial levels (< 1 log CFU/mL). Statistical comparisons were performed between control and phage-treated groups at each time point (C2 vs P2; C4 vs P4) using unpaired t-tests. Significance levels: $^{*}p < 0.01$, $^{**}p < 0.001$

reduced. Song et al. (2021) found phage stability between pH 4.0 and 11.0, with notable loss of activity at pH 3.0 and complete inactivation at pH 12.0 and above. Their phage also remained stable at 4 °C, 25 °C, and 37 °C, but gradually lost activity at 60 °C and was inactivated at ≥ 70 °C. Similarly, Kwak et al. (2023) showed that their phage retained activity across a wide pH range (5.0–11.0) and temperatures between 4 °C and 55 °C for one hour. Activity dropped substantially at 65 °C and above. Barasuol et al. (2022) also confirmed that phages maintained high activity at 37 °C but were completely inactivated after 60 min at 60 °C and 70 °C. Our results fall within the expected stability range, further supporting their applicability in real-world processing and storage conditions.

Long-term storage is also crucial because the stability of phages directly affects their viability, efficacy, and practical usability in biocontrol applications. Preserving phages in appropriate media and at optimal temperatures ensures the maintenance of their infectivity and structural integrity over time. This is particularly important for industrial-scale formulations, where consistent activity and shelf life are essential for regulatory approval and field application (Gonzalez-Menendez et al. 2018). We demonstrated full stability at 4 °C and −80 °C in TSB and TBS medium supplemented with 20% glycerol. Similar findings have been reported in previous studies. Hamza et al. (2016) observed superior phage stability at 4 °C over two months compared to −20 °C and room temperature, emphasizing the effectiveness of

refrigeration, particularly without cryoprotectants. Likewise, Hsieh et al. (2011) showed that phage *Stau2* retained infectivity for up to 36 months at 4 °C and −85 °C, with SM buffer and simple saline solutions providing sufficient stability.

Whole-genome analysis of phages SA1298KAY and SA1306KAY revealed genomic features that further support their biosafety and potential for food-related biocontrol applications. Both genomes exhibited high-quality assemblies with > 90% completeness, terminal repeats, and no detectable integrase elements, tRNAs, or CRISPR arrays with each phage contig measuring approximately 17.5 kb in length. Importantly, no antimicrobial resistance genes and harbored typical structural and lytic genes. Taxonomically, both isolates were assigned to the class *Caudoviricetes* and showed close relatedness to *Staphylococcus* phages associated with the genus *Rosenblumvirus*, based on whole-genome similarity and clustering analyses, and these findings were consistent with the morphological characteristics observed by TEM, which revealed icosahedral capsids with short, non-contractile tails. The whole-genome features of SA1298KAY and SA1306KAY closely parallel those described in other *Rosenblumvirus* phages isolated from food or clinical sources. For example, ESa4 (*Rosenblumvirus*) was shown to have a compact, lineer complete lytic genome (~ 18 kb), absence of integrases, tRNAs, or virulence-related genes, and 22 well-annotated protein-coding genes (Peters et al. 2025). Similarly, Sharifi et al. (2024) reported two food-derived lineer *Rosenblumvirus* phages (~ 17 kb) with terminal repeats, strictly lytic lifestyle, and genomes lacking lysogeny or AMR-associated loci, further confirming the safety profile for biocontrol. Banar et al. (2025) described vB_SauR_SW21 (17,369 bp; 19 CDSs), again matching the gene content and modular organization found here, including conserved structural, packaging, lytic, and DNA replication modules, with most ORFs in the positive orientation.

Phylogenetic analyses based on both whole-genome and major capsid protein sequences clearly support the close evolutionary relationship between SA1306KAY and SA1298KAY, evidenced by their clustering on a distinct *Rosenblumvirus* sub-branch and their high (~ 92%) genomic identity. Similar findings have been reported for other *Rosenblumvirus* phages, where variants cluster tightly with moderate to strong bootstrap support, reflecting recent divergence and shared biological features among members of the genus (Cunha et al. 2025; Peters et al. 2025; Banar et al. 2025). The congruent clustering observed in the major capsid protein phylogeny further strengthens this interpretation, indicating that structural genes evolve in parallel with the rest of the genome and that the observed relationship is not an artefact of whole-genome alignment alone (Smith et al. 2013). The VIRIDIC heatmap adds an additional line of evidence by showing that both phages share the highest intergenomic similarity with established *Rosenblumvirus* references and form a coherent block within the *Rosenblumvirus* similarity space, clearly separated from other staphylococcal podoviruses (Cunha et al. 2025). Taken together, these concordant genomic, proteomic, and intergenomic signals support the view that SA1298KAY and SA1306KAY represent closely related *Rosenblumvirus* variants with potentially overlapping biology and host range.

