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Evaluation of Novel Virulent Phages Infecting the *Aeromonas hydrophila* and *Escherichia coli* Isolated from Sewage Water Samples

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MULTIDRUG-resistant bacteria are now emerging for almost all the present-day antibiotics. *Aeromonas hydrophila* D2007 and *Escherichia coli* W102 were isolated from fresh food and drinking water samples and they were resistant to 57.14% and 85.71% of tested common antibiotics respectively. Three bacteriophages (phages) were isolated from sewage samples. Morphological examinations suggested that phage ΦAHP7, which infects *A. hydrophila* D2007, belongs to the Myoviridae family and other phages ΦECP8 and ΦECP9 capable of lysing *E. coil* W102 belongs to Siphoviridae and Podoviridae families, respectively. For the three phages, the optimal multiplicity of infection (MOI) was calculated to be 0.001. Phages were characterized by determining their host range and stability in pHs, temperatures, and salinity. The latent periods of phages ΦAHP7, ΦECP8, and ΦECP9 were 10, 20 and 10min with average burst sizes of 53.5±0.5, 26.5±0.5 and 67.5±0.5 phages per infected cell, respectively. The three phages gradually reduced OD_{600} and are able to stop the growth of *A. hydrophila* D2007 and *E. coli* W102 *in vitro* at a low MOI of 0.001. Phages ΦAHP7, ΦECP8, and ΦECP9 treatments achieved 1.55, 1.68 and 2.28 log CFU/g (P< 0.01) reduction of viable bacterial number in red cabbage and 1.48, 1.38 and 1.68 log CFU/g (P< 0.01) reduction in tomato after 30 min at room temperature (28°C) respectively. Applications of lytic ΦAHP7, ΦECP8, and ΦECP9 bacteriophages lead to a rapid reduction of *A. hydrophila* D2007 and *E. coli* W102 counts in fresh food for human consumption.

Keywords: *Aeromonas hydrophila*, *Escherichia coli*, Bacteriophage, ΦAHP7, ΦECP8, ΦECP9.

Introduction

Throughout developing and developed countries, food-and waterborne diseases correlated with microbial pathogens pose a serious health threat. World Health Organization (WHO) reports 600 million foodborne diseases and 420000 deaths throughout 2010 (Fung et al., 2018). Food products become polluted at various stages from growing or development to consumption (CDC, 2011), and water pathogens deposit to surface water in different ways as a direct connection to animal faeces or manure which carry pathogens. A Food and Drug Administration (FDA) report estimates that the risk of foodborne diseases each year contributes to $152 billion (FDA, 2012).

Aeromonas hydrophila (*A. hydrophila*) represents an interesting food and waterborne pathogens as causative agent of severe gastrointestinal infections. Gastroenteritis and wound infections are two main diseases caused by *Aeromonas*. Gastroenteritis typically occurs following consumption of infected water or food (Teunis & Figueras, 2016), whereas wound infections result from exposure to contaminated water. The organism is resistant to chlorine, cold temperature (Garcia-Gimeno et al., 1996), and common antibiotics, such as penicillin, ampicillin, and colistin. *A. hydrophila* has been isolated from many sources. *A. hydrophila is* also linked with soil (Brandi et al., 1996). Foods in which *A. hydrophila* had been isolated were most possibly polluted with water, dirt or animal faeces (Daskalov, 2006).

Escherichia coli is one of the most significant

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and common food and waterborne pathogens and it was a major global public health concern (Ahmed & Shimamoto, 2014). This microorganism is the most important agent in humans for urinary and gastrointestinal infections. This triggers a number of disorders, including moderate diarrhea, hemorrhagic colitis, uremic syndromes and reflects the most significant water, foods pollution index. The rise of multi-drug resistant *E. coli* varieties in recent years is growing (Ansari et al., 2015) and causes multiple incidents of foodborne disease due to the ingestion of contaminated food worldwide.

