Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 22 No.1 Ser. No. 74 2021

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

High efficacy of a characterized lytic bacteriophage in combination with thyme essential oil against multidrug-resistant *Staphylococcus aureus* in chicken products

Abdallah, K.[1]; Tharwat, A.[1] and Gharieb, R.[2*]

[1]Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt (current address); [2]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt

[*]***Correspondence:*** *R. Gharieb, Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, 44511, Zagazig, Egypt. E-mail: ghariebrasha@gmail.com*

10.22099/ijvr.2020.38083.5543

(Received 8 Aug 2020; revised version 27 Oct 2020; accepted 9 Nov 2020)

Abstract

Background: The emergence and spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in the food industry have led to using alternative natural bioagents for controlling *S. aureus* in food. **Aims:** The current work aimed to isolate and characterize a lytic phage specific to *S. aureus* and evaluate its efficacy with thyme essential oil for controlling *S. aureus* growth in chicken fillets. **Methods:** Twenty *S. aureus* strains previously isolated from ready-to-eat chicken products were tested for antimicrobial susceptibility and used for phage isolation. **Results:** All *S. aureus* strains were multidrug-resistant (MDR). The isolated phage (vB_SauM_CP9) belonged to the family *Myoviridae* and maintained its stability at pH (4-9) and temperature (30-70°C). The phage showed lytic activity on ten *S. aureus* strains and had a burst size (228 PFU/infected cell), latent period (45 min), and rise period (15 min). A combination of *S. aureus* phage multiplicity of infection (MOI) 10 + thyme oil 1% caused a higher significant reduction in *S. aureus* growth (87.22%) in artificially inoculated chicken fillets than individual treatment with bacteriophage or thyme essential oil. **Conclusion:** To our knowledge, this is the first report to evaluate the efficacy of bacteriophage and thyme oil for controlling the growth of MDR *S. aureus* in chicken products and recommending application of *S. aureus* phage and thyme oil combination in the food industry to achieve food safety goals and consumer protection as well as mitigate the antimicrobial resistance crisis.

Key words: Bacteriophage, Biocontrol, Chicken products, *Staphylococcus aureus*, Thyme essential oil

Introduction

Raw and ready-to-eat poultry products have gained popularity in recent times because they are considered excellent sources of high-quality protein, minerals, and vitamins that are essential to optimal human growth and development. Contamination of these products with foodborne pathogens such as enterotoxigenic *Staphylococcus aureus* remains an important public health concern, causing human food poisoning that varies in severity from mild to fatal disease when a sufficient amount of one or more performed enterotoxins were consumed in contaminated food (Le Loir *et al*., 2003). Enterotoxigenic *S. aureus* strains can contaminate food products at different stages of the food chain from production until consumption due to improper handling of these products (Crago *et al*., 2012). The use of antibiotics and other antimicrobial agents throughout the food chain contributes to the growth of resistant bacteria that could be transferred directly to humans. The emergence and spread of methicillin-resistant *S. aureus* (MRSA) from food and food products to consumers put humans at potential risk of acquiring the strains that resist antibiotic treatment (Apata, 2009; Hammerum *et al*., 2010). A combination of natural antimicrobial agents represents one of the most promising approaches for combating multidrug-resistant (MDR) bacteria in food. Hence, application of potential antimicrobial alternatives such as essential oils and host-specific bacteriophages (phages) in food industry can reduce bacterial load and increase the safety and shelf life of food products (Burt, 2004; Bajpai *et al*., 2012; Ghosh *et al*., 2016). Essential oils are natural plant extracts composed mainly of bioactive components such as terpenes (monoterpenes and sesquiterpenes), terpenoids (oxygenated compounds as phenols, alcohols, aldehydes, ketones, or ethers), and aromatic compounds (Tongnuanchan and Benjakul, 2014). These bioactive compounds possess biological, antioxidant, and antimicrobial properties which are particularly attributed to the phenolic compounds (Bakkali *et al*., 2008; Bassolé and Juliani, 2012). Among the wide variety of essential oils, thyme oil (*Proteus vulgaris*), is widely used in the food industry mainly for its flavor, aroma, as well as the strongest antimicrobial activity against MDR pathogenic and spoilage bacteria in food (Anžlovar *et al*., 2014). The main constituents of

thyme oil are phenolic components such as thymol (23-60%), γ-terpinene (18-50%), p-cymene (8-44%), carvacrol (2-8%), and linalool (3-4%) (Pecarski *et al*., 2016).

Bacteriophages are viruses that specifically adsorb to the target bacteria, multiply and exhibit either lytic (results in cell lysis and the release of new progeny phages) or lysogenic (the phage DNA is incorporated into a host cell without causing cell lysis) life cycle (Ackermann and Prangishvili, 2012). The increasing incidence of foodborne illnesses and prevalence of antibiotic-resistant bacteria have led to the use of lytic (virulent) phages as an alternative biocontrol agent in food industry to assure the safety of food products and maintain the One-Health strategy (Sulakvelidze, 2013; Kittler *et al*., 2017).

Since there are no previous studies that focused on the biocontrol of *S. aureus* in poultry industry using bacteriophage or essential oils, the current study aimed to isolate and characterize a lytic bacteriophage that targets the MDR *S. aureus.* The activity of bacteriophage and thyme oil on the growth of *S. aureus* in artificially inoculated chicken fillets was evaluated.

Materials and Methods

Staphylococcus aureus strains

Twenty-one *S. aureus* strains (20 strains from chicken products and one reference ATCC 25923 strain) were obtained from Food Control Department, Faculty of Veterinary Medicine, Zagazig University and were used as host strains in this study. *Staphylococcus aureus* strains from chicken products were previously recovered from ready-to-eat products (shawarma, pane, and burger) that were randomly collected from different restaurants and street vendors at different sanitation levels in Sharkia Governorate, Egypt. The isolation and identification procedures of *S. aureus* followed the guidelines of ICMSF (1996). The suspected colonies were examined for the colony morphology and identified based on Gram staining, motility, coagulase, thermostable nuclease production, catalase, oxidase tests. The identified colonies were confirmed by polymerase chain reaction (PCR) and kept in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) containing 20% glycerin at -80°C for further use.