The results of this study demonstrate that the phage cocktail provided significant reductions in *S. aureus* counts and a complete suppression of SEA production in medium. In tryptic soy broth medium, the rapid and pronounced lytic effect at low inoculum levels suggests efficient phage adsorption and replication, while more sustained activity at higher inocula reflects robust host-phage interactions, consistent with previous reports on *S. aureus* phages such as KMSP1 and mSA4, which showed high stability and broad host range activity in dairy environments (Kwak et al. 2023). Studies have also emphasized the need for high MOI and optimization of incubation conditions to achieve maximum lytic effect in milk matrices, where environmental factors such as low temperature, high fat content, and protein-rich composition may limit phage propagation compared to laboratory conditions (O'flaherty et al. 2005; Son & Duc 2024).

Recent research in pasteurized and raw milk verified that lytic phage cocktails can achieve up to 5 log reductions in *S. aureus* counts, with consistent suppression of enterotoxin production, strongly supporting the potential of phage biocontrol for food safety and public health. Titze et al. (2020) demonstrated that a three-phage mixture, applied to both pasteurized and raw bovine milk inoculated with mastitis isolates, produced reductions of up to 3 log CFU/mL within eight hours, even under complex milk conditions and with only modest efficacy loss compared to pasteurized milk. Similarly, Kwak et al. (2023) reported the KMSP1 phage could decrease viable *S. aureus* cells to below detection limits in milk at room temperature, while similar efficacy was shown using PSA2 in raw milk at both 24 °C and 4 °C with no toxin detected in phage-treated samples. Sharifi et al. (2024) also documented *Rosenblumvirus* phages isolated from milk efficiently inhibiting milk-isolated *S. aureus*, highlighting their biocontrol relevance. In summary, the data in this study confirm and extend previous work showing that lytic phage cocktails represent a safe and highly effective biocontrol strategy against *S. aureus* in dairy products, able to substantially reduce both bacterial loads and toxin hazards. These findings contribute to the growing consensus that phage-based biopreservation holds promise as a practical intervention for controlling staphylococcal contamination in milk and improving food safety.

On the other hand, this study has several limitations that should be acknowledged. First, phage stability was assessed only under a restricted set of pH and temperature conditions, without including neutral or alkaline pH values or temperatures outside the 4–40 °C range; future work should extend these assays to a broader environmental and processing spectrum to more comprehensively define the stability profiles of these phages. Second, biocontrol experiments in broth and in milk were performed at different temperatures (37 °C vs. 4 °C), so the reduced phage efficacy observed in milk cannot be clearly separated from the effect of lower temperature on both bacterial growth and phage replication. Finally, milk biocontrol experiments were conducted only at 4 °C, which represents proper refrigeration and does not cover intermediate temperatures (e.g. 10–15 °C) that better mimic cold-chain failures or poor refrigeration; therefore, future studies should also assess phage efficacy under these more permissive temperature conditions, where S. aureus can grow and the need for effective biocontrol is likely to be highest.

In conclusion, this study presents a comprehensive evaluation of two phages, SAKAY1298 and SAKAY1306, demonstrating their high efficacy and biosafety as biocontrol agents against *S. aureus* in milk. Their strict lytic behavior and the absence of antimicrobial resistance or virulence genes further underscore their safety and suitability for food-related applications and may also be of interest for future research in the context of antibiotic-resistant pathogens. In light of these findings, future research should focus on integrating these phages into hurdle technology strategies across diverse and complex food matrices, as well as under industrial processing conditions. Overall, this work provides scientific evidence supporting the application of SAKAY1298 and SAKAY1306 as effective, safe, and adaptable tools for reducing *S. aureus* contamination, thereby enabling future phage-based applications in food safety and public health.

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Author contribution N.D.A. conceived and designed the study. Methodology was developed by N.D.A., G.C., M.G., and I.E. The investigation was carried out by G.C., G. Cengiz, B.O.A., N.E.O., and the scholars and consultants of the TÜBİTAK-121Z447 project. Formal analysis was conducted by G.U. and N.D.A. The original draft of the manuscript was written by G.C. and N.D.A., while N.E.O., M.G., I.E., and N.D.A. contributed to review and editing. Data visualization was performed by G.C. and G.U. Supervision was provided by M.G., I.E., and N.D.A. Project administration was undertaken by N.D.A.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Declaration of generative AI and AI-assisted technologies in the writing process During the preparation of this work the authors used ChatGPT in order to edit the grammar of the paper. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Competing interests The authors declare no competing interests.

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