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Potential use of SAP-4 as a destructive and biofilm stripper against disobedient *Staphylococcus aureus* (SA) and methicillin-resistant *S. aureus* (MRSA) isolates

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ABSTRACT

In Egypt, treating SA/MRSA infections using prescribed antibiotics are becoming increasingly challenging. Not just in Egypt, but globally as well, the crisis of antibiotic-resistant bacteria must be addressed immediately; consequently, it is now imperative to look for alternatives. One of the most promising solutions in this context is bacteriophage. Finding a lytic phage that would allow us to target these bacteria and their biofilm was therefore our goal. Our research revealed that while resistance to other antibiotics varied, all strains of *S. aureus* exhibited resistance to aminoglycoside. Among isolated phages, SAPI-4 phage (member of *Myoviridae* family) has the widest host range, as it was able to infect 100% of *S. aureus* with high efficiency of plating (EOP). SAPI-4 phage significantly ($p < 0.05$) stable in the acidic circumstances, but at pH 11, after 24 h, about 50% of its infectivity was decreased. Furthermore, SAPI-4 shown superior ability to significantly ($p < 0.05$) reduce *S. aureus* in TSB media, raw milk and in both short and long-time storage with high phage stability. In short-time experiment, SAPI-4-treated samples showed a count of 0.46 ± 0.04 Log_{10} CFU/cm^2, compared to 6.21 ± 0.97 Log_{10} CFU/cm^2 in untreated samples. Furthermore, in the long-term experiment (at day 21), the count of treated sample was 0.97 ± 0.03 Log_{10} CFU/cm^2, compared to 5.71 ± 0.09 Log_{10} CFU/cm^2 of untreated sample. At 10^7 PFU/mL, SAPI-4 was able to eradicate 87%, 93%, 84%, and 91% of the biofilms of SAC9, SAM7, ASC14, and SAC1, respectively. This suggests that the phage's effectiveness against *S. aureus* biofilms. These results suggest that SAPI-4 might be able to lessen the bad effects of *S. aureus* and stop biofilm from forming.

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Introduction

In Egypt, *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) infections are becoming more difficult to treat with widely given antibiotics (Tadesse et al. 2017). In addition, antibiotic resistance is a worldwide concern that is getting worse for the economy and for people's health in general (Tondi 2021). The World Health Organization forecasts that if viable replacements to present antibiotics are not found, antimicrobial resistance (AMR) might result in up to 10 million annual deaths and a global cost of USD 100 trillion by 2050 (O'Neill 2014). This would surpass the number of deaths from cancer and heart diseases.

S. aureus is an important prevalent pathogen that can cause a variety of infectious diseases in both humans

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and animals through different pathways (Uchiyama et al. 2014, Olsen et al. 2018). *S. aureus* can cause a wide range of illnesses in humans, including suppurative infections, pneumonia, pericarditis, and meningitis (Chessa et al. 2015). In animals, it can cause a variety of local or systemic infections, including avian arthritis, bovine mastitis, and septicemia (Kaźmierczak et al. 2014).

Methicillin-Resistant *S. aureus* (MRSA) causes a wide range of illnesses, from skin and soft tissue infections to potentially fatal conditions like sepsis, pneumonia, and bacteremia (Boswihi and Udo 2018).

Even though antibiotics have been used extensively for a long time, the prevalence of infectious diseases is continually rising. In particular, the number of drug-resistant *S. aureus* strains is rising quickly (Alonzo and Torres 2014, Ganaie et al. 2018). Additionally, Laverty et al. (2013) shown that certain genes in *S. aureus* allow the bacteria to stick to implant surfaces, form groups, and build an extracellular polymeric substance (EPS) matrix, known as biofilm.

Biofilms allow bacterial cells to adhere to uncolonized portions of a host and then spread throughout those areas (Kaplan 2010). Because biofilms are more immune-resistant than planktonic cells, treating biofilm infections can be particularly challenging (Costerton et al. 1999, Leid 2009). Furthermore, studies have demonstrated that using antibiotics to treat chronic infections is less effective against biofilm bacteria (Sharma et al. 2019, Sofy et al. 2020b).

In biofilms, antibiotic resistance mechanisms are conferred by the following mechanisms, according to Stewart and Costerton (2001): (1) reduced antibiotic penetration through the biofilm matrix; (2) differentiation of particular biofilm cells into a protected phenotype; (3) upregulation of antibiotic-efflux pumps; and (4) antibiotic inactivation from beta-lactamase enzymes. Consequently, it's critical to find novel treatments that can either complement or completely replace the use of antibiotics. The use of bacteriophages to infect and destroy pathogenic multidrug-resistant (MDR) bacteria is known as phage therapy, an underutilized alternative to antibiotic treatment (Lu and Koeris 2011).

Phages, which can be used alone or in conjunction with other agents, have recently been recognized as natural, safe, highly specific, and effective alternatives to antibiotics in the prevention and treatment of bacterial infections caused by *S. aureus* (Chang et al. 2015).

As a result, phages can sterilize bacteria (Chang et al. 2015, Sharma et al. 2017) and seem like a good substitute for disinfectants and antimicrobials in the fight against bacteria. Furthermore, phages are safe to use in food and clinical items because they only infect bacteria and do not damage humans (Gutiérrez et al. 2016). According to Ahn et al. (2013) and Knezevic and Petrovic (2008), phages are very successful at reducing and controlling bacterial biofilms on a variety of surfaces generated by different species, including *S. aureus*.

Therefore, the goal of this study was to find lytic bacteriophage so that it could be used to disrupt *S. aureus* and lessen the production of their biofilm.

Materials and Methods

Bacterial isolates used in phage isolation and Growth Conditions

The investigation was employed on 17 out of 59 presumed *S. aureus* (only purified isolates) that had been previously recovered from samples of meat products, called (SAM7 to MPSA-14) and dairy products isolates called (SAC9 to SAC17) as reported by Quinn et al. (2002). First, the isolates were cultivated at 37°C for 24 hours on peptone water (Sigma, Germany). Subsequently, they were cultivated on mannitol salt agar (Difco, Detroit, Mich., USA) and Baird Parker medium (Oxoid, England). Colonies were discovered after the plates were incubated for 24 to 48 hours at 37°C. The colonies have featured such a 2-3 mm diameter, convex appearance, smooth texture, circular shape, and moist surface.

Their color ranged from gray to jet-black, often with a white border and surrounded by a dark area. Furthermore, a clear zone outside was frequently observed. In order to confirm their identification, biochemical assays (Gram staining, catalase, nitrate reduction, citrate, urease, glucose, mannitol fermentation, and coagulase activities) were used to identify all likely colonies (Holt et al., 2000; Cheesbrough, 2006).

For subsequent research, typical pure and identified isolates were stored at −80 °C in trypticase soy broth (TSB, DifcoTM, MI, USA) medium supplemented with 45% v/v glycerol.

Antibiotic Susceptibility test of S. aureus Isolates

In order to find multi-drug resistant isolates for phage isolation, the antibiotic susceptibility test of 16 isolates of *S. aureus* was evaluated. For this analysis, the Kirby-Bauer disk diffusion method (Bauer et al. 1966) was applied. Thirteen distinct antibiotics (Oxoid,UK) from different categories were employed. The assay included the following antibiotics: Kanamycin (30 mcg), Aztreonam (1 mcg), Levofloxacin (5mc g), Ciprofloxacin (5 mcg), Ampicillin (10 mcg), Erythromycin (15 mcg), Gentamicin (10 mcg), Streptomycin (10 µg), Tetracycline

(30 mcg), Tobramycin (10 mcg), Flucloxacillin (5 mcg), Clindamycin (2 mcg), and Ofloxacin (1 mcg). The results were interpreted following the protocols established by the National Committee for Clinical Laboratory Standards (NCCLS/CLSI 2007), and categorized as S (sensitive), I (intermediate), and R (resistant).

Confirm Identification of S. aureus Isolates

Out of the 16 isolates, were initially identified as *S. aureus* through biochemical methods, 4 isolates with the highest level of antibiotic resistance, 25% (n = 8/19) were re-identified. These identifications are subsequently confirmed using the Biomerieux VITEK 2 identification system, following the protocols established by Bannerman et al. (1993) and Funke and Funke-Kissling (2005).

Biofilm Formation Features of MDR-S. aureus Isolates

The quantification of biofilm production activity in four multi-drug resistant *S. aureus* isolates was carried out using the Tissue Culture Plate (TCP) method, following the protocol outlined by Bekir et al. (2011). The assay was conducted in a 96-well flat-bottom polystyrene microtiter plate obtained from Sigma-Aldrich, Costar, USA. Each well, except background wells, contained 0.2 mL of bacterial suspension (2×10^6 CFU/mL) in TSB with 0.25% glucose. The plate was then placed in a shaker and incubated overnight at 37 °C. Following incubation, the wells were washed with 0.2 mL/well PBS (pH = 7.2) to remove any unattached cells. To fix the biofilm to the polystyrene wells, each well received 200 µL of 95% ethanol. Subsequently, the biofilm was stained with 0.1% crystal violet (125 µL/well) from Sigma-USA for duration of 20 minutes. After staining, the plate was washed with PBS to remove excess stain and then treated with a solution containing solubilized dye dissolved in 1% w/v SDS to obtain a measurable optical density at OD_{570} nm using an ELISA reader (Sunrise™-TECAN, Zürich, Switzerland). Each assay was performed three times to ensure accuracy and reliability. Based on these measurements and following methodological guidelines established by (StepanoviĆ et al. 2007), we identified the strength of biofilm formation as either strong, moderate, or low for each tested *S. aureus* isolate.

Detection of S. aureus Phages

Phages Isolation Source

According to Bibi et al. (2016), samples of sewage water (8 samples/20 mL) were collected from the Wastewater Treatment Plant (WWTP), located in the northern part of Egypt's Kafr El-Sheikh governorate (N 30° 56' 45"E"42'06°31). This area was specifically chosen because of the alarming rise in water pollution caused by two major sources – the main Gharbia drain, also known as "Kitchener," and the "Number 8" drain. These drains contain a significant amount of health, industrial, and agricultural pollutants, which contribute to the pollution in this region.