Antimicrobial susceptibility of *S. aureus* strains

The antimicrobial susceptibility test of *S. aureus* strains was performed using the disk diffusion method. The bacterial suspension adjusted to the turbidity of 0.5 McFarland standard was uniformly spread on the surface of Muller Hinton agar plates (Oxoid Limited, Basingstoke, UK) and the antibiotic discs with variable concentrations (Oxoid Limited, Basingstoke, UK) were used. *Staphylococcus aureus* ATCC 25923 was used as the positive control strain. The zone diameter breakpoints and interpretive categories represent sensitive, intermediate, and resistant strains and followed the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2019). The isolates were categorized as MDR according to Magiorakos *et al*. (2012) and the multiple antibiotic resistance (MAR) index was calculated for each isolate (Pual *et al*., 1997).

Isolation of bacteriophage specific to *S. aureus*

Twenty wastewater samples collected from eight poultry slaughterhouses at different locations in Egypt were used for phage isolation using *S. aureus* host strains. Fifty ml of each sample were filtered through a 0.45 µm membrane filter and mixed with 50 ml Luria Bertani (LB) broth (Oxoid, USA) containing the log phase cells of *S. aureus* (10^8 CFU/ml) followed by overnight incubation at 37°C. The cultured broth was centrifuged at 8000 × g for 10 min and filtered through a 0.45 µm membrane filter. The presence of lytic phage in the filtrate was examined by spot test (Chang *et al*., 2005) using double layer method with some modifications (Sambrook and Russel, 2001). Briefly, 0.1 ml aliquot of overnight broth culture from each *S. aureus* isolate was individually mixed with melted soft nutrient agar 0.7% (47°C) and poured onto solid LB agar (1.5%) to form double layer agar plates. The phage suspension (10 µL) was added to bacterial lawns on double layer agar plates followed by overnight incubation at 37°C. The phage lytic activity was observed on the bacterial lawns and represented by plaques.

Purification of *S. aureus* phage

The presence of lytic phage was confirmed by plaque assay (Schnabel and Jones, 2001) using double layer method. The phage filtrate (10^9 PFU/ml) was added to the overnight broth culture of each *S. aureus*, then mixed with 3.5 ml warm molten top agar (0.7%) and poured on solid LB agar (1.5%) plates. The plates were checked for the presence of lytic phage in the form of plaques after overnight incubation at 37°C. A single plaque was picked with a sterile glass Pasteur pipette, mixed with log phase *S. aureus* culture and incubated at 37°C for 24 h. The incubated mixture was centrifuged at 12,000 × g for 10 min and filtered through a 0.45 µm membrane filter. The filtrate was subjected to three repeated rounds of single plaque isolation using the double layer method as described above. The phage was eluted by adding 5 ml of saline solution (0.85% NaCl) to the plate followed by overnight incubation at 4°C with shaking. The phage suspension was centrifuged at 12,000 × g for 10 min, filtered through a 0.45 µm membrane filter, then purified by dextran sulfate-polyethylene glycol system, and stored at 4°C until used.

Determination of *S. aureus* phage morphology by electron microscopy

A drop of the phage suspension (10^{10} PFU/ml) was placed on 200 mesh copper grids with carbon-coat form var films and the excess was drawn off with filter paper. A saturated solution of 2% uranyl acetate (pH = 4) was then placed on the grids and the excess was drawn off. Specimens were examined by a transmission electron microscopy (TEM; JEOL 100 cx) at 80 KV and

magnification (×10,000-140,000). The isolated phage was named and classified according to Kropinski *et al*. (2009) and Adriaenssens and Brister (2017).

Restriction enzyme analysis of phage genomic DNA

The phage DNA was extracted by the phenol/ chloroform method. The extracted DNA was then digested by *Eco*RI, and *Hind* III restriction enzymes (Sigma, USA) according to the instructions of the suppliers and the mixtures were incubated at 37°C for 3 h. The digested and undigested products were separated on an ethidium bromide stained agarose gel (1%) at 100 V for 2-3 h and visualized by an ultraviolet trans-illuminator.

Titer and multiplicity of infection (MOI) of *S. aureus* phage

The phage suspension was serially diluted in phosphate buffer saline (PBS; pH = 7.2) and each dilution was subjected to a single plaque assay. The plaques were counted in plates containing 50-300 plaques and the MOI for *S. aureus* phage was calculated as the ratio of phage plaque forming unit per milliliter (PFU/ml) to bacterial colony forming unit per milliliter (CFU/ml) (Lu *et al*., 2003).

The effect of pH and temperature on *S. aureus* phage stability

The effect of pH and temperature on phage stability and viability was studied following the methods of Pajunen *et al*. (2000). The filtered phage suspension was diluted to 10^6 PFU/ml in broth liquid medium adjusted to various pH degrees (3-10). The residual phage activity was determined after overnight incubation of the mixtures at 4°C using plaque assay. Moreover, the effect of temperature on phage stability was determined by incubating the phage suspension at different temperature degrees (30, 40, 50, 60, 70, 80, and 90°C) for 10 min. The phage survival was determined by plaque assay.

Host range of *S. aureus* phage

The phage host range was determined against 21 *S. aureus* strains and other 11 bacterial strains (8 *Escherichia coli*, 2 *Salmonella enteritidis*, and 1 *Listeria monocytogenes*) using spot assay and confirmed by plaque assay. The lytic activity was represented by plaques on the bacterial lawns.

Infection parameter and bacteriolytic activity of *S. aureus* phage

The ability of phage at different MOI (1 and 10) to infect *S. aureus* in liquid culture (OD_{600}=0.2) was assessed at 37°C for a period of 36 h (Lu *et al*., 2003). The growth of *S. aureus* was estimated in an infected and non-infected culture (control) by measuring the optical density (OD) at 600 nm every 4 h up to 36 h using a spectrophotometer. The experiment was performed in triplicate.

One-step growth curve assay

The diluted phage suspension was mixed with *S. aureus* cells (10^8 CFU/ml) and allowed to adsorb for 30 min at 37°C followed by centrifugation at 10,000 × g for 30 s. The pellets were resuspended in LB broth and incubated at 37°C. During incubation, 100 µL of the sample was taken at 15 min intervals up to 90 min and the titer was determined by double-layer plaque assay. The experiments were done three times. The burst size, latent period, and rise period were calculated from the growth curve as described previously (Lu *et al*., 2003).