A phage is a virus that infects bacteria. Lytic phages treat human bacterial infections (Maura and Debarbieux, 2011), and were used for the decontamination of processed food and agricultural products (Maura & Debarbieux, 2011; El-Shibiny et al., 2017; Huang et al., 2018; Bai et al., 2019). Moreover, El-Dougdoug et al. (2020) used lytic phages to control antibiotic resistant *Salmonella* Typhi in the tap water and milk. Phages have certain benefits, such as high host specificity and self-replication, as long as they are host-present. For phage therapy, phage must be isolated from the environment and show a relatively wide host range with strong lytic activity (Hyman, 2019). In this study, tailed phages contain dsDNA with strong lytic activities were isolated and characterized. The phage-based biocontrol of *Aeromonas* infection has been documented in several studies. Previously, *Aeromonas* phages ΦZH1 and ΦZH2 were isolated and their biology was characterized briefly. ΦZH1 and ΦZH2 were classified in the family Podoviridae with strong virulence against *Aeromonas hydrophila* infecting Nile tilapia (El-Araby et al., 2016). Recently, *Aeromonas* phage Akh-2 was molecularly characterized and classified in the Siphoviridae family, phage Akh-2 was used as a therapy against *Aeromonas* infections in fish (Akmal et al., 2020).

Several studies have reported on the phage-based biocontrol of pathogenic *E. coli.* An *E. coli* phage vB_EcoS_HSE2 controls pathogenic *E. coli* effectively which was classified in the family Siphoviridae with limited host range (Peng & Yuan, 2018). The phage P.E1 isolated from sewage in hospital reduced *E. coli* counts. The P.E1 phage had shown a narrow host range appropriate for use in phage therapy. Phage displayed lytic activity up to 70°C and under alkaline conditions but its activity decreased under higher acidic conditions (Bibi et al., 2016).

Several studies (Leverentz et al., 2003; Bigwood et al., 2008; Bai et al., 2019) stated that phage therapy significantly reduced foodborne pathogens in different foods, with decreases varying from 1.8 and 4.6 logs relative to the untreated ones.

The main objective of this work was to isolate and characterize specific lytic bacteriophages for multidrug-resistant (MDR) *A. hydrophila* D2007 and *E. coli* W102 and to evaluate the potential use of these phages to control *A. hydrophila* D2007 and *E. coli* W102 in fresh food in Egypt.

Materials and Methods

Sample collection, bacterial isolation, and identification

Sixty three fresh food samples (Cucumber, Broccoli, Pepper, Parsley, Dill, Celery, Red cabbage, Rocca and Tomato) were collected from street shops in Benha City, Qalyubia Governorate, Egypt. As well as sixty chlorinated tap water samples were aseptically collected from three different places in Benha city, Egypt. After that, *Aeromonas hydrophila* D2007 and *Escherichia coli* W102 were isolated from leafy herb (Dill) and drinking tap water samples respectively. The isolation was done by serial dilution plate method (Jett et al., 1997) and the isolated bacterial species were identified by morphological and biochemical tests (Buchanan & Gibbons, 1974; Das et al., 2012). The selected bacterial isolates were confirmed by VITEK® 2 COMPACT automated instrument for ID/AST testing (Biomerieux).

Antibiotic susceptibility testing

Antibiotic susceptibility of *A. hydrophila* D2007 and *E. coli* W102 was performed on Mueller Hinton Agar by disc diffusion method (Bauer et al., 1966). Antibiotics used were Levofloxacin (LE 5µg), Ciprofloxacin (CIP 5µg), Cephalexin (CL 30µg), Ofloxacin (OF 5µg), Ampicillin–Sulbactam (A/S 10/10µg), Amoxicillin-clavulanic acid (AMC 20/10µg), Imipenem (IPM 10 µg), Co-Trimoxazole (COT 25µg), Gentamicin (GEN 10µg), Ampicillin (AMP 10µg), Amikacin (AK 30µg), Ceftazidime (CAZ 30µg), Cefotaxime (CTX 30µg) and Amoxicillin (AX 25µg). Interpretation of the results was carried out according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2016). The test performed in triplicate to confirm the results.