Phages Isolation and Enrichment

To enrich the sewage water sample in the TSB medium for phage isolation, an equal volume of 10 mL was added. Each *S. aureus* isolate was inoculated into a separate sterile flask containing the mixture, along with a mid-logarithmic phase of fresh culture (100 µL) for each strain. The flasks were then placed in a shaker incubator at 37°C and set to shake at 270 rpm for 16-20 hours. After incubation, the mixtures were centrifuged at 7000× g for 20 minutes at 4 °C. They were then filtered using sterile disposable filters with a pore size of 0.45 µm and transferred to clean flasks, which were stored at 4°C (Gudina et al. 2018).

Phages Detection

Spot-Test Assay

Spot testing was conducted based on the method described by (2018). In this process, a soft layer consisting of 4 mL of TSB with 0.7% agar was inoculated with a 100 µL overnight culture of each MDR-*S. aureus* isolate. Subsequently, solidified TSA media was poured into petri-plates to create a hard layer. The soft layer was then carefully added on top and allowed to solidify for approximately 15 minutes, forming a double layer. To determine phage activity, 10 µL of the enriched samples were spotted onto the surface of the soft layer and incubated overnight at 37 °C. Following this period, any lysis zones observed on the TSA plates indicated partial or complete cell lysis. These lysis zones were individually transferred to CM phage buffer using sterilized wire loops (0.735 gm/L $CaCl_2 \cdot 2H_2O$: 2.5 g/L $MgSO_4 \cdot 7H_2O$; 0.05 g/L gelatin; 6 mL/L Tris buffer at pH 7.2).

Plaque Assay

For the assessment of phage plaque formation, we employed the double agar overlay technique as described by Kaur et al. (2012) and Sangha et al. (2014). Serial dilutions (ten-fold) were performed for each phage lysate. Then, each dilution (100 µL) was combined with an overnight culture host in separate tubes and incubated at 37°C for duration of 24 hours. Afterward, the tubes were mixed thoroughly before adding a soft agar layer (4 mL) to the suspension created earlier. This mixture was promptly poured onto solidified TSA plates and subsequently incubated at 37°C for another day. Plaques of various sizes and shapes were carefully collected and transferred into 1 mL of CM phage buffer. They were

then allowed to incubate at 25°C for 24 hours, allowing the phages to diffuse into the buffer.

Phages Purification, Propagation, and Titration

The purification and propagation of phages were carried out using the double agar overlay method, as described by Kaur et al. (2012) and Sangha et al. (2014). To purify isolated phages, a single plaque was picked from previous plates, and the double agar overlay procedure was repeated three times consecutively. Phage propagation, 100 µL of the original *S. aureus* lawn was mixed with 100 µL of phage suspension in TSB medium and incubated at 37°C for 24 hours. Ten double overlay agar plates were prepared for each isolated phage. Three mL of CM phage buffer was added to each plate. The top area of the soft layer was scratched and transferred to sterile clean tubes (50 mL), followed by the addition of 1 mL of buffer for plate and phage washing before being poured into the collection tubes. These tubes were left undisturbed for 15 minutes and then vortexed (Vortex-Genie-2; Inc., Bohemia, NY, USA) for 5 minutes. Afterwards, centrifugation (7000× *g*) was performed at 4°C for 15–20 minutes to separate the pellet from the supernatant. The pellet was discarded while the supernatant was filtered and transferred into sterile clean tubes, which were subsequently stored at 4°C. To determine each phage titer, a ten-fold serial dilution of the phage suspension was conducted and counted using the double agar overlay assay as previously described.

Host Range of S. aureus Phages

The host range of *S. aureus* phages was determined through spot testing, following the method described by Gudina et al. (2018). The lytic activity of all phages was assessed against a total of 24 bacterial strains, including 16 isolates of *S. aureus* used in phage isolation and 8 other strains consisting of MRSA (1), VRSA (1), and six of non-*Staphylococcus* strains, includes *Salmonella* sp. (2), *Escherichia coli* (2), *Klebsiella pneumoniae* (1), and *Bacillus cereus* (1). To conduct the spot test, 10 µL of each isolated phage was spotted onto a soft agar layer inoculated with each bacterial strain at a concentration of 1×10^8 CFU/mL. The plates were then poured onto solidified TSA plates and incubated overnight at 37°C. Clear spots observed on the agar surface were examined after incubation to determine the presence of lytic activity. To verify the host range further, the efficiency of plating (EOP) method, modified from Pujato et al. (2017), was also employed. Phage suspension was serially diluted to a concentration of 1×10^7 PFU/mL. In double-layer agar plate assays, 100 µL of phage lysate and 100 µL of an overnight culture were added. The plates were subsequently incubated based on optimal conditions specific to each target bacteria being tested. At the end of the incubation period, plaques formed were counted and expressed as PFU/mL. All experiments were conducted in triplicates for both tested and target hosts to ensure accuracy and repeatability. The efficiency of EOP was determined by calculating the average PFU on test bacteria and comparing it to the average PFU on lawns, with the standard deviation (±SD) for triplicates. The results of the EOP were analyzed in four categories: high efficiency EOP ranging from 0.5 to 1.0, moderate efficiency EOP ranging from 0.2 to less than 0.5, low efficiency EOP ranging from 0.001 to less than 0.2, and inefficient EOP below 0.001.

One Step Growth Curve and Multiplicity of Infection (MOI) Assays

The determination of latency period and burst size of SAPI- 4 phage were carried out through a one-step growth curve methodology. Initially, a culture host consisting of 4 mL with a concentration of 10^7 CFU/mL was inoculated with 50 µL of phage lysate at an optimal MOI. To ensure proper adsorption, the phages were incubated in a shaker at 37°C for 5 minutes, followed by a centrifugation step (12,000× *g*) for 1 minute to eliminate any free phages. The resulting pellet was then suspended in 4 mL of fresh TSB medium, marking the starting point (time zero), and subsequently incubated at 37°C. During the incubation period, samples of 100 µL were collected at 5-minute intervals following the addition of the phages, up to duration of 200 hours. These samples were used to calculate the plaques using the overlay method, as described by Sangha et al. (2014). The burst size was determined by calculating the ratio between the final count of liberated phage particles and the initial count of phage particles. To assess Multiplicity of Infection (MOI) and conduct single-step growth tests, we followed the methods outlined by Sangha et al. (2014). In separate sterile tubes, each containing a phage lysate (10^8 PFU/mL), we combined 5 mL of TSB with the original bacterial host (1.5×10^8 CFU/mL) to achieve various MOIs (10, 1, 0.1, 0.01, 0.001, or 0.0001). The mixture was then incubated at 37°C with shaking (220 rpm) for 4 hours. Following incubation, the tubes underwent centrifugation at 8000 ×g for 20 minutes. The resulting pellet was discarded, and the supernatant was filtered using a 0.22 mm syringe filter. To determine the titers, we employed the double overlay agar method described by Kaur et al. (2012) and Sangha et al. (2014). The MOI corresponding to the highest phage titer was identified as the optimal MOI for the phage.

Transmission Electron Microscopy (TEM)

One mL of high titer stock phages was undergoing centrifugation (16,000× *g*) for 60 minutes at 4°C and then washed using a phage buffer. After gently suspending the

particle in 20 µL of CM phage buffer, the supernatant was disposed of. The 200 mesh carbon grids were filled with five microliters of the suspended phages under examination, covered with formvar, and left for two minutes. Phages were negatively stained for 30 seconds with 2% uranyl acetate, and any excess stain was removed with filter paper Ackermann (2012). Electron microscopy (Model Beckman 1010) at 60 KV was utilized to evaluate the samples at the Regional Center for Mycology and Biotechnology, Al-Azhar University in Cairo, Egypt Accolas and Spillmann (1979).

pH Stability and Thermostability of S. aureus Phage

SAPI- 4 stability was tested at intervals of 1, 3, 5, 7, 12, and 24 hours at different pH values of 3,5, 7, 9, and 11 and various temperatures of –20°C, 4°C, 25°C, 37°C, 45°C, 55°C, 65°C, and 75°C, according to Jamalludeen et al. (2007). Using the double overlay agar method, the phage titer was measured for each sample (Kaur et al. 2012, Sangha et al. 2014). Three duplicates of the experiment were run, and the long standard deviation (±SD) was recorded.

Bacteriolytic activity of SAPI- 4

In vitro Challenge Assay

Potential of Bacterial Culture Clearance

The potential contribution of the SAPI- 4 phage to the suppression of bacterial growth in the broth medium was assessed using a method used by Khawaja et al. (2016). The host culture (7 $\log_{10}$ CFU/mL) was incubated for 24 hours at 37°C before being transferred into two 100 ml sterile, clean flasks. A SAPI- 4 phage (9 $\log_{10}$ PFU/mL) was added to one flask (test flask), whereas the second flask (positive control) did not change. It is indicated in the experiment observations along with the fact that there is a third flask (negative control) that is the only one that contains the TSB medium. The flasks were shaken at a rate of 220 rpm while being incubated for the entire night at 37°C. At intervals of 2, 4, 6, 8, 10, 12, and 24 hours, the optical density (OD_{620}) was measured. The experiment was performed in triplicate.

Using the 96-Well Microtiter Plate

The lytic activity of the SAPI- 4 phage was measured using a 96-well flat-bottom polystyrene microtiter plate (Sigma-Aldrich, Costar, USA) and optical density ($O.D_{620}$ nm), with some minor changes, in accordance with Anany et al. (2011). This test made use of the same bacterial lawn that was utilized for phage isolation. A series of wells are present in the microtiter plate: test wells, which contain bacteria treated with phage, negative control wells, which contain only TSB medium, and positive control wells, which contain overnight cultures of the tested bacteria untreated by phage. The absorbance was measured at 2 h, 4 h, 6 h, 390 min, 7 h, 450 min, 8 h, 12 h and after 24-hours incubation (37°C) period in a shaker (160 rpm) using an ELISA reader (SunriseTM-TECAN, Switzerland) with a long standard deviation (±SD) for triplicates.

In vivo Challenge Assay

The bactericidal activity of the phage against *S. aureus* was examined in raw milk and beef burger sections in order to establish a progressive adaptation to the in vivo circumstances.