Application of bacteriophage and thyme oil for reducing *S. aureus* in artificially contaminated chicken fillets

Preparation of thyme oil and bacteriophage

The thyme oil (*Thymus vulgaris*) was purchased from the National Research Centre, Egypt. Different concentrations (0.5 and 1%) were prepared by diluting the oil in sterile distilled water and tween 80. Moreover, the phage titer was determined as previously described and two concentrations (MOI 1 and MOI 10 PFU/ml) were prepared from the phage suspension using the double overlay method with *S. aureus.*

Preparation of chicken fillets and S. aureus inoculum

Chicken carcasses (2000 ± 45 g) were aseptically obtained from a local abattoir and the breast muscle was removed and sliced into fillets (4 × 6 × 1 cm). Twenty-five g of each fillet were tested for the presence of *S. aureus* and *S. aureus* free fillets were used for artificial inoculation and decontamination trials. Furthermore, *S. aureus* inoculum was prepared by centrifugation of an overnight incubated *S. aureus* culture in BHI broth at 4000 × g for 5 min. The supernatant was removed, and the pellet was resuspended in sterile normal saline (0.85%) followed by centrifugation. The pellet was then resuspended in saline (0.85%) and adjusted to the turbidity of 0.5 McFarland standard (10^8 CFU/ml).

Inoculation of chicken fillets with S. aureus

A 0.1 ml of the diluted *S. aureus* culture (10^8 CFU/ml) was distributed over the surface of each chicken fillet in a sterile ziploc plastic bag using a sterile insulin syringe. The ziploc was sealed and massaged from outside for distribution of *S. aureus*. The inoculated fillets were left for 25-30 min at room temperature to allow attachment and adsorption of the inoculated bacteria.

Treatment of inoculated chicken fillets with thyme oil and bacteriophage

Chicken fillets were divided into six groups each containing five samples: a control group (untreated), 2nd group (treated with thyme oil 0.5%), 3rd group (treated with thyme oil 1%), 4th group (treated with *S. aureus* phage MOI1), 5th group (treated with phage MOI 10) and 6th group (treated with thyme oil 1% + phage MOI 10). The samples were treated with thyme oil, bacteriophage, or both for 30, 60, and 120 min. The

thyme oil was applied through dipping, while the phage suspension was sprayed on both sides of the chicken fillet samples with a hand-operated plant spray. The control group was dipped in sterile distilled water. The viable bacterial count was determined on Baird Parker agar plates after 30, 60, and 120 min in control and treated groups as mentioned above and expressed as colony forming unit per gram (CFU/g). The experiment was performed in triplicate. The percentage of reduction in CFU/g was calculated as follows:

(A-B)/A×100

Where,
A: The mean of *S. aureus* CFU/g in a sample before treatment (control)
B: The mean of *S. aureus* CFU/g in a sample after treatment

Statistical analysis

The data were represented as means±standard deviation (SD) from at least three experiments. The differences between means were determined by one-way analysis of variance (ANOVA) and analyzed by SPSS statistics software for windows version 24.0 (IBM Corp. 2016, Armonk, NY). The differences were considered statistically significant at $P<0.05$. Duncan's multiple range test (DMRT) was used as a post hoc test to differentiate between means at $P<0.05$.

Results

Antimicrobial susceptibility of *S. aureus* strains

The results of the disc diffusion test declared a higher resistance of *S. aureus* to nalidixic acid (100%), methicillin (95.2%), sulfamethoxazole (90.5%), amoxicillin (71.4%), doxycycline (57.1%), cefotaxime (47.6%), erythromycin (42.9%), and norfloxacin (38.1%). However, lower resistance rates to gentamicin (19%) and ciprofloxacin (9.5%) were observed (Table 1). All *S. aureus* isolates in this study displayed multidrug-resistance patterns (resistant to at least one drug in ≥ three antimicrobial classes). The MAR index of the isolates ranged from 0.3-1 (average=0.57).

Morphological characterization and restriction enzyme analysis of *S. aureus* phage

The isolated *S. aureus* phage named vB_SauM_CP9 had an icosahedral head (92 × 50 nm) and long contractile tail (100 nm) and was categorized as a member of the *Myoviridae* family based on TEM micrograph (Fig. 1A). The phage had a double-stranded DNA that was susceptible to digestion with *Hind* III restriction enzyme and not susceptible to *Eco*RI digestion (Fig. 1B).

Phage stability at different pH and temperature degrees

The stability of vB_SauM_CP9 phage was investigated at pH range (3-10) and temperature range (30-70°C). The results showed that the phage titer was stable at pH range 4-9 with maximum stability at pH = 6-7. However, the phage was completely inactivated at pH = 3 and 10, and no plaques were produced (Fig. 2A). The phage was heat stable and survived a wide range of temperatures (30-70°C). However, the phage was completely inactivated at 80 and 90°C, and no plaques were produced (Fig. 2B).

Table 1: Antimicrobial interpretive criteria of *S. aureus* host strains used in this study and their susceptibility to phage

Strain	NA	MA	SMX	AMX	DO	CTX	E	NOR	G	CP	MAR index	Spot test
S1	R	R	R	R	R	R	R	R	R	R	1	+
S2	R	R	R	S	I	I	S	I	I	S	0.3	-
S3	R	R	R	R	R	I	S	I	S	S	0.5	-
S4	R	R	R	R	S	R	R	R	S	S	0.7	+
S5	R	R	R	I	S	R	R	R	I	S	0.6	+
S6	R	R	S	R	R	S	S	S	S	S	0.4	-
S7	R	R	R	R	I	S	S	I	S	S	0.4	-
S8	R	R	R	R	R	S	S	I	S	S	0.5	-
S9	R	R	R	I	S	R	R	R	S	I	0.6	+
S10	R	R	R	R	S	R	R	R	S	S	0.7	+
S11	R	R	R	I	I	R	R	R	R	S	0.7	+
S12	R	R	I	R	R	S	S	S	S	S	0.4	-
S13	R	R	R	R	I	R	I	I	R	S	0.6	+
S14	R	I	R	R	R	I	S	I	S	S	0.4	-
S15	R	R	R	I	I	R	R	R	S	I	0.6	+
S16	R	R	R	R	R	R	R	S	S	S	0.7	+
S17	R	R	R	R	R	S	S	I	S	S	0.5	-
S18	R	R	R	I	R	I	S	S	S	S	0.4	-
S19	R	R	R	R	R	S	S	S	S	S	0.5	-
S20	R	R	R	R	R	S	S	S	S	S	0.5	-
ATCC 25923	R	R	R	R	R	R	R	R	R	R	1	+