Bacteriophages isolation and purification

Several waste water samples from Sandanhoor Village, Qalyubia Governorate, Egypt were collected. In the screening of bacteriophages, identified individual bacteria (*A. hydrophila* D2007 and *E. coli* W102) were used as hosts. Phage isolation was conducted according to Pereira et al. (2011) method. The sewage samples collected had been centrifuged to remove solid impurities at 6000rpm for 15min. The supernatants had been purified using a membrane filter of 0.45μm (Millex GP, Merck Millipore, Darmstadt, Germany) to eliminate bacterial cells. Ten milliliter (10mL) aliquots of the filtrates were mixed with 30mL of Luria-Bertani media (LB) to enrich the phages, inoculating 0.4mL of an overnight bacterium culture and incubated for overnight (37ºC; 250rpm. 24hrs). After incubation, cultures were centrifuged at 6000rpm for 10min at 4ºC, and supernatants were filtered using a 0.45μm membrane filter (Millex GP, Merck Millipore, Darmstadt). Enriched filtrates for phage activity have been checked using the spot test (Mirzaei & Nilsson, 2015). Then a filtrate containing enriched phages (100μL) has been mixed in LB medium and 3mL molten top of soft nutrient agar (0.7%), using 0.3mL of host cells (OD^{600}= 0.3). The mixture was overlaid onto solidified agar (1.5%) (Adams, 1959; Mihara et al., 2016). Clear phage plaques were picked from the plate after 8-12hrs at 37ºC incubation. Phages have been purified by standard methods (Gencay et al., 2017). Following plaque purification, high titer phage stocks were generated and diluted to know their titers. By using NaCl (1M) and PEG 8000 (10 percent w/v) isolated phages were concentrated, and purified by chloroform extraction (1:1 v/v). Double Agar Layer (DAL) method was used to evaluate the titre of phages. For further analysis, purified phages were stored at 4°C.

Transmission electron microscopy (TEM) analysis of bacteriophages

Purified phage particles were stained with 2% (w/v) phosphotungstic acid and were detected under transmission electron microscope (JOEL-JM-100-C, Japan Electron Optics Laboratory Co., Ltd), at the Electron Microscope Unit, University of Al-Azhar, Cairo, Egypt.

Determination of optimal multiplicity of infection (MOI)

The optimum infection ratio between the propagated phages and the host bacterial cells was calculated (*A. hydrophila* D2007 and *E. coli* W102) using the method described by Yang et al. (2010). The bacterial cells grown in LB medium at 10^8 CFU/mL and infected with phages at MOI 1, 0.1, 0.01, 0.001, 0.001, 0.0001, and 0.00001. After incubation, the optimal MOI was determined which produced the highest phage titer (maximum PFU/mL). The experiment was repeated three times and the mean results were determined for all repetitions.

Host range of bacteriophages

To evaluate the lytic spectrum of the obtained bacteriophages, twenty two pathogenic bacterial isolates from different sources were used. The spot test was used on, 3 *Aeromonas hydrophila* isolates, 7 *Escherichia coli* isolates, *Streptococcus* spp., *Serratia* spp., *Staphylococcus aureus, Proteus* spp., 2 *Acinetobacter baumanni* isolates, 4 *Klebsiella pneumoniae* spp. isolates, *Salmonella* spp. and *Shigella* spp. (Bielke et al., 2007). Briefly, double -layer agar plates were prepared with different bacterial strains. On each agar plate, the lysis range of isolated phages was determined by spotting 10μL of each phage lysate. The plates were incubated at 37°C overnight and examined for clearing areas (Bielke et al., 2007). All spot tests had been performed three times to validate the findings.

One step growth curve

A. hydrophila D2007 and *E. coli* W102 were separately grown in LB broth at 37°C and optical density measurements at 600nm were estimated at the mid exponential phases (OD^{600}= 0.3). Centrifugation at 6000rpm for 5min was done for 1mL of the culture to harvest the bacterial cells. Then 100μL phage lysate (10^6 PFU/mL) (MOI= 0.001) were mixed with them. The complex of phage bacteria was collected after 10min of centrifugation and resuspended in 10mL LB to allow the phage to adsorb to bacteria. During the subsequent incubation, samples were taken at 5-min intervals for 60min at 37°C. After 24hrs of incubation at 37°C., plaques were counted (Lee & Park, 2015) and expressed by PFU per infected cell.

Bacteriophages stability assay

The effect of the temperature on the stability of the phages was determined according to Lu et al. (2003). Phages lysates were taken and incubated at 25°C, 37°C, 45°C, 55°C, 65°C and 70°C for 30, 60 and 90min in Eppendorf tubes. Upon treatment, tubes were slowly cooled and the surviving phages were detected. The stability of the phage at different pH values was tested by incubating the phages in an appropriate pH broth varying from 3 to 11 according to Capra et al. (2006). In addition, the effect of NaCl concentration on the viability of phages activity was performed according to Capra et al. (2006). The samples were examined using a double agar overlay method for determining the number of phage surviving particles and the surviving phage counts were represented as PFU/mL.