Challenge in Raw Milk

The presence of *S. aureus* and antibiotic residues were previously checked in raw milk. At a concentration of 7 $\log_{10}$ CFU/mL *S. aureus* isolate was added to raw milk (100 mL). In addition, we introduced 1 mL of phage at a concentration of 8 $\log_{10}$ PFU/mL. Prior to being incubated at 37°C without shaking, the preparations were mixed using a Vortex Mixer (Infitek Co., Ltd. China). Then, using 100 µL of sample material, the preparations were tested using the spatula method (Standardization 1995), for *S. aureus* germ density at zero time, 30 minutes (t = 30m), two hours (t = 2h), and eight hours (t = 8h). This was accomplished, in short, by plating serial dilutions (Ringer's solution; Al Gomhoria Trading comp. Egypt), and after 24 hours of incubation at 37°C, the colony forming units per milliliter (CFU/mL) were calculated. For every data point, three measurements were taken. Raw milk that had been inoculated with *S. aureus* (SAC9) served as the positive control culture without phages, while raw milk free of *S. aureus* was employed as the negative control. The study was conducted in triplicate, including positive and negative controls.

Challenge in Beef Burger Cuts

After being purchased from a nearby grocery, beef burger slices were aseptically sliced and brought into the lab in an icebox. After slicing the beef burger into little pieces (2 cm × 2 cm square), 70% ethanol was used to disinfect it for three hours. Following that, it was taken out of the alcohol solution, given three rounds of sterile water washings, and let to dry in the Petri plate for half an hour. The FDA (2002) states that total aerobic bacterial count was used to screen the microbial burden on TSA plates. For the experiment, only bacteria-free cuts were used, and they were split into three equal groups.

Short-Term Storage Efficacy and Stability

S. aureus (SAC9) overnight cultures that had been previously incubated and suspended in TSB were produced. Just two groups were given one milliliter of the

bacterial culture (6 $\log_{10}$ CFU/cm^2), which was then dried for one hour to allow the bacteria to adhere to the beef burger parts. The other group, which was not contaminated and was not treated, was utilized as a negative control. It was inoculated with CM buffer alone.

For *S. aureus* phage efficacy, beef burger slices were sprinkled with SAPI-4 phage lysate (8 $\log_{10}$ PFU/cm^2) at the ideal MOI, ensuring that only one of the two groups—the contaminated, treated group—was artificially infected with bacteria. To give the phage time to adsorb on the beef burger pieces, it was left for 40 minutes. While the remaining group—the contaminated, untreated group—remains untreated after being infected with bacteria. For a week, all Petri plates were kept at 4°C with sterile stretchable plastic covering them. On TSA plates, the bacterial $\log_{10}$ reduction was counted on days 0, 2, 3, 4, 5, 6, and 7 of the storage periods.

For SAPI-4 phage stability, the double agar overlay technique was used to monitor the SAPI-4 phage titer in order to ascertain the virion stability in the food throughout the storage period (Kaur et al. 2012, Sangha et al. 2014).

Long-Term Storage Efficiency and Stability

Over the course of three weeks, the effectiveness of SAPI-4 phage for controlling *S. aureus* in food was also evaluated. Three additional sets of two-centimeter-thick beef burger slices were made in the same manner and arranged individually on plates. Following a suitable and hygienic covering, the plates were frozen for 21 days at -20°C.

For SAPI-4 phage efficacy and Stability; both the $\log_{10}$ count of bacteria (CFU/mL) and phage (PFU/mL) was performed at days 0, 2, 4, 8, 10, 14, 18, and 21. The SAPI-4 phage's stability was simultaneously tested by Kaur et al. (2012) and Sangha et al. (2014) using the twofold agar overlay method.

Recovery of S. aureus

According to Quinn et al. (2002), the artificially injected *S. aureus* were retrieved by calculating the $\log_{10}$ reduction in order to assess the effectiveness of the *S. aureus* phage in suppressing *S. aureus* in food. The sample was added and combined with one milliliter of PBS in a sterile and clean bag. To prevent bacteriophage plating, homogenize (for beef burger slices only), vortex (Spricigo et al. 2013), then centrifuge (3000× g) for 10 min (Tomat et al. 2018). After discarding the supernatant, the pellet was combined with peptone water and incubated for 24 hours at 37ºC. Subsequently, a portion was collected and cultivated on mannitol salt agar, followed by Baird Parker medium. Following a 24- to 48-hour incubation period at 37ºC, all inoculation plates were examined for colonies. The colonies are smooth, convex, round, 2-3 mm in diameter, moist, gray to jet-black, and usually have off-white, light-colored edge that is encircled by an opaque zone and often has an outside transparent zone. Biochemical and coagulase activity tests are performed on all suspected colonies (Holt et al. 2000, Cheesbrough 2006).

Anti-Biofilm Activity of SAPI- 4 Phage

The SAPI-4 phage's anti-biofilm activity was measured in relation to the isolates that produce the greatest biofilm, namely SAC9, SAM7, ASC14, and SAC11. In this experiment, the Tissue Culture Plate (TCP) method was employed, adhering to the procedure described by Bekir et al. (2011).

A 96-well flat-bottom polystyrene microtiter plate was purchased from Sigma-Aldrich in Costar, USA, and used for the test. A 0.2 mL bacterial solution (2×10^6 CFU/mL) in TSB with 0.25% glucose (+ve control well) was present in every well, with the exception of background wells. The plate had anti-biofilm wells, or wells loaded with 0.2 mL of bacterial suspension and supplemented with 10^7 PFU/mL of SAPI-4 phage suspension.

After that, the plate was put in a shaker and kept at 37°C for the entire night. After the incubation period, any unattached cells were removed from the wells by washing them with 0.2 mL/well PBS (pH = 7.2). 200 µL of 95% ethanol was added to each polystyrene well in order to adhere the biofilm to the surface. The biofilm was then stained for 20 minutes using 0.1% crystal violet (125 µL/well) from Sigma-USA.

Following staining, the plate was treated with a solution containing solubilized dye dissolved in 1% w/v SDS to obtain a detectable optical density at OD570 nm using an ELISA reader (SunriseTM-TECAN, Zürich, Switzerland). Excess stain was then removed from the plate using PBS washing. Three runs of each assay were conducted to guarantee precision and dependability. Using these data and the methodological principles set forth by StepanoviĆ et al. (2007), we classified each tested *S. aureus* isolate's biofilm formation strength as strong, moderate, or low. The biofilm reduction percentages were computed in accordance with Else et al. (2003) and Kostaki et al. (2012).

Statistical Analysis

Food studies and biofilm tests were carried out in triplicate, with three samples used in each replication for each assay. The effectiveness of *S. aureus* phage in controlling *S. aureus* in TSB medium and food, as well as its stability at pH and temperature, were reported in triplicate with standard deviation (±SD) included. The

data was transformed into log_{10} units representing the outcomes of the bacteria and phage. The statistical analysis was carried out at a probability level of 0.05 using SPSS (Social Science version 26.00) software, with a totally randomized experimental design. Levene's study's parametric distribution and one-way ANOVA with least significant difference (LSD) test variance analysis were used to produce quantitative analyses. A 95% confidence interval and a 5% agreed-upon error margin were established. GraphPad Prism 8 was used to create the graphs.

Results

Isolation and Identification of S. aureus

Out of 17 possible *S. aureus* isolates that were previously identified by biochemical and culture techniques. There was only one isolate that tested negative for *S. aureus*. However, every single one of the remaining sixteen isolates tested positive for *S. aureus*; under a microscope, they were found to be clusters of Gram-positive cocci, biochemically were negative for oxidase and indole and positive for coagulase, urease, citrate, nitrate, and glucose fermentation. In addition, colonies have a smooth texture, circular form, convex appearance, 2-3 mm diameter, and moist surface. Their color was different, ranging from gray to jet-black, and they were often surrounded by an opaque area and had a pale border. Moreover, an external clear zone was frequently observed. Additionally, following complete biochemical identification using the VITEK2 system, the *S. aureus* isolates demonstrated great probability (92%) for verifying the identification.

Antibiotic Susceptibility test of S. aureus Isolates

The antibiotic sensitivity of the *S. aureus* isolates was assessed using a variety of antibiotic classes, including aminoglycosides, quinolones, macrolides, penicillin, and monobactam groups. The results presented in Table (1) indicate that the *S. aureus* isolates exhibited differing levels of selectivity towards specific antibiotic classes. While most *S. aureus* isolates were resistant to the aminoglycoside group, all isolates were found to be susceptible to the monobactam class, which is represented by 100% of the isolates of aztreonam, followed by the groups of macrolides, penicillin, and quinolones. A maximum of 31% of isolates (5/16) in the macrolides group were resistant to erythromycin, while 25% (4/16) were resistant to both tetracycline and clindamycin. Regarding penicillin antibiotic classes, ampicillin and flucloxacillin were found to be resistant in seven (53.8%) and six (37.5) isolates, respectively (Figure 1). The isolates of *S. aureus* exhibited resistance to ciprofloxacin 25% (4/16), levofloxacin 53.8% (7/16), and ofloxacin 31% (5/16) when tested against members of quinolones groups (Figure 1). The strongest resistance was shown by all *S. aureus* isolates against three aminoglycoside antibiotics: gentamicin (31% (5/16), tobramycin (37.5%), streptomycin (81% (13/16), and kanamycin 87.5% (14/16), (Figure 1). The results showed that SAC9 which was resistant to 10 of the 13 (77%) antibiotics, was the most resistant isolate, followed by SAM7 and SAC14, both of them are resistant to 9 of 13 (69%) antibiotics, then SAC11 was resistant to 8 out of 13 (61.5%), (Figure 2).

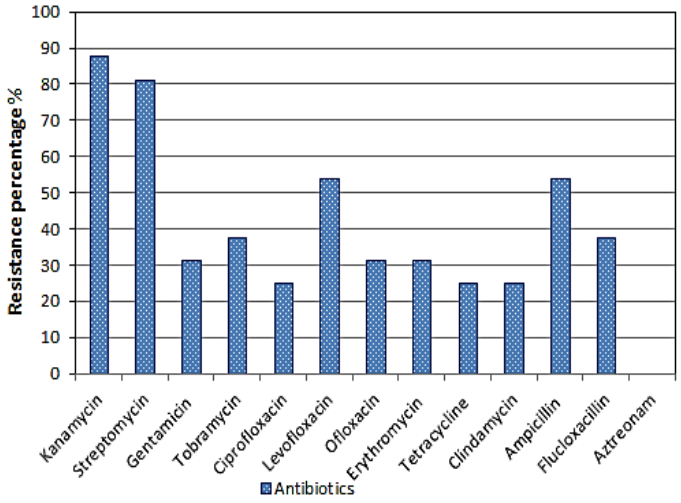

Fig 1. Overall percentages of resistance to different antibiotics (N=13) of *S. aureus* isolates.