NA: Nalidixic acid, MA: Methicillin, SMX: Sulfamethoxazole, AMX: Amoxicillin, DO: Doxycycline, CTX: Cefotaxime, E: Erythromycin, NOR: Norfloxacin, G: Gentamicin, CP: Ciprofloxacin, MAR: Multiple antibiotic resistance index (=0.57), S: Sensitive, R: Resistant, and I: Intermediate

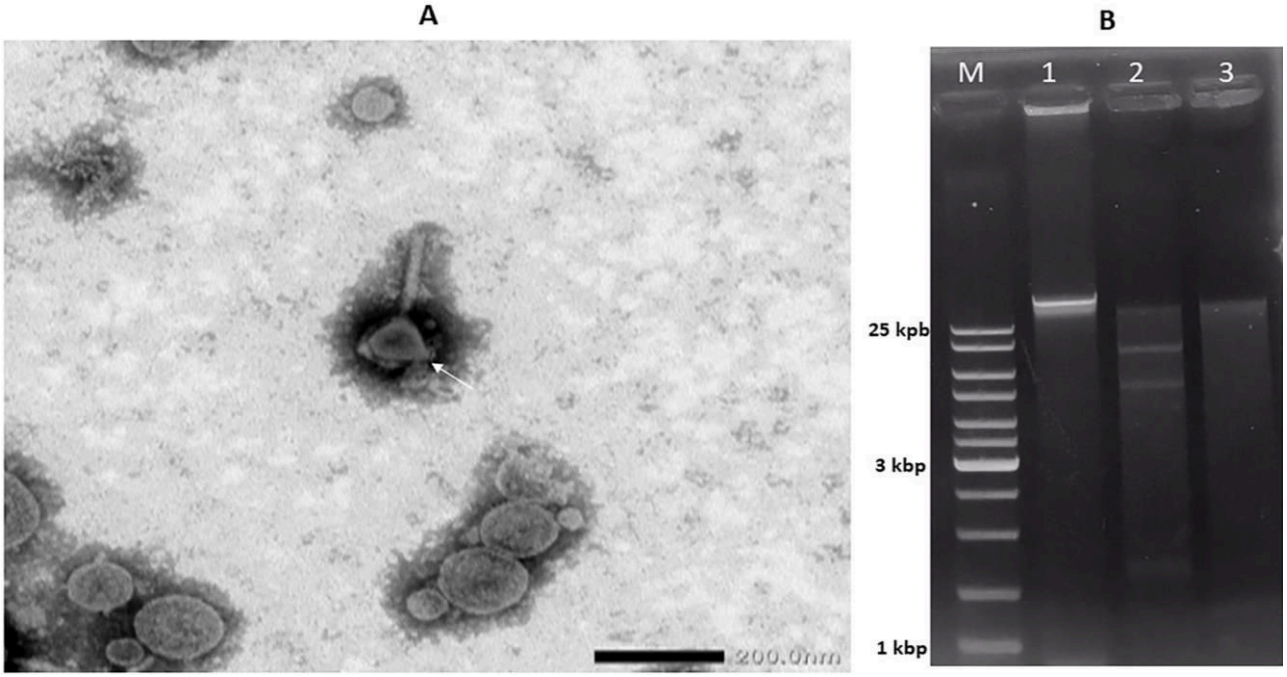

Fig. 1: Morphological characterization and restriction enzyme analysis of vB_SauM_CP9 phage. **A**: The arrow shows phage particles under TEM. The virions were negatively stained with uranyl acetate (scale bar, 200 nm), and **B**: Restriction enzyme digestion analysis of phage DNA. Lane M: DNA marker (New England Biolabs), Lane 1: Undigested DNA, Lane 2: The phage DNA was digested with *Hind* III, and Lane 3: No digestion with *Eco*RI

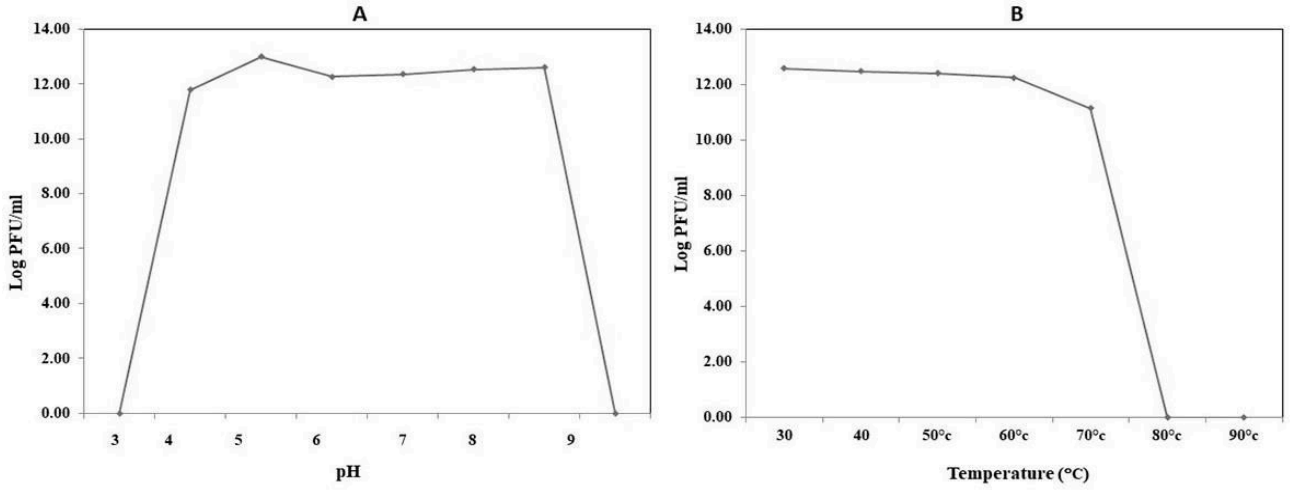

Fig. 2: The effect of different pH (3-10), and temperature (30-90°C) on the stability of *S. aureus* vB_SauM_CP9 phage. The data were presented for three independent experiments. **A**: The phage titer was stable at pH range 4-9 with maximum stability at pH = 6-7, and **B**: The phage titer was stable at temperature range 30-70°C