The bacterial growth reduction assay

The bacterial growth reduction tests were performed with bacteria in LB broth. The medium (100mL) in every 6 beaker flasks (3 flasks for each phage, and 3 flasks as a control) were inoculated with a fresh overnight bacterial culture and then incubated at 37°C with shaking. Bacterial culture OD_{600} in these flasks adjusted to OD_{600} of 0.3 by dilution with free media. Flasks were incubated in a shaker for refreshing of bacterial cells then phage was added to special flasks, maintaining a multiplicity of infection MOI at 0.001 (optimum MOI). By taking samples every hour, bacterial growth was examined by monitoring the OD_{600} using a spectrophotometer (Genway 6300, UK) (Elhalag et al., 2018). The growth experiments for each strain were repeated three times.

Application of bacteriophages in artificially contaminated fresh vegetables

Samples of tomatoes and red cabbage were bought in the local market (Benha, Egypt). Samples of the tomatoes were cut into 10mm slices with a sterile knife and divided into squares of equal size 10mm thick and 10mm in diameter. Each square was weighted and the weights were recorded. Red cabbage samples were cut into $10mm^2$ with a sharp knife, each leaf section was weighted and the weights were recorded. The food surfaces as well as a knife were disinfected with 70% ethanol immediately before cutting. The bacterial cells (*A. hydrophila* D2007 and *E. coli* W102) were prepared for inoculation of the food squares. Bacteria were grown overnight in LB broth, the concentration of cultures were adjusted to 10^5 CFU/mL by measuring OD_{600} and confirmed by plate counts. The food squares were placed in serialized plastic plates and then were separately inoculated with 25 µL of the bacterial suspension (*A. hydrophila* D2007 and *E. coli* W102) containing approximately 10^5 CFU/mL. The sample squares took about 10 minutes. Then a phage (10^6 PFU/mL) treatment was applied as a runoff spray on the entire square at MOI of 100 (< 250µL/ square). Sample squares incubated at room temperature to allow bacteria and phage interaction at incubation times (30, 60, 90min and 24hrs) were then homogenized in a tube containing 2mL of serialized distilled water. Then 100µL of homogenous mixtures were serial diluted to 10^{-5} and from the final dilution (50µL) were taken and plated in triplicate to determine CFU/g (Conway et al., 2000). The decreases in bacterial counts were calculated by the differences between control and phage treated samples in viable counts.

Statistical analysis:

Means and standard deviations for treatments have been determined. Tests were conducted with paired samples to determine whether there were statistical differences between phage-treated samples and controls. Statistical analysis of SPSS software was carried out (IBM Corp. Published 2011. Windows IBM SPSS Statistics, version 20.0. IBM, Armonk, NY, USA). Statistically significant differences were considered at a P value < 0. 05 and highly significant at P value < 0.01.

Results

Isolation and identification of bacteria

Thirty bacterial isolates (47.61%) were isolated from fresh food (raw vegetables) samples (n= 63) and among these isolates, bacterial isolate No D2007 was selected and characterized through morphological and biochemical analysis, this isolate exhibited strongest antibiotic resistance, phage sensitivity and identified as *Aeromonas hydrophila* D2007, it was further confirmed by VITEK® 2 COMPACT system. On the other hand, seven bacterial isolates (11.66%) were obtained from 60 chlorinated drinking tap water samples and the isolate No W102 was selected according to the phage and antibiotic sensitivity test and characterized through morphological and biochemical analysis and identified as *Escherichia coli* W102; it was further confirmed by VITEK® 2 COMPACT system.