Biofilm Formation Behavior of S. aureus Isolates

Most *S. aureus* isolates had a high capacity for biofilm formation. In contrast, Table (2) indicates that 10 out of 16 (62.5%) isolates had a considerable ability to build biofilm. This is due to the fact that *S. aureus* are regarded as the quintessential example of Gram-positive bacteria that may produce biofilms. The results showed that five isolates (31.25%) developed a moderate biofilm, while only one isolate (1/16; 6.25%) was a poor biofilm producer (Table 2). It is important to note that the capacity to build biofilm and antibiotic resistance are positively correlated. As a result, table (2) indicates that whereas weak biofilm makers had reduced antibiotic resistance, strong biofilm-producing *S. aureus* isolates showed antibacterial resistance against a range of antibiotics.

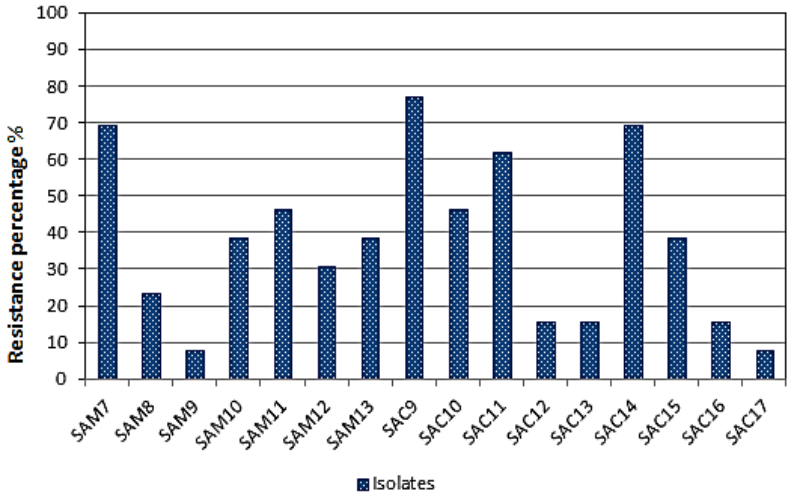

Fig 2. Overall percentages of resistance of different *S. aureus* isolates (N=16) to antibiotics.

Table 1 Antibiotic sensitivity profiles of different *Staphylococcus aureus* isolates.

	S. aureus isolates															
Antibiotics groups	SAM7	SAM8	SAM9	SAM10	SAM11	SAM12	SAM13	SAC9	SAC10	SAC11	SAC12	SAC13	SAC14	SAC15	SAC16	SAC17
Aminoglycoside																
Kanamycin	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R	I
Streptomycin	I	I	R	R	R	R	R	R	R	R	R	R	S	R	R	R
Gentamicin	R	I	I	I	R	I	I	R	R	I	I	I	R	I	I	I
Tobramycin	R	S	I	S	I	I	R	R	R	R	S	S	R	S	I	S
Quinolones																
Ciprofloxacin	S	R	S	I	R	I	I	R	S	I	S	I	R	S	I	I
Levofloxacin	R	S	S	R	R	S	R	R	S	R	I	I	R	I	S	S
Ofloxacin	R	I	S	R	I	I	S	R	S	R	S	I	I	R	S	I
Macrolides																
Erythromycin	I	I	I	S	R	S	I	R	S	R	S	I	R	R	I	S
Tetracycline	R	I	S	S	I	S	S	R	S	R	S	S	R	S	S	S
Clindamycin	R	I	S	R	I	R	R	I	S	S	S	I	I	S	S	S
Penicillin																
Ampicillin	R	R	I	I	I	R	S	R	R	R	I	I	R	S	I	I
Flucloxacillin	R	S	S	S	S	I	I	I	R	S	I	S	R	R	I	S
Monobactam																
Aztreonam	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 2 *Staphylococcus aureus's* pattern of biofilm formation

	Optical Density (O.D.)			
S. aureus isolates	Negative control (O.D.620nm)	Growth (O.D.620nm)	Biofilm (O.D.570nm)	Biofilm Interpretation
SAM7	0.121	2.127±0.63	1.117±0.23	Strong productive
SAM8		1.570±0.32	0.310±0.02	Moderate Productive
SAM9		1.904±0.52	0.544±0.11	Strong productive
SAM10		1.870±0.61	0.477±0.16	Strong productive
SAM11		1.950±0.43	0.554±0.21	Strong productive
SAM12		1.880±0.44	0.411±0.11	Strong productive
SAM13		1.887±0.39	0.637±0.17	Strong productive
SAC9		2.313±0.63	1.361±0.31	Strong productive
SAC10		1.529±0.41	0.307±0.08	Moderate Productive
SAC11		1.991±0.46	0.988±0.09	Strong productive
SAC12		1.490±0.29	0.441±0.10	Strong productive
SAC13		1.640±0.47	0.398±0.07	Moderate Productive
SAC14		2.274±0.59	1.056±0.21	Strong productive
SAC15		1.446±0.55	0.310±0.06	Moderate Productive
SAC16		1.604±0.47	0.307±0.08	Moderate Productive
SAC17		1.393±0.38	0.245±0.05	Weak productive

S. aureus Bacteriophages

Isolation, Purification, and Propagation of S. aureus phages

Out of the eight sewage water samples, three yielded positive results for the *S. aureus* phage. Spot test was used to identify several phages isolates on *S. aureus* isolates (SAM7, SAC9, SAC11, and SAC14). Non-lytic phages were not included in this investigation since we were solely interested in choosing lytic phages. Only five phages were present in the transparent (clear) plaques produced by the double overlay plating technique; nine phages that produced turbid plaques were disregarded. The phages that were chosen included two for SAM7, one for SAC9, SAC11, and SAC14. Purified, all five of the recovered lytic phages were propagated at titers of 10^9 to 10^{10} PFU/mL.

Host Range and EOP Pattern of S. aureus Phages

As stated in the Methods, spot tests were carried out on plates containing lawns of different isolates of 16/SA, 1/MRAS, 1/VRSA, 2/*Salmonella*, 2/*E. coli*, 1/*K. pneumoniae*, and 1/*B. cereus*. Each phage isolate's lytic ability was determined by checking the plates for positive spot tests on bacterial lawns (Table 3). All spot tests were conducted again to ensure the accuracy of the results, and the EOP method was used to detect the range of lysis in terms of high, moderate, or no. All the bacterial isolates displayed in Table (3) underwent host range testing; however, a majority of the lytic phage was selected because numerous isolates had varying lysis outcomes when subjected to five phages. The 18 *S. aureus* strains were susceptible to varied degrees of infection from the 5 phages, exhibiting monovalent behavior, but none of them were able to infect any of the six non-*S. aureus* bacterial strains. The fact that the SAPI-4 phage could infect both MRSA and VRSA is noteworthy. In addition to its capacity to lyse MRSA and VRSA, the SAPI-4 phage was the most widely distributed in its host range, having the ability to infect 100% of the *S. aureus* isolates with 77.77% (14/18) of high EOP (Table 3). The next phage, SAPI-1, has a high EOP of 38.88% (7/18) and can lyse 10 out of 18 (55.55%) *S. aureus* strains (Table 3). The phage's ability to infect 8 out of 18 (44.44%) *S. aureus* bacteria with 27.77% (8/18) of high EOP was demonstrated in SAPI-3 (Table 3), which came next. The lysis of 6 (33.33%) *S. aureus* strains with 22.22% (4/18) of high EOP was demonstrated by both SAPI-2 and SAPI-5 (Table 3).

One Step Growth Curve and Multiplicity of Infection (MOI) Assays

Because SAPI-4 phage is the broadest host range; it was selected in this study. The highest number of plaques was formed when the SAPI-4 phage was at its ideal MOI of 0.1. At different MOI values, the number of SAPI-4 plaques rapidly decreased, reaching its lowest point at a MOI of 10 (Figure 3A). Furthermore, it was discovered that the SAPI-4 phage had a latency time of 40 – 120 minutes and a burst size of 215 PFU/infected cell (Figure 3B).

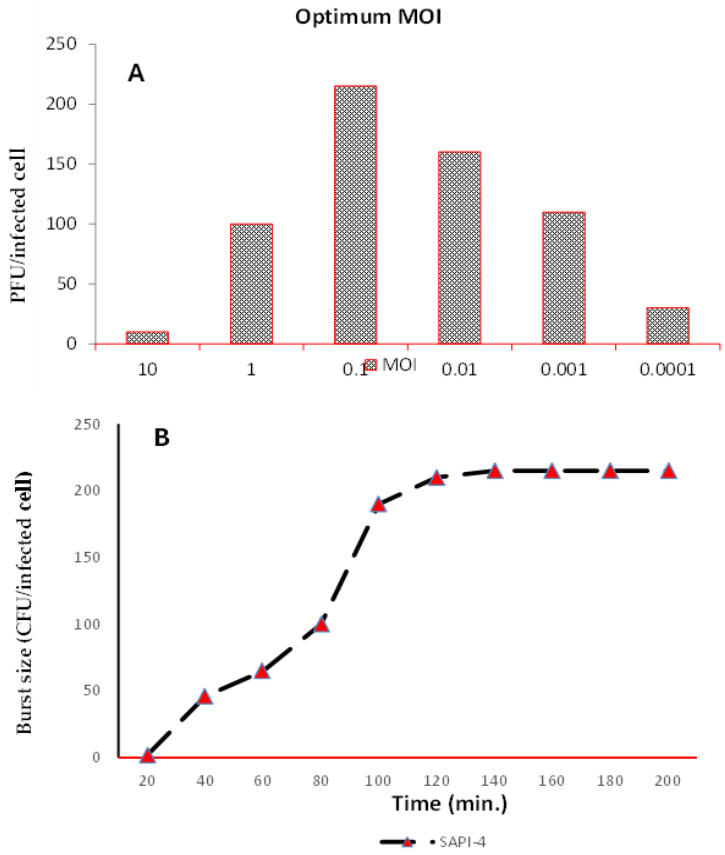

Fig 3. A- MOI of SAPI-4 phage, B- values of burst size and latent period.