Table 2: Host range of vB_SauM_CP9 phage

Tested strains	No.	No. susceptible to phage lysis
Staphylococcus aureus	21	10 (+)
Escherichia coli (O91, O125, O78, O142, O26, O114, O126, and O128)	8	-
Salmonella enteritidis	2	-
Listeria monocytogenes	1	-

+ The isolates were lysed by phage and clear plaques were produced, and - no plaques were produced

Host range and bacteriolytic activity of *S. aureus* phage

The host range of vB_SauM_CP9 phage revealed that this phage was species specific, caused lysis to ten *S. aureus* strains and produced clear uniform plaques (2 mm) but showed no lytic activity on the other bacterial strains (Tables 1 and 2). The activity of vB_SauM_CP9 phage (MOI 1 and 10) on the growth of *S. aureus* in a

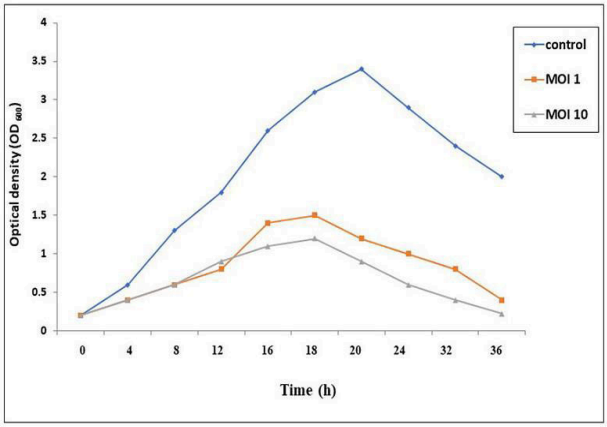

Fig. 3: The activity of vB_SauM_CP9 phage (MOI 1 and 10) on the growth of *S. aureus* in an infected culture at 37°C compared with control. The growth was measured at OD_{600}. The data were presented for three independent experiments

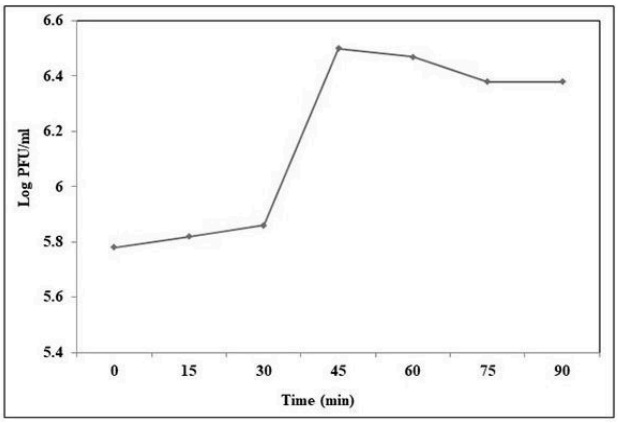

Fig. 4: One-step growth curve of vB_SauM_CP9 phage. The plaque forming units (PFU) per infected cell were shown at different times post infection. The samples were taken every 15 min up to 90 min

liquid culture at 37°C was shown in Fig. 3, the phage displayed rapid lytic activity on *S. aureus* and the OD of the infected culture decreased rapidly compared with control but did not reach zero after 36 h of infection.

One-step growth curve of *S. aureus* phage

As shown in Fig. 4, the burst size of the vB_SauM_CP9 phage is 228 PFU/infected cell, latent period (45 min) and rise period (15 min).

The activity of thyme oil and phage on the growth of *S. aureus* in chicken fillets

The results clarified a significant reduction in *S. aureus* CFU/g in artificially inoculated chicken fillets after 30, 60, and 120 min treatment with different concentrations of thyme oil (0.5 and 1%) and bacteriophage (MOI 1, MOI 10) compared with controls ($P<0.05$). The reduction percentages were 7.89, 23.68, 36.84, 37.72, and 43.86% after 30 min exposure to thyme oil 0.5%, thyme oil 1%, phage MOI 1, phage MOI 10, and thyme oil 1% + phage MOI 10, respectively compared with controls (Fig. 5). Moreover, *S. aureus* CFU/g were significantly reduced by 32.61, 40.58, 58.70, 66.67, and 80.43% after treatment with thyme oil 0.5%, thyme oil 1%, phage MOI 1, phage MOI 10, and thyme oil 1% + phage MOI 10, respectively for 60 min (Fig. 5). However, the higher percentages of reduction in *S. aureus* CFU/g was observed after treatment of chicken fillets with thyme oil 1% + phage MOI 10 (87.22%) for 120 min compared with phage MOI 10 (76.94%), phage MOI 1 (73.06%), thyme oil 1% (63.84 %), thyme oil 0.5% (56.67%) and control ($P<0.05$).

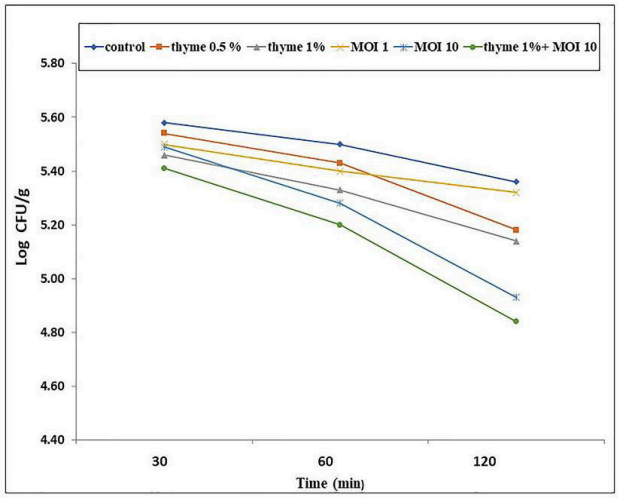

Fig. 5: The effect of thyme oil (0.5 and 1%), phage (MOI 1, MOI 10), and thyme oil 1% + phage MOI 10 on the growth of MDR *S. aureus* in chicken fillets after 30, 60, and 120 min. The log_{10} CFU/g was presented for three independent experiments. The higher reduction in CFU/g was observed after treatment with a combination of phage MOI 10 and thyme 1% ($P<0.05$).