Determination of antibiotic resistance/ susceptibility

Isolated *A. hydrophila* D2007 and *E. coli* W102 showed 57.14% (8/14) and 85.71% (12/14) resistant to antibacterial agents (antibiotics) respectively. *A. hydrophila* D2007 was resistant to Ampicillin (AMP 10µg), Ampicillin-Sulbactam (A/S 10/10µg), Amoxicillin (AX 25µg), Amoxicillin-clavulanic acid (AMC 20/10µg), Cefotaxime (CTX 30µg), Cephalexin (CL 30µg), Amikacin (AK 30µg) and Co-Trimoxazole (COT 25µg) (Table 1). Whereas, *E. coli* W102 was found to be resistant to Ampicillin (AMP 10µg), Ampicillin-Sulbactam (A/S 10/10µg), Amoxicillin (AX 25µg), Amoxicillin-clavulanic acid (AMC 20/10µg), Cefotaxime (CTX 30µg), Ceftazidime (CAZ 30µg), Cephalexin (CL 30µg), Imipenem (IPM 10µg), Co-Trimoxazole (COT 25µg), Levofloxacin (LE5µg), Ciprofloxacin (CIP 5µg) and Ofloxacin (OF 5µg) (Table 1).

Isolation and morphology of bacteriophages

In total three morphologically different phages were isolated following enrichment of sewage, one for *A. hydrophila* D2007 and two phages for *E. coli* W102 each of them had strong lytic activity (Fig. 1 b, e and h). Phage of *A. hydrophila* designated as ΦAHP7 and phages of *E. coli* designated as ΦECP8 and ΦECP9. Plaques produced by the phage ΦAHP7 were small circular, regular and clear without center and halo with plaque diameter 2mm. Plaques produced by the phages (ΦECP8 and ΦECP9) were circular, regular and clear without center and halo. Phage ΦECP8 produced plaques of 4 mm in diameter while phage ΦECP9 produced plaques of about 6mm in diameter (Fig. 1 g and Table 2). After phages propagation, the final concentrations were generally $2.9x10^7$, 2.3×10^6 and 6.5×10^6 PFU/mL for ΦAHP7, ΦECP8, and ΦECP9, respectively.

The Transmission Electron Microscopy (TEM) morphological analysis revealed that the three phages had tails and were thus belonged to the Caudovirales order. The phages were assigned to three families based on their morphological features (Fig. 1). Phage AHP7 has an icosahedral head with a contractile tail, capsid measurements were 88.55±0.32 x114±3nm), tail length (77.25±0.51nm) and tail width (26.35±1.35nm) (Fig. 1 c), It belongs to the Myoviridae family. Phage ECP8 has a non-contractile long tail, capsid diameter (60.22±1.13nm), tail-length (202.25±2.25nm) and tail-width (23.86±1.14nm) based on morphology, it belongs to the family Siphoviridae (Table 2). Phage ΦECP9 has an icosahedral head with a very short tail; the diameter of capsid was (66.95±0.45nm) and tail length (16.8±0.2nm), the typical morphology of members of the family Podoviridae (Fig. 1 i).

Multiplicity of infection

The MOI with the highest phage progeny development (2.5 $x10^7$, 1.93×10^6 and 4.77×10^6 PFU/mL) has been designed as ideal MOI for ΦAHP7, ΦECP8 and ΦECP9 respectively and has been selected for the following experiments. The optimal MOI of Φ ECP7, Φ ECP8 and ΦECP9 was 0. 001as showed in Fig. 2.

TABLE 1. Antibiotic susceptibility patterns of isolated *A. hydrophila* D2007 and *E. coli* W102 to different antibiotics.

Bacteria	AMP	A/S	AX	AMC	CAZ	CTX	CL	AK	GEN	IPM	COT	LE	CIP	OF
A. hydrophila D2007	R*	R	R	R	I*	R	R	R	S*	S	R	S	I	S
E. coli W102	R	R	R	R	R	R	R	I	S	R	R	R	R	R

* Denotes for Resistant (R), Intermediate (I) and Susceptible (S).

TABLE 2. Characteristics of bacteriophages against *A. hydrophila* D2007 and *E. coli* W102.

Phage name	Bacterial host	Plaque morphology	Plaque diameter (mm)	Prospected family	Head dimension (nm)	Tail length (nm)	Tail width (nm)	Concentration (PFU/mL)
ΦAHP7	*A. hydrophila* D2007	Small, punctiform, clear, without center, without halo	2mm	*Myoviridae*	88.55±0.32× 114±3	77.25±0.51	26.35±1.35	$2.9x10^7$
ΦECP8	*E. coli* W102	Circular, clear, without center, without halo	4mm	*Siphoviridae*	60.22±1.13	202.25±2.25	23.86±1.14	2.3×10^6
ΦECP9	*E. coli* W102	Circular, clear, without center, without halo	6mm	*Podoviridae*	66.95±0.45	16.8±0.2	16.7±0.1	6.5×10^6