Morphological Characteristics of SAPI-4 Phage

The features of SAPI-4 plaques were transparent, large and spherical in shape, with diameters ranging from 3 to 4 mm (Figure 4A). Additionally, the SAPI-4 phage's morphological appearance was revealed by electron microscopy. The phage had a hexagonal head that measured 63.27 nm in diameter, a long, contractile tail that measured 161.52 nm in length and 14.26 nm in width, and a terminal spike that measured 108.61 nm (Figure 4B). The International Committee on Taxonomy of Viruses created the current classification scheme, which places phage SAPI-4 in the *Myoviridae* family of the *Caudovirales* order. The usual morphology of phage aggregates, which organize into clusters, was also depicted (Figure 4C).

pH Stability and Thermostability of SAPI-4 Phage

The SAPI-4 phage's ability to survive was evaluated throughout a broad pH range (3, 5, 7, 9, and 11) and over various time intervals (1, 3, 5, 7, 12, and 24 hours). Figure 4A demonstrated unequivocally that SAPI-4 phage remained significantly ($p < 0.05$) stable in the face of acidic conditions. Whereas there is no a notable drop in titer at different pH values after 1 h, while after 24 h of incubation roughly 50% (4.72±0.71 $\log_{10}$ PFU/mL of 9±0.63 $\log_{10}$ PFU/mL) of phage infectivity is declined at pH 11 (Figure 5A). In another way the SAPI-4 phage also showed the highest levels of activity following a one-hour treatment at -20, 4, 25, 37, 45, and 55 °C. At 65 °C, there was a discernible start to the significant ($p < 0.05$) titer decline, with titers falling to 4.0±0.65 and 3.1±0.72 at 12 and 24 hours, respectively. After 12 hours at 75°C, a significant ($p < 0.05$) titer reduction was observed, with just 1.0 ± 0.41 $\log_{10}$ PFU/mL remaining from the original phage titer of 9 ± 0.74 $\log_{10}$ PFU/mL. Full inactivation was detected after 24h at 75°C (Figure 5B).

Table 3: Evaluation of host range using spot test and the EOP profile

Strains	Five Virulent *S. aureus* Phages									
	SAPI-1		SAPI-2		SAPI-3		SAPI-4		SAPI-5	
	ST	EOP	ST	EOP	ST	EOP	ST	EOP	ST	EOP
S. aureus strains										
SAM7	+	H	+	H	+	H	+	H	-	N
SAM8	-	N	-	N	-	N	+	M	-	N
SAM9	-	N	-	M	-	N	+	H	+	M
SAM10	+	H	+	M	+	H	+	H	-	N
SAM11	+	M	-	N	+	M	+	H	-	N
SAM12	+	H	+	H	+	H	+	H	+	H
SAM13	-	N	-	N	-	N	+	M	-	N
SAC9	+	H	-	N	+	M	+	H	+	M
SAC10	+	M	+	H	_	N	+	H	-	N
SAC11	-	N	-	N	+	H	+	H	-	N
SAC12	+	H	+	H	-	N	+	H	+	H
SAC13	+	H	+	M	+	H	+	H	-	N
SAC14	-	N	-	N	-	N	+	M	+	H
SAC15	+	H	-	N	+	H	+	H	-	N
SAC16	-	N	+	M	-	N	+	H	-	N
SAC17	+	M	-	N	-	N	+	H	+	H
MRSA	-	N	-	N	-	N	+	M	-	N
VRSA	-	N	-	N	-	N	+	H	-	N
Non-*S. aureus* strains										
Salmonella sp.	-	N	-	N	-	N	-	N	-	N
Salmonella sp.	-	N	-	N	-	N	-	N	-	N
Escherichia coli	-	N	-	N	-	N	-	N	-	N
Escherichia coli	-	N	-	N	-	N	-	N	-	N
Klebsiella pneumoniae	-	N	-	N	-	N	-	N	-	N
Bacillus cereus	-	N	-	N	-	N	-	N	-	N

EOP stands for efficiency of plating, ST stands for spot test, + for positive lysis by the phage, - for negative lysis by the phage, H: high EOP between 0.5 and 1.0 N: no EOP (inefficient) <0.001, L: low EOP from 0.001-0.1, and M: moderate EOP from 0.2-0.4.

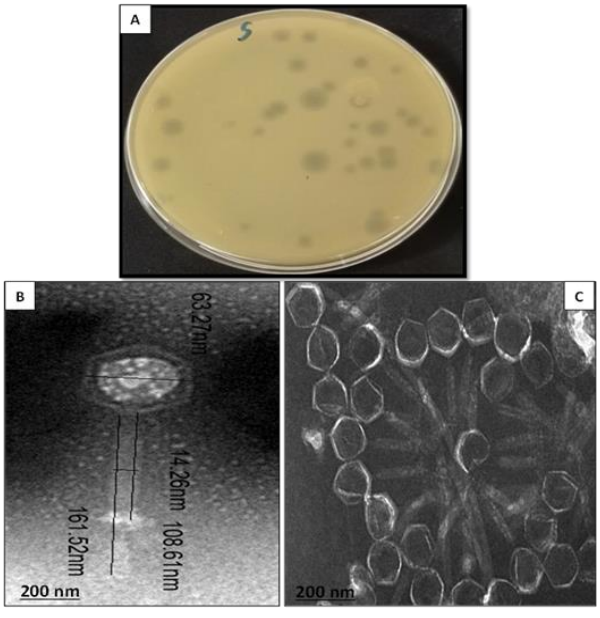

Fig 4. Morphological features of SAPI, A; transparent, large and spherical plaques. Transmission Electron Microscopy (TEM) at 80,000× of, SAPI-4 virion shape, and C; SAPI-4 phage aggregation.

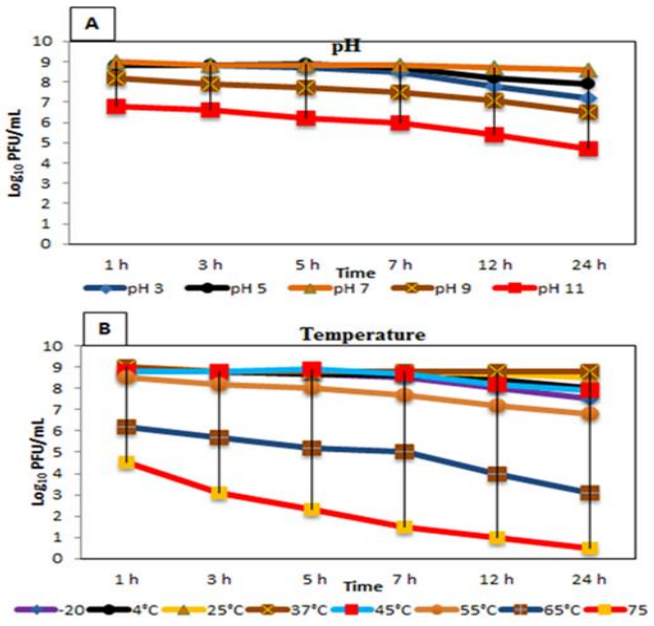

Fig 5. Virion persistence of the SAPI-4 phage throughout 1, 3, 5, 7, 12, and 24 hours (**A**) at various pH values (3, 5, 7, 9, and 11) (**B**) at various temperatures (-20, 4, 25, 37, 45, 55, 65 and 75 °C).

Bacteriolytic Challenge activity of SAPI-4 phage in vitro study

Potential of Bacterial Culture Clearance

It was found that the host bacteria (SAC9) that had been phage treated was growing less quickly than the host that had not been phage treated. Without the phage, SAC9 grew as usual and increased progressively over the course of a day (2.232±0.24 O.D.620). On the other hand, growth in the flask that was exposed to phage started out slowly and was impacted by the phage addition. By the eighth hour of incubation, however, growth had totally stopped (Figure 6A). Using the agar plates and colony counting method, this experiment was confirmed. After eight hours of incubation, we found no bacterial colonies on the plates treated with the phage, whereas after 24 hours, there were 7.22 ± 1.04 Log_{10} bacterial colonies in the dishes that were not treated with the phage (Figure 6B).

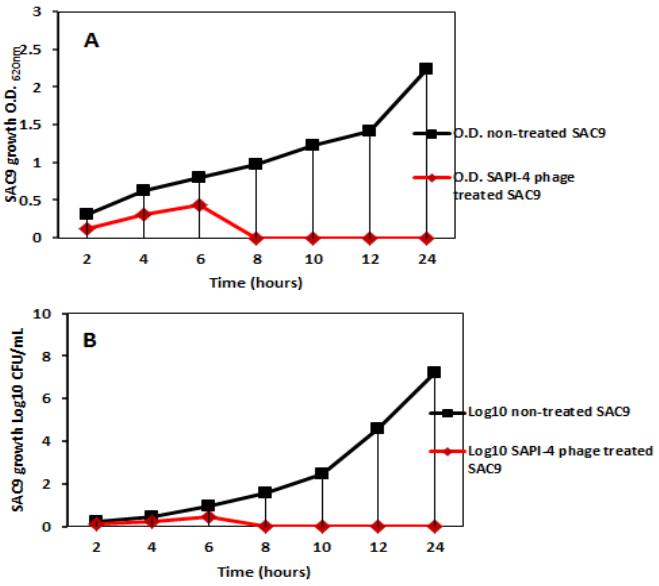

Fig 6. *S. aureus* (SAC9) growth over different time periods, **A**; SAC9 growth $O.D._{620}$, **B**; SAC9 growth Log_{10} CFU/mL. Black line; represent non-treated SAC9; red line; represent phage-treated SAC9.