Discussion

Staphylococcus aureus is considered the primary cause of foodborne intoxication and its presence in raw and ready-to-eat food products represents an important problem for food processors, food service workers and consumers. The current work aimed to study the antibiotic resistance profile of *S. aureus* strains as well as isolation and characterization of lytic phage able to infect and lyse these strains. The reduction level of *S. aureus* in artificially inoculated chicken products was evaluated after treatment with the isolated phage and thyme oil.

In this study, the tested *S. aureus* strains previously recovered from ready-to-eat chicken products showed a higher prevalence of MDR and higher MAR index (>0.2) which indicated contamination from high risk sources where antibiotics or growth promotors are overused (Crago *et al*., 2012). The frequent use of antibiotics in the poultry industry for therapeutic purposes or as growth promoters is the main factor responsible for the dissemination of antibiotic-resistant bacteria. This should sound the alarm and increase awareness because poultry products may constitute a reservoir for disseminating MRSA or other antibiotics resistant *S. aureus* into the community (Fan *et al*., 2015; Teramoto *et al*., 2016).

The isolated phage in this study had the

morphological characteristics of the *Myoviridae* family. Most lytic *S. aureus* phages that belonged to the *Myoviridae* family have previously been isolated (Kwan *et al*., 2005; Synnott *et al*., 2009; Kvachadze *et al*., 2011; Vandersteegen *et al*., 2011; Alves *et al*., 2014; El Haddad *et al*., 2014; Li and Zhang, 2014; Takemura-Uchiyama *et al*., 2014; Gutierrez *et al*., 2015; Ganaie *et al*., 2018; Gharieb *et al*., 2020). However, *S. aureus* phages which belonged to the family *Siphoviridae* (Yoon *et al*., 2013; Marek *et al*., 2019) and *podoviridae* (Ganaie *et al*., 2018) were isolated in former studies. In this study, the phage had genomic DNA that was susceptible to *Hind* III restriction enzyme. The nucleotide sequence of the phage will be performed in a further study.

The phage stability is essential for a successful application of phage for controlling foodborne pathogens in the food industry. The survival and persistence of bacteriophages are affected by different factors such as pH, ions, and temperature (Jończyk *et al*., 2011). Temperature plays a major role in phage survival and storage as well as attachment, penetration and multiplication of the phage in the target bacteria (Tey *et al*., 2009). Of interest, the isolated vB_SauM_CP9 phage was stable and maintained its activity at a wide range of pH (4-9) and temperature (30-70°C). The host range showed that the isolated phage was specific to *S. aureus* and possessed strong lytic activity on *S. aureus* in an infected culture at 37°C, resulting in a rapid decline in the growth of *S. aureus*. These features indicate that the isolated phage could be a useful biocontrol agent in the food industry, because it can withstand the different environmental stresses during processing, handling, and storage of food as well as maintaining its infectivity in controlling the proliferation of *S. aureus* in food (Kazi and Annapure, 2016). Previous studies stated the stability of *S. aureus* phages: SPW (Li and Zhang, 2014), SAJK-IND, and MSP (Ganaie *et al*., 2018) at pH range (4-9) and temperature (40-50°C). However, they were completely inactivated at pH (<4 or >10) and temperature above 50°C.

Interestingly, the high burst size of the isolated phage as shown in the one-step growth curve favors its use as a biocontrol agent because phages with high burst size and short latent period are good candidates for practical applications in the food industry (Jun *et al*., 2013). The burst size of the vB_SauM_CP9 phage in this study was higher than the burst size of *S. aureus* phages isolated in previous studies (Alves *et al*., 2014; Li and Zhang, 2014; Ganaie *et al*., 2018; Gharieb *et al*., 2020).

A notable feature is that the thyme oil and vB_SauM_CP9 phage caused a significant reduction in *S. aureus* inoculated in chicken fillets. However, the higher reduction (87.22%) was achieved after treatment with a combination of phage MOI 10 and thyme oil 1% compared with treatment with each one alone. The bactericidal activity of thyme oil against pathogenic and spoilage bacteria in food could be related to the highest percentage of phenolic components that affect cell membrane lipids leading to increasing permeability, leakage of ions and molecules resulting in cell death (Burt, 2004; Nazzaro *et al*., 2013). Besides essential oils, the lytic (virulent) phages are preferred as biocontrol agents in the food industry because they usually lyse target bacterial cells (Atterbury *et al*., 2003; Goode *et al*., 2003; Marek *et al*., 2019). The infection cycle of lytic phages could be initiated by adsorption to the bacterial cell wall through binding between the virion-associated peptidoglycan hydrolases (VAPGHs) and cell wall receptors followed by insertion of phage genetic material in the host cell cytoplasm and synthesis of viral components. Consequently, phage produce endolysins at the end of the lytic cycle that lyse the bacterial cell wall peptidoglycan layer, and new phages were released (Fischetti, 2010). The phage suspension can be applied on food surfaces or mixed with processed food by spraying or through packaging material (Sulakvelidze, 2013; Lone *et al*., 2016).

This study evaluated for the first time the efficacy of a lytic *S. aureus* bacteriophage and thyme oil in controlling the growth of MDR *S. aureus* inoculated in chicken products. However, previous studies reported the higher inhibitory activity of thyme oil on *S. aureus* inoculated in minced beef (Ibrahim *et al*., 2016; Salem *et al*., 2017). On the other hand, a combination of a commercial *Salmonella* bacteriophage cocktail (Salmonelex) (MOI 1) and thyme oil (0.8 and 1.6%) showed a higher significant reduction in *S. enteritidis* inoculated in chicken breast samples than individual treatment with bacteriophage or thyme oil (Moon *et al*., 2020).

This study concluded that *S. aureus* strains recovered from chicken products that were MDR displayed a higher MAR index and constituted a potential threat to food consumers. The lytic phage isolated in this study was specific to *S. aureus* strains and showed higher activity in reducing *S. aureus* in chicken fillets especially when used in combination with essential thyme oil. Therefore, this study recommends that a combination of thyme oil and lytic *S. aureus* phage is a promising biocontrol agent and antimicrobial alternative in the food industry to control and reduce the MRSA or other antibiotic resistant *S. aureus* contamination in food.

Acknowledgement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Ackermann, HW and Prangishvili, D (2012). Prokaryote viruses studied by electron microscopy. Arch. Virol., 157: 1843-1849.