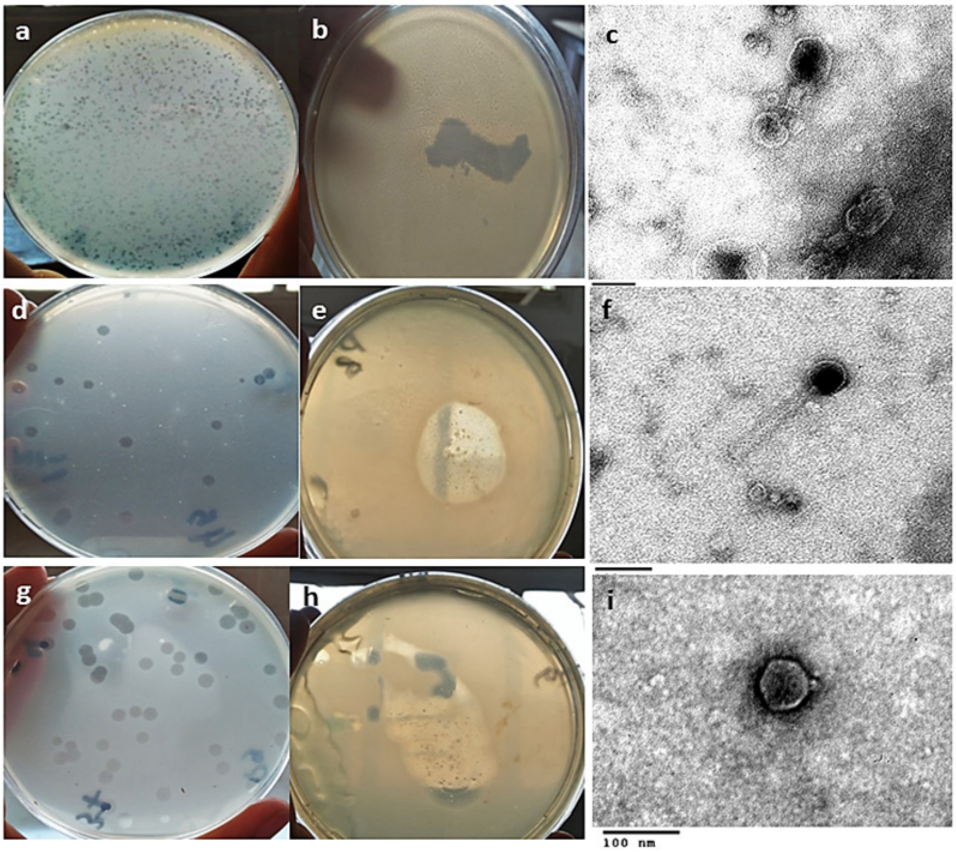

Fig. 1. (a, d, g) plaque morphology of phages ΦAHP7, ΦECP8 and ΦECP9 respectively and (b, e, h) spot test showing lytic activity of phages ΦAHP7, ΦECP8 and ΦECP9, respectively. (c, f, i) TEM analysis showing phages morphology ΦAHP7 (the family Myoviridae), phage ΦECP8 (the family Siphoviridae) and phage ΦECP9 (the family Podoviridae) [The scale bar represents 100nm].