Using a 96-Well microtiter tissue culture plate, spectrophotometry ($O.D_{620}$nm) was used to measure the lytic activity of SAPI-4 against SAC9. We can precisely ascertain the period at which the phage inhibits bacterial growth because of the way the time was divided. It was discovered that SAC9 without phage treatment (+ve control wells) showed a steady increase in absorbance over the course of the incubation period (24 hours), whereas the optical density of SAC9-phage treated wells showed no growth at 390 min or at any other time (Figure 7). The results obtained made it abundantly evident that SAPI-4 might be applied as a biological disinfectant to manage *S. aureus* in the environment.

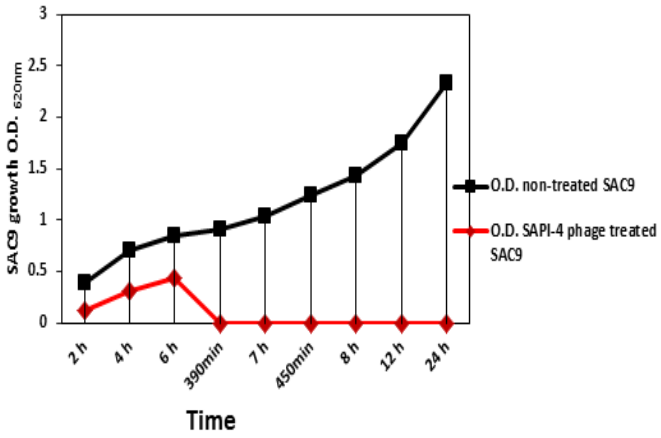

Fig 7. Lytic activity ($O.D._{620}$) of SAPI-4 against SAC9 at different time periods, using a 96-well microtiter tissue culture plate. Black line; represent non-treated SAC9; red line; represent phage-treated SAC9.

In vivo Study

Challenge in Raw Milk

In a simulation of SAPI-4 phage application in preserving food samples, it has been demonstrated that milk samples inoculated with bacteria (SAC9) and treated with SAPI-4 phage can effectively combat these germs and lessen their impact. Since there were no bacterial colonies after 6 hours, while SAPI-4 phage- free plates (+ ve control) showed a count of 1.08 ± 0.16 Log_{10} CFU/mL (Figure 8), it was evident that the phage had a remarkable capacity to totally stop bacterial development. Furthermore, no growth was observed throughout the remaining incubation periods, and the naturally occurring expulsion of bacteria that had not been treated with phage was observed (7.1 ± 1.12 Log_{10} CFU/mL at 24 hours), (Figure 8).

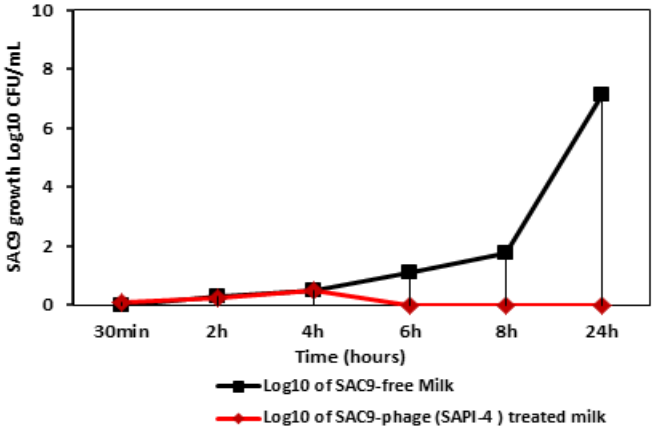

Fig 8. Preservative efficacy of SAPI-4 phage against inoculated SAC9-raw milk. Black line; represents SAPI-4 phage- free SAC9; red line; represents SAPI-4 phage-treated SAC9.

Challenge in Beef Burger Cuts

Short-Term Storage Efficacy and Stability

As established by examining the beef burger samples at the experiment's specified intervals. When compared to the samples that were not treated with the SAPI-4 phage, the growth of SAC9 was 1.64 ± 0.22 Log_{10} CFU/cm2 on day two of the experiment and grew to 6.21 ± 0.97 Log_{10} CFU/cm2 on the seventh and final day (Figure 9). Conversely, samples that were treated with the SAPI-4 phage then injected with SAC9 demonstrated extremely encouraging outcomes. It was discovered that there were significant effects on the growth of the bacteria, with growth on the second day being 0.19 ± 0.07 Log_{10} CFU/cm^2, and growth reaching a maximum of just 0.46 ± 0.04 Log_{10} CFU/cm^2 on the seventh and final day (Fig. 3B). Measuring phage stability throughout the experiment revealed that the starting phage count (8 log_{10} PFU/cm^2) was not entirely impacted until the seventh and final day (7.65± 1.14 log_{10} PFU/cm^2) (Figure 9).

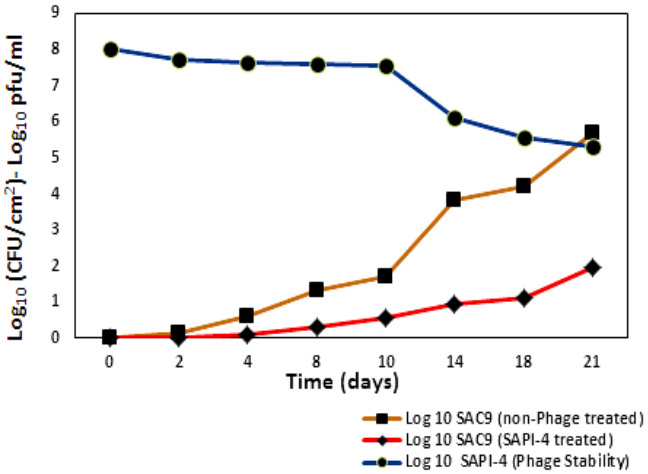

Fig 9. Short-term (7 days) efficacy and stability of SAPI-4 phage against inoculated SAC9- beef burger cuts. Brown line; represents non-phage treated SAC9, red line; represent SAPI-4 phage-treated SAC9, and blue line; represent SAPI-4 phage virions stability.

Long-Term Storage Efficacy and Stability

On day two of the experiment, the growth of SAC9 in the beef burger samples that were not treated with the SAPI-4 phage was 0.14 ± 0.01 Log_{10} CFU/cm^2, and on day eight, it had grown to 1.34 ± 0.17 Log_{10} CFU/cm^2. The number of bacteria was 1.34 ± 0.09 Log_{10} CFU/cm^2 two weeks after the experiment began and 5.71 ± 0.09 Log_{10} CFU/cm^2 on the experiment's final day. Conversely, samples treated with the SAPI-4 phage did not exhibit any growth on day two. On day 8, there was hardly much growth, with a count of 0.32 ± 0.05 Log_{10} CFU/cm^2. SAC9 count was 1.98 ± 0.15 Log_{10} CFU/cm^2 and 0.97 ± 0.03 Log_{10} CFU/cm^2 on days 14 and 21, respectively (Figure 10). After two weeks of measurement (6.11 ± 1.04 log_{10} PFU/cm^2), the count of SAPI-4 phage was very faintly influenced. It was found that the count was not affected until day 10 (7.54 ± 1.17 log_{10} PFU/cm^2) of the experiment. The phage was not significantly altered at the remaining times; at 18 and 21 days, the counts were 5.57 ± 1.15 log_{10} PFU/cm2 and 5.33 ± 1.11 log_{10} PFU/cm^2, respectively (Figure 10).

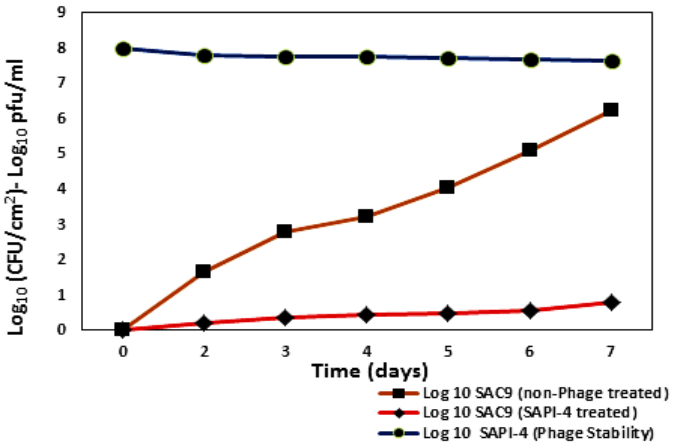

Fig 10. Long-term (21day) efficacy and stability of SAPI-4 phage against inoculated SAC9- beef burger cuts. Brown line; represents non-phage treated SAC9; red line; represent SAPI-4 phage-treated SAC9, and blue line; represent SAPI-4 phage virions stability.

Biofilm Attenuation Action of SAPI-4 Phage

For each of the four isolates that were examined, the SAPI-4 phage demonstrated a potent effect against biofilm; the effect varied from 93% at the highest dose (10^7 PFU/mL) to 21% at the lowest dose (10^4 PFU/mL), it showed an effect. The impact appeared to be dependent on the phage dose, since the phage was able to eliminate 87%, 93%, 84%, and 91% of the biofilms of SAC9, (Figure 11A) SAM7 (Figure 11B), ASC14 (Figure 11C) and SAC11 (Figure 11D), respectively, at a concentration of 10^7 PFU/mL.

The phage showed a significant ($p < 0.05$) reduction effect against the biofilm of the four isolates at concentration 10^6 PFU/mL and 10^5 PFU/mL. However, at concentration of 104 PFU/mL the effect on the biofilm was moderate to weak, with percentages of 23%, 34%, 21%, and 24% against SAC9, (Figure 11A) SAM7 (Figure 11B), ASC14 (Figure 11C) and SAC11 (Figure 11D), respectively. None of the four isolates' biofilms showed any effects at the final concentration (10^3 PFU/mL).