Adriaenssens, E and Brister, JR (2017). How to name and classify your phage: an informal guide. Viruses. 9:70. doi:10.3390/v9040070.

Alves, DR; Gaudion, A; Bean, JE; Perez Esteban, P; Arnot, TC; Harper, DR; Kot, W; Hansen, LH; Enright, MC; Tobias, A and Jenkins, AT (2014). Combined use of bacteriophage K and a novel bacteriophage to reduce *Staphylococcus aureus* biofilm formation. Appl. Environ. Microbiol., 80: 6694-6703.

Anžlovar, S; Baričevič, D; Ambrožič Avguštin, J and Dolenc Koce, J (2014). Essential oil of common thyme as a natural antimicrobial food additive. Food Technol. Biotechnol., 52: 263-268.

Apata, D (2009). Antibiotic resistance in poultry. Int. J. Poult. Sci., 8: 404-408.

Atterbury, RJ; Connerton, PL; Dodd, CE; Rees, CE and Connerton, IF (2003). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. Appl. Environ. Microbiol., 69: 6302-6306.

Bajpai, VK; Baek, KH and Kang, SC (2012). Control of *Salmonella* in foods by using essential oils: a review. Food Res. Int., 45: 722-734.

Bakkali, F; Averbeck, S; Averbeck, D and Idaomar, M (2008). Biological effects of essential oils – a review. Food Chem. Toxicol., 46: 446-475.

Bassolé, IHN and Juliani, HR (2012). Essential oils in combination and their antimicrobial properties. Molecules. 17: 3989-4006.

Burt, S (2004). Essential oils: their antibacterial properties and potential applications in foods–a review. Int. J. Food Microbiol., 94: 223-253.

Chang, HC; Chen, CR and Lin, JW (2005). Isolation and characterization of novel giant *Stenotrophomonas maltophilia* phage phiSMA5. Appl. Environ. Microbiol., 71: 1387-1393.

Clinical and Laboratory Standards Institute (CLSI) (2019). *Performance standards for antimicrobial susceptibility testing*. 29th Edn., CLSI Supplement M100. Wayne, PA: CLSI.

Crago, B; Ferrato, C; Drews, S; Svenson, L; Tyrrell, G and Louie, M (2012). Prevalence of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) in food samples associated with foodborne illness in Alberta, Canada from 2007 to 2010. Food Microbiol., 32: 202-205.

El Haddad, L; Abdallah, NB; Plante, PL; Dumaresq, J; Katsarava, R; Labrie, S; Corbeil, J; St-Gelais, D and Moineau, S (2014). Improving the safety of *Staphylococcus aureus* polyvalent phages by their production on a *Staphylococcus xylosus* strain. PLoS One. 9: e102600. https://doi: 10.1371/journal.pone.0102600.

Fan, Y; Li, S; Deng, B and Zhao, Y (2015). Prevalence and relevance analysis of multidrug-resistant *Staphylococcus aureus* of meat, poultry and human origin. Indian J. Anim. Res., 49: 86-90.

Fischetti, VA (2010). Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. Int. J. Med. Microbiol., 300: 357-362.

Ganaie, MY; Qureshi, S; Kashoo, Z; Wani, SA; Hussain, MI; Kumar, R; Maqbool, R; Sikander, P; Banday, MS; Malla, WA; Mondal, P and Khan, RIN (2018). Isolation and characterization of two lytic bacteriophages against *Staphylococcus aureus* from India: Newer therapeutic agents against bovine mastitis. Vet. Res. Commun., 42: 289-295.

Gharieb, RMA; Saad, MF; Mohamed, AS and Tartor, YH (2020). Characterization of two novel lytic bacteriophages for reducing biofilms of zoonotic multidrug-resistant *Staphylococcus aureus* and controlling their growth in milk. LWT-Food Sci. Technol., 124:109145. https://doi.org/10.1016/j.lwt.2020.109145.

Ghosh, A; Ricke, SC; Almeida, G and Gibson, KE (2016). Combined application of essential oil compounds and bacteriophage to inhibit growth of *Staphylococcus aureus in vitro*. Current Microbiol., 72: 426-435.

Goode, D; Allen, V and Barrow, P (2003). Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. Appl. Environ. Microbiol., 69: 5032-5036.

Gutierrez, D; Vandenheuvel, D; Martinez, B; Rodriguez, A; Lavigne, R and Garcia, P (2015). Two phages, phiIPLA-RODI and phiIPLA-C1C, lyse mono- and dual-species staphylococcal biofilms. Appl. Environ. Microbiol., 81: 3336-3348.

Hammerum, AM; Lester, CH and Heuer, OE (2010). Antimicrobial-resistant enterococci in animals and meat: a human health hazard? Foodborne Pathog. Dis., 7: 1137-1146.

Ibrahim, HM; El Sabagh, RA; El-Roos, A; Nahla, A and Abd El Fattah, H (2016). Antimicrobial effect of some essential oils on *Staphylococcus aureus* in minced meat. Benha Vet. Med. J., 30: 183-191.

ICMSF (International Commission of Microbiological Specification for Foods) (1996). Microorganisms in Food. Their significance and methods of enumeration. 3rd Edn., Canada, University of Toronto.

Jończyk, E; Kłak, M; Międzybrodzki, R and Górski, A (2011). The influence of external factors on bacteriophages. Folia Microbiol., 56: 191-200.

Jun, JW; Kim, JH; Shin, SP; Han, JE; Chai, JY and Park, SC (2013). Characterization and complete genome sequence of the *Shigella* bacteriophage pSf-1. Res. Microbiol., 164: 979-986.

Kazi, M and Annapure, US (2016). Bacteriophage biocontrol of foodborne pathogens. J. Food Sci. Technol., 53: 1355-1362.

Kittler, S; Wittmann, J; Mengden, RALP; Klein, G; Rohde, C and Lehnherr, H (2017). The use of bacteriophages as One-Health approach to reduce multidrug-resistant bacteria. Sustain. Chem. Pharm., 5: 80-83.

Kropinski, AM; Prangishvili, D and Lavigne, R (2009). Position paper: the creation of a rational scheme for the nomenclature of viruses of bacteria and archaea. Environ. Microbiol., 11: 2775-2777.