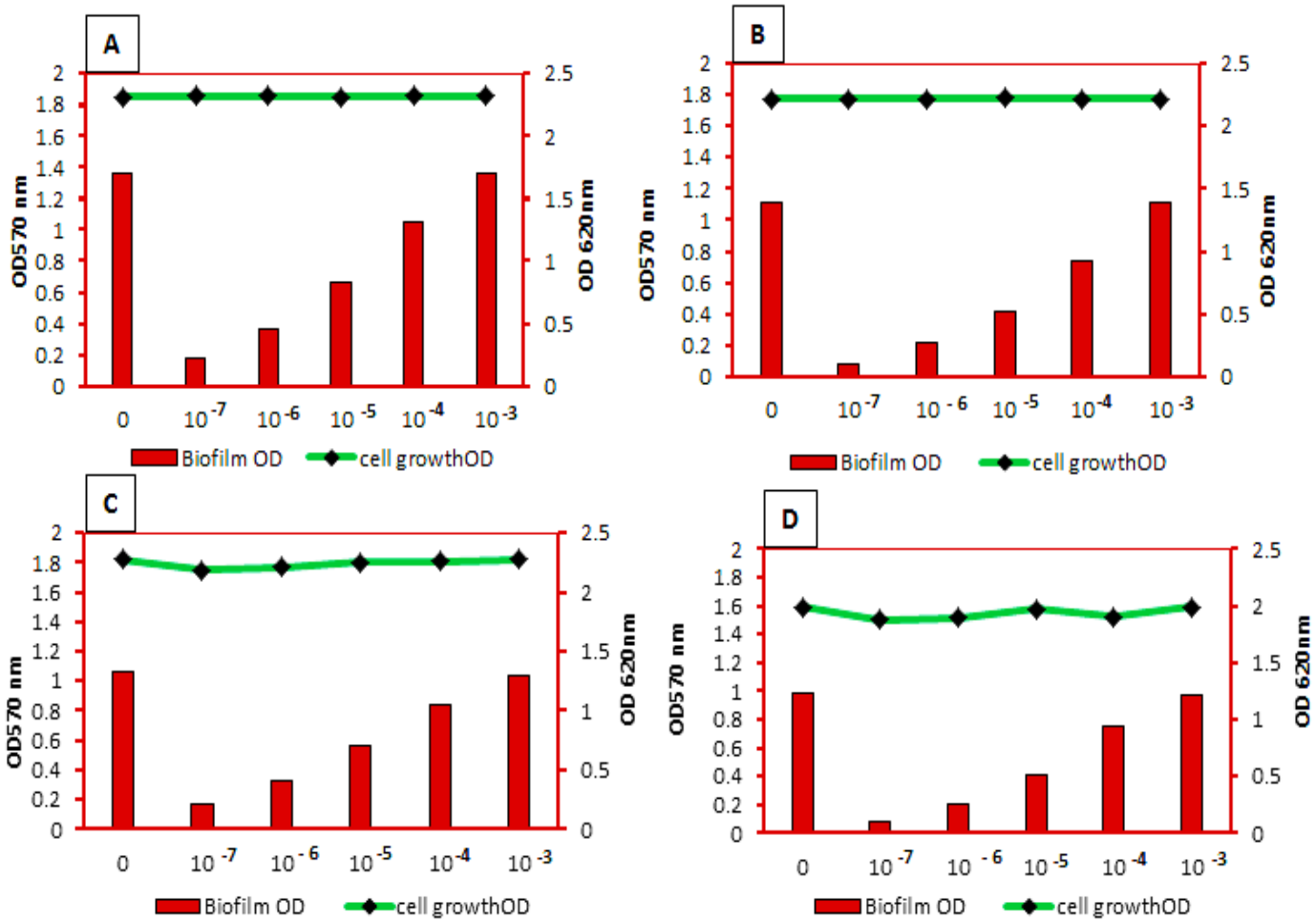

Fig 11. SAPI-4's qualitative anti-biofilm impact against *S. aureus* isolates utilizing the Tissue Culture Plate (TCP) method. SAC9 biofilm (**A**); SAM7 biofilm (**B**); ASC14 biofilm (**C**), and Biofilm SAC11 (**D**). The test was conducted using a 96-well microtiter plate, with 0.2 mL of bacterial solution (2×10^6 CFU/mL) in TSB containing 0.25% glucose in each well. There were two types of control wells on the plate: -ve control (media only) and +ve control (media with bacteria). Anti-biofilm wells (Media with bacteria and phage) were also present on the plate. The plate was washed with PBS (pH = 7.2) after being incubated at 37 °C for the entire night while being shaken. After fixing the biofilm with 95% ethanol, the biofilm was stained with 0.1% crystal violet. For solubilization, the dye was dissolved in 1% w/v SDS, and the optical density was measured at 570 nm.

Discussion

Health and the economy are facing increasing challenges due to the worldwide problem of antibiotic resistance (Tondi 2021). The World Health Organization forecasts that if effective replacements to present medicines are not found, antimicrobial resistance (AMR) might result in up to 10 million annual deaths and a global cost of USD 100 trillion by 2050 (O'Neill 2014). *Staphylococcus aureus* is regarded as one of the most significant pathogens, causing nosocomial infections that impact the soft tissues, respiratory system, skin, and bones. Given that antibiotic multidrug resistance is a common occurrence among the isolates, these infections often pose a difficult challenge (Chambers and DeLeo 2009). According to many previous studies (Stewart and Costerton 2001), biofilm increases and strengthens bacterial antibiotic resistance in many. Phage therapy can be a suitable alternative therapy, particularly for bacteria that are resistant to many drugs. It has numerous potential uses in both human and veterinary medicine (Golkar et al. 2014). This work emphasizes the potential utility of phage therapy as a treatment for Staphylococcal infections, which have proven difficult to cure with commonly used antibiotics. Additionally, phages are very successful at

reducing and controlling bacterial biofilms (Knezevic and Petrovic 2008).

Based on the current data, most *S. aureus* isolates were resistant to the Aminoglycoside group, while were susceptible to the Monobactam class. Followed by groups of macrolides, penicillin, and quinolones. The most resistant isolate, SAC9, was found to be resistant to 10 of the 13 (77%) antibiotics. SAM7 and SAC14, on the other hand, were both found to be resistant to 9 of the 13 (69%) antibiotics, and SAC11 was found to be resistant to 8 of the 13 (61.5%). Our results are consistent with a prior investigation by Akya et al. (2020), which revealed that *S. aureus*, a pathogen of great importance in clinical and community settings, possesses a well-known resistance to penicillin and other antimicrobials. In addition to, the current study corroborated those of another study (Islam et al. 2021) that demonstrated *S. aureus*'s increased resistance to the majority of antibiotics, particularly penicillin.

This resistance is brought on by the β-lactamase enzymes that are produced; the first record of a *S. aureus* strain resistant to penicillin was published in 1945 (Willis et al. 2022). Moreover, the primary causes of *S. aureus*'s development of antibiotic resistance have been identified as the alterations of drug binding sites on molecular targets, the up regulation of efflux pumps, and the acquisition of genetic determinants through the horizontal gene transfer of mobile genetic elements (Mlynarczyk-Bonikowska et al. 2022). However, in recent years, only a small number of antibiotics with unique chemical classes have become available. Here, isolates of *S. aureus* demonstrated its sensitivity. These antibiotics attach to the dipeptide D-Ala4-D-Ala5 of lipid II, preventing the transglycosylation and transpeptidation that are carried out by PBP2 and PBP2a, two proteins that are necessary for the synthesis of bacterial cell walls and that have the ability to stop peptidoglycan remodeling (Zeng et al. 2016, Akya et al. 2020). Additional factors that impact and exacerbate this issue in the same setting include the over prescription of antibiotics, drug purchases made over the counter, medications advised by unofficial health care practitioners, polypharmacy practices, etc (Ansari et al. 2014). Due to restricted access to and availability of regular health care, such illogical antimicrobial usage is much more prevalent in remote and rural locations (Yau et al. 2021).

Most *S. aureus* isolates in this study had a high capacity for biofilm formation. We found that 10 out of 16 (62.5%) isolates had a considerable ability to build biofilm. This is because *S. aureus* is thought to be the classic example of a Gram-positive bacterium with the ability to form biofilms. It is noteworthy that there exists a positive correlation between the ability to form biofilm and antibiotic resistance. The earlier data aligned with our prior understanding gained from reading numerous scholarly publications and from our hands-on experience.

Numerous investigations corroborate our findings, demonstrating that *S. aureus* can stick to implant surfaces, aggregate, and create an extracellular polymeric substance (EPS) matrix due to the activation of certain genes (Laverty et al. 2013). Furthermore, it has been discovered that using antibiotics to treat persistent infections is less effective against biofilm bacteria (Sharma et al. 2019). According to Neopane et al. (2018), 86.7% of *S. aureus* samples showed the capacity to form biofilm, which is in line with our data. Even while their investigations yielded a higher percentage of biofilm-producing *S. aureus* isolates than our current study, we are definitely moving in the same direction.

Furthermore, the previously mentioned study supported our assertion that bacteria that form biofilms can exhibit greater resistance than those that do not. Remarkably, all *S. aureus* isolates that did not produce biofilm were non-MDR, but 86.7% of those that did produced biofilm (Neopane et al. 2018). Similar research was conducted on fever patients by Gupta et al. (2015), who discovered that 84.28% of the *Staphylococci* isolates were biofilm-producing bacteria. According to Gupta et al. (2015), of those, 21.4%, 62.8%, and 15.8% were strong, moderate, and non-biofilm producers, respectively.

Many bacteria that are resistant to drugs create biofilms as a defense mechanism and a means of surviving in harsh environments. According to Young et al. (2002), they are connected to the development of virulence factors and antibiotic tolerance.

Because biofilms are more resilient to the host's immune responses than planktonic cells, biofilm infections are particularly challenging to treat (Leid 2009). Furthermore, it has been discovered that using antibiotics to treat persistent infections is less effective against biofilm bacteria (Sharma et al. 2019). According to (Stewart and Costerton 2001), biofilm-conferred antibiotic resistance mechanisms include: (1) decreased antibiotic penetration through the biofilm matrix; (2) differentiation of specific biofilm cells into a protected phenotype; (3) up regulation of antibiotic-efflux pumps; and (4) antibiotic inactivation from beta-lactamase enzymes.

From all sewage-isolated Staph phages, selecting lytic phages was our goal; non-lytic phages were excluded from our investigation. Two phages for SAM7 and one each for SAC9, SAC11, and SAC14 were selected. When the Staph phages were exposed to the bacterial isolates utilized in the host range assay, the lysis results varied.

With a high EOP of 77.77% (14/18) and the potential to infect 100% of the *S. aureus* isolates, the SAPI-4 phage was the most extensively disseminated across its host range. It is known that sewage water contains a large number of bacteriophages. Hospital sewage and waste water treatment plants provide a very specific habitat that is home to resistant bacteria (Mattila et al. 2015).

According to earlier research, many phages were identified from wastewater specific to a variety of bacteria, such as *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Acinetobacter* sp., *Klebsiella* sp., *Enterococcus faecalis*, and coagulase-negative *Staphylococci* strains (Kwiatek et al. 2012, Sofy et al. 2020, Abdelhadi et al. 2021, Sofy et al. 2021)

According to Wang et al. (2016), fecal sewage included significant levels of staphylophages. Tan et al. (2020) demonstrated the ease of separating bacteriophages from sewage in another investigation. Conversely, a study by Mattila et al. (2015) demonstrated that phages against Staph were difficult to separate from sewage drains. The host density and surrounding environmental factors affect the effectiveness of recovering bacteriophages that can destroy specific pathogenic bacteria (Echeverría-Vega et al. 2019).

The morphological characteristics of the SAPI-4 phage assigned to the *Myoviridae* family within the *Caudovirales* order. The SAPI-4 phage was shown to have a burst size of 215 PFU/infected cells and latency duration of 40 to 120 minutes. Several phages from the order *Caudovirales* are obligatorily lytic on *S. aureus* and cannot transfer bacterial DNA. Most of them have a complex virion structure comprising a head and long contractile tail (Barylski et al. 2020).

According to a previous study, the best candidates for phage therapy are Staphylococcal phages belonging to the *Myoviridae* family since they typically have lytic properties and a wide host range (Melo et al. 2018, Ingmer et al. 2019).

Phage variations in host range could result from altered restriction endonucleases, non-specific binding receptors (Pires et al. 2016), and the development of phage resistance via a number of different mechanisms, referred to as insensitive bacterial mutants (BIM) (O'Flynn et al. 2006).

The high replication efficiency and short latent time are most likely caused by the high processivity of phage DNA polymerase (Lavigne et al. 2009). The SAPI-4 phage was clearly stable ($p < 0.05$) in the presence of acidic conditions, as demonstrated by its pH and thermal persistence; nevertheless, after 24 hours of incubation, at pH 11, about 50% (4.72 ± 0.71 log10 / 9 ± 0.63 $\log_{10}$ PFU/mL) of its infectivity reduced. The thermostability of the *Caudovirales* bacteriophages was demonstrated by a prior study (Jończyk et al. 2011), which is consistent with our findings. Likewise, the environment's acidity and alkalinity are significant variables impacting the stability of the phage. In agreement with other research, phages exhibit stability across a range of temperatures, pH values, and biotic environments (Ahmadi et al. 2017, Sommer et al. 2019). Furthermore, our results concur with those of Jamalludeen et al. (2007). It was determined that most phages were structurally unaffected by pH values ranging from 5 to 9. On the other hand, high concentrations might cause the phages to thicken, deposit, or aggregate (Jepson and March 2004).

According to our research, the SAPI-4 phage exhibited potent bacteriolytic activity against the inoculated SAC9 isolate in TSB media, where the growth completely stopped after the eighth hours of incubation. Furthermore, no Staph growth was observed at 390 minutes or at any other point during the experiment, according to spectrophotometric measurements of SAPI-4's lytic activity in 96-Well microtiter tissue culture plates. Our findings were in line with those found in earlier research, which claimed that phage K, a well-known *S. aureus* phage, had 79% lytic activity on the strains tested (Abatángelo et al. 2017). In one section of our work, we concur with O'Flaherty et al. (2005), who did in fact find two lytic Staph phages; however, in another section, we disagree, since his isolates were linked to the Siphoviridae family.

In addition, we looked at the SAPI-4 phage's bacteriolytic activity in raw milk and beef burger sections to determine a gradual adaptation to the in vivo conditions. It has been shown that milk samples treated with the SAPI-4 phage and injected with bacteria (SAC9) can successfully fight off this bacterium and decrease their effects. After six hours of treatment, there was no bacterial growth, showing that the phage had an amazing ability to completely inhibit bacterial development. Moreover, during the remaining incubation times, no growth was seen. Conversely, samples of beef burgers injected with SAC9 and treated with the SAPI-4 phage showed very promising results. With high significant stability of phage virions, it was found that there were notable effects on the growth of the bacteria in both short (7 days) and long-term storage periods.

In order to assess the lytic activity of the SAPI-4 phage in food samples with regard to a potential application in the food sector, it was important to consider the complex medium of proteins, lipids, carbohydrates, and other elements that make up beef burgers. Moreover, milk is a polydisperse medium that is complex and contains lipids, enzymes, lactose, caseins, whey proteins, minerals, trace elements, and vitamins (García et al. 2009). Therefore, in order to prepare for a potential

intramammary application in mastitis therapy, it was required to investigate the lytic activity of SAPI-4 phage in milk.

Similar to our investigation, but with different findings, a prior study (Krömker 2006) looked at the bactericidal action of bacteriophages against *S. aureus* in milk. The phage titer or phage bactericidal activity in raw milk decreased, according to earlier studies, however our data demonstrated high lytic activity with phage stability. This could be explained by a reduction in the phage's capacity to bind to the host cells in raw milk (O'Flaherty et al. 2005, García et al. 2009).

Wall teichoic acid (WTA) is necessary for the adsorption and infection of all staphylococcal phages that are currently known (Xia et al. 2011, Li et al. 2015). The majority of gram-positive bacteria have a peptidoglycan layer that is connected to WTA, an anionic glycopolymer (Brown et al. 2013). The majority of *S. aureus* strains' WTA is composed of 40–60 phosphodiester-linked polyribitol phosphate units, which are further embellished with D-alanine, α-GlcNAc, or β-GlcNAc alterations (Takeuchi et al. 2016). While Myoviridae have the widest host range because, like phage K, they attach to both the WTA backbone and α-GlcNAc-modified WTA (Xia et al. 2011, Takeuchi et al. 2016). Furthermore, during infection, peptidoglycans in the bacterial cell walls are locally broken down by phage structural elements called virion-associated peptidoglycan hydrolases (VAPGHs) (Rodríguez-Rubio et al. 2012). N-acetylmuramoyl-L-alanine amidase and holin were also included in the lysis cassette of the phages. Similar to signal peptides, holin is a tiny phage encoding protein that modifies permeability by creating big holes in the cell membrane (White et al. 2011). Three subtypes can be distinguished from it: class I, class II, and class III (Shi et al. 2012). Furthermore, three domains were found in several phage ORFs predicted to encode N-acetylmuramoyl-L-alanine amidase: the N-terminal CHAP endopeptidase domain, the PGRP super-family conserved domain, and the SH3 peptidoglycan-binding domain. Endolysin's role is responsible for the particular lysis of N-acetylmuramoyl-L -alanine amidase, while holin devotes itself to the amidase activation at a predetermined moment (Zhang et al. 2017). It is believed that the holin-endolysin lytic system, which is shared by practically all dsDNA phages, lyses the host cell to release the progeny of the phage (Oliveira et al. 2013, Zhang et al. 2017).

The phage demonstrated a significant ($p < 0.05$) decrease in biofilm formation from the four isolates when applied at concentrations of 106 and 10^5 PFU/mL. On the other hand, the biofilm of all four isolates exhibited no effects at the final concentration (10^3 PFU/mL), while at a concentration of 10^4 PFU/mL; the effect was moderate to mild.

A number of previous studies have shown the potential utility of bacteriophages in combating bacterial infections and their capacity to break up biofilms, given the global rise in antibiotic resistance brought on by usage (Gorski et al. 2009, Kraushaar et al. 2013).

Complementing our results, Lungren et al.'s study (Lungren et al. 2013) showed a noteworthy reduction in *S. aureus* biofilm formation on the surface of bacteriophage-treated central venous catheter material when compared to untreated controls, indicating the efficacy of bacteriophage K against staphylococci.

Additionally, it has been shown that *P. aeruginosa* biofilms can be successfully eliminated by phage therapy in a model of animals with cystic fibrosis (Waters et al. 2017). Additionally, it has been shown that phages are efficient against oral biofilms, such as *Fusobacterium nucleatum*, *Enterococcus faecalis*, and *Streptococcus* spp., that cause periodontal, peri-implant, and caries infections (Szafrański et al. 2017).

Phages have the ability to aggressively infiltrate and disrupt biofilms in the natural world, which makes them useful for obtaining targeted and enhanced treatments against biofilms (Domingo-Calap and Delgado-Martínez 2018). In the fight against biofilms, phages expressing EPS-degrading enzymes are particularly interesting. Phage-encoded depolymerases are enzymes that selectively break down elements of the EPS matrix, facilitating phage penetration (Cornelissen et al. 2011). The phage carriage of EPS depolymerases is important to the interpretation of the impact of phages on biofilms as these enzymes can disrupt biofilms without associated phage-induced bacterial lysis (Tait et al. 2002).

EPS depolymerases have the ability to break down the structure of biofilms (Latka and Drulis-Kawa 2020) or prevent biofilm development (Guo et al. 2017). According to Olszak et al. (2017), EPS depolymerases can also create tunnels across the biofilm matrix, increasing diffusion through the biofilm structure without significantly reducing biofilm biomass. By increasing the availability of bacteria for phage adsorption, for example, EPS depolymerase activity may also increase the risk of phage infection and related bacterial lysis (Darch et al. 2017). A related problem is that decreased biofilm bacterial viability is the cause of cell count reductions in the presence of phage EPS depolymerase activity. Phage biofilm removal results can be analyzed from an ecological and therapeutic standpoint.

Conclusion

We may conclude from the above findings that the SAPI-4 phage, which is specific to SA/MRSA isolates

and possesses a strong bacteriolytic (infect 100% of *S. aureus*) and anti-biofilm ability (eradicate 87%, 93%, 84%, and 91% of the biofilms of SAC9, SAM7, ASC14, and SAC1, respectively.), was effectively isolated from sewage. Belonging to the *Myoviridae* family, the phage exhibited a broad host range and a high efficiency of plating (EOP). Research on phage physiology has yielded positive indicators for phage tolerance over a wide pH range and at various temperatures. We also intend to use next-generation sequencing (NGS) and in-vivo in animal infection models to validate the phages' therapeutic potential.

Conflict of interest

The authors declare that they have no conflict of interest.

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