Kvachadze, L; Balarjishvili, N; Meskhi, T; Tevdoradze, E; Skhirtladze, N; Pataridze, T; Adamia, R; Topuria, T; Kutter, E; Rohde, C and Kutateladze, M (2011). Evaluation of lytic activity of staphylococcal bacteriophage Sb-1 against freshly isolated clinical pathogens. Microb. Biotechnol., 4: 643-650.

Kwan, T; Liu, J; DuBow, M; Gros, P and Pelletier, J (2005). The complete genomes and proteomes of 27 *Staphylococcus aureus* bacteriophages. Proc. Natl. Acad. Sci. U. S. A., 102: 5174-5179.

Le Loir, Y; Baron, F and Gautier, M (2003). *Staphylococcus aureus* and food poisoning. Genet. Mol. Res., 2: 63-76.

Li, L and Zhang, Z (2014). Isolation and characterization of a virulent bacteriophage SPW specific for *Staphylococcus aureus* isolated from bovine mastitis of lactating dairy cattle. Mol. Biol. Rep., 41: 5829-5838.

Lone, A; Anany, H; Hakeem, M; Aguis, L; Avdjian, AC; Bouget, M; Atashi, A; Brovko, L; Rochefort, D and Griffiths, MW (2016). Development of prototypes of bioactive packaging materials based on immobilized

bacteriophages for control of growth of bacterial pathogens in foods. Int. J. Food Microbiol., 217: 49-58.

Lu, Z; Breidt, JrF; Fleming, H; Altermann, E and Klaenhammer, T (2003). Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, ΦJL-1, from a cucumber fermentation. Int. J. Food Microbiol., 84: 225-235.

Magiorakos, AP; Srinivasan, A; Carey, R; Carmeli, Y; Falagas, M; Giske, C; Harbarth, S; Hindler, JF; Kahlmeter, G; Olsson-Liljequist, B; Paterson, DL; Rice, LB; Stelling, J; Struelens, MJ; Vatopoulos, A; Weber, JT and Monnet, DL (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect., 18: 268-281.

Marek, A; Pyzik, E; Stepien-Pysniak, D; Urban-Chmiel, R and Nowaczek, A (2019). Characterization of bacteriophages and their carriage in *Staphylococcus aureus* isolated from broilers in Poland. Br. Poult. Sci., 60: 373-380.

Moon, SH; Waite-Cusic, J and Huang, E (2020). Control of *Salmonella* in chicken meat using a combination of a commercial bacteriophage and plant-based essential oils. Food Control. 110: 106984. https://doi.org/10.1016/j. foodcont.2019.106984.

Nazzaro, F; Fratianni, F; De Martino, L; Coppola, R and De Feo, V (2013). Effect of essential oils on pathogenic bacteria. Pharmaceuticals. 6: 1451-1474.

Pajunen, M; Kiljunen, S and Skurnik, M (2000). Bacteriophage φYeO3-12, specific for *Yersinia enterocolitica* serotype O: 3, is related to coliphages T3 and T7. J. Bacteriol., 182: 5114-5120.

Pecarski, D; Ketin, S; Omerovic, I; Mirkovic, M; Jugovic, Z and Biocanin, R (2016). Chemical compositions and antimicrobial activities of oregano and thyme essential oils. Bulg. Chem. Commun., 48: 678-683.

Pual, S; Bezbarauh, RL; Roy, MK and Ghosh, AC (1997). Multiple antibiotic resistance index and its reversion in *Pseudomonas aeruginosa.* Lett. Appl. Microbiol., 24: 169-171.

Salem, AM; Zakaria, EM and Abd El-Raheem, KA (2017). Efficiency of some essential oils in control of methicillin resistant *Staphylococcus aureus* (MRSA) in Minced Beef. Benha Vet. Med. J., 32: 177-183.

Sambrook, JW and Russell, D (2001). *Molecular cloning: a laboratory manual*. 3rd Edn., NY, USA, Cold Spring Harbor Press.

Schnabel, EL and Jones, AL (2001). Isolation and characterization of five *Erwinia amylovora* bacteriophages and assessment of phage resistance in strains of *Erwinia amylovora*. Appl. Environ. Microbiol., 67: 59-64.

Sulakvelidze, A (2013). Using lytic bacteriophages to eliminate or significantly reduce contamination of food by foodborne bacterial pathogens. J. Sci. Food Agric., 93: 3137-3146.

Synnott, AJ; Kuang, Y; Kurimoto, M; Yamamichi, K; Iwano, H and Tanji, Y (2009). Isolation from sewage influent and characterization of novel *Staphylococcus aureus* bacteriophages with wide host ranges and potent lytic capabilities. Appl. Environ. Microbiol., 75: 4483-4490.

Takemura-Uchiyama, I; Uchiyama, J; Kato, SI; Ujihara, T; Daibata, M and Matsuzaki, S (2014). Genomic and phylogenetic traits of *Staphylococcus* phages S25-3 and S25-4 (family Myoviridae, genus Twort-like viruses). Ann. Microbiol., 64: 1453-1456.

Teramoto, H; Salaheen, S and Biswas, D (2016). Contamination of post-harvest poultry products with multidrug resistant *Staphylococcus aureus* in Maryland-Washington DC metro area. Food Control. 65: 132-135.

Tey, BT; Ooi, ST; Yong, KC; Ng, MYT; Ling, TC and Tan, WS (2009). Production of fusion m13 phage bearing the di-sulphide constrained peptide sequence (C-WSFFSNI-C) that interacts with hepatitis B core antigen. Afr. J. Biotechnol., 8: 268-273.

Tongnuanchan, P and Benjakul, S (2014). Essential oils: Extraction, bioactivities, and their uses for food preservation. J. Food Sci., 79: 1231-1249.

Vandersteegen, K; Mattheus, W; Ceyssens, PJ; Bilocq, F; De Vos, D; Pirnay, JP; Noben, JP; Merabishvili, M; Lipinska, U; Hermans, K and Lavigne, R (2011). Microbiological and molecular assessment of bacteriophage ISP for the control of *Staphylococcus aureus*. PloS One. 6: e24418. doi: 10.1371/journal.pone. 0024418.

Yoon, H; Yun, J; Lim, JA; Roh, E; Jung, KS; Chang, Y; Ryu, S and Heu, S (2013). Characterization and genomic analysis of two *Staphylococcus aureus* bacteriophages isolated from poultry/livestock farms. J. Gen. Virol., 94: 2569-2576.