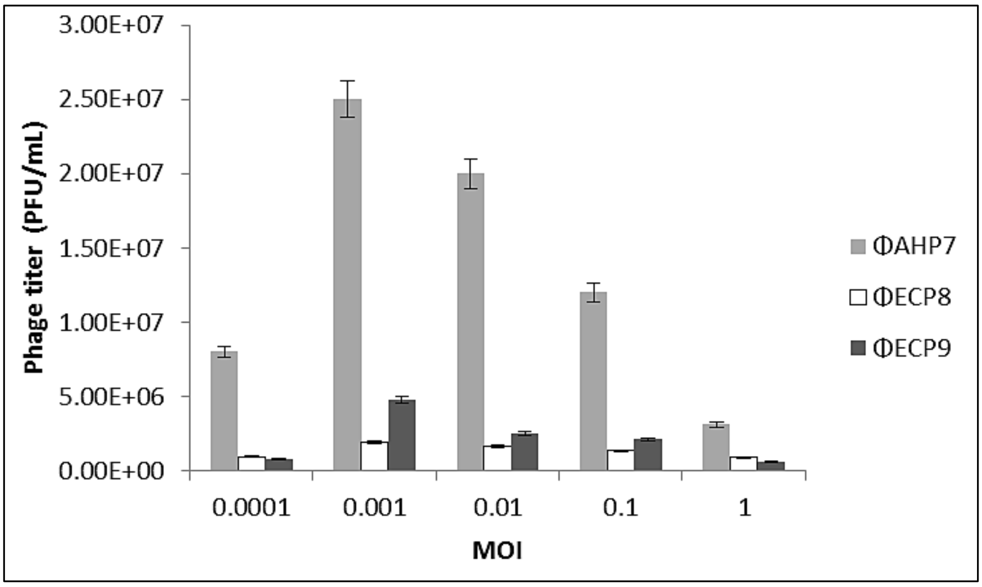

Fig. 2. Optimal multiplicity of infection (MOI) of phages ΦAHP7, ΦECP8 and ΦECP9. Comparison of phages titer after incubation at five ratios of MOI (0.0001, 0.001, 0.01, 0.1 and 1 in LB medium.

Bacteriophages host range

To evaluate the phages (ΦAHP7, ΦECP8, and ΦECP9) host range, their infectivity was tested against 22 different bacterial isolates (Table 3), and the selected isolates had multidrug resistance (data not shown). Phage ΦAHP7 infected 3 *Aeromonas hydrophila* isolates, *Acinetobacter baumanni* complex 15 and *Klebsiella pneumoniae* spp. pneumoniae14 while the two *E. coli* phages (ΦECP8 and ΦECP9) were able to infect all multidrug-resistant *E. coli* isolates and Gram-positive *Streptococcus* spp. However, the three phages were not able to lyse the other bacterial strains used in this study (Table 3).

One step growth curve

The obtained one-step growth curve of the three phages (ΦAHP7, ΦECP8, and ΦECP9) indicated that the latent periods of infection were 10, 20 and 10min with average burst sizes of 53.5±0.5, 26.5±0.5 and 67.5±0.5 virions per infected bacterial cell respectively (Fig. 3). The complete infection cycles of ΦAHP7, ΦECP8, and ΦECP9 were 35, 55 and 30min respectively.

TABLE 3. Lytic activity of the *A. hydrophila* D2007 and *E .coli* W102 phages (ΦAHP7, ΦECP8 and ΦECP9) on different bacterial species.

Name of bacterial isolate	Source of bacterial isolate	Name of phage		
		ΦAHP7	ΦECP8	ΦECP9
Aeromonas hydrophila D2007	This study	+	-	-
Aeromonas hydrophila (1)	Lab isolate	+	-	-
Aeromonas hydrophila (2)	Lab isolate	+	-	-
Escherichia coli W102	This study	-	+	+
Escherichia coli S1	Benha children's Hospital	-	+	+
Escherichia coli S2	Benha children's Hospital	-	+	+
Escherichia coli Af	Nasser Institute Hospital	-	+	+
Escherichia coli W	Nasser Institute Hospital	-	+	+
Escherichia coli A	Nasser Institute Hospital	-	+	+
Escherichia coli U	Nasser Institute Hospital	-	+	+
*Streptococcu*s spp.	Benha University Hospital	-	+	+
Serratia spp.	Benha University Hospital	-	-	-
Staphylococcus aureus	Benha University Hospital	-	-	-
Proteus spp.	Benha University Hospital	-	-	-
Salmonella spp.	Benha University Hospital	-	-	-
Shigella spp.	Benha University Hospital	-	-	-
Acinetobacter baumanni complex 15	Sohag University Hospital	+	-	-
Acinetobacter baumanni complex 20	Sohag University Hospital	-	-	-
Klebsiella pneumoniae spp. pneumoniae13	Sohag University Hospital	-	-	-
Klebsiella pneumoniae spp. pneumoniae14	Sohag University Hospital	+	-	-
Klebsiella pneumoniae spp. pneumoniae 21	Sohag University Hospital	-	-	-
Klebsiella pneumoniae spp. pneumoniae23	Sohag University Hospital	-	-	-

(+ means lysis) (- means no lysis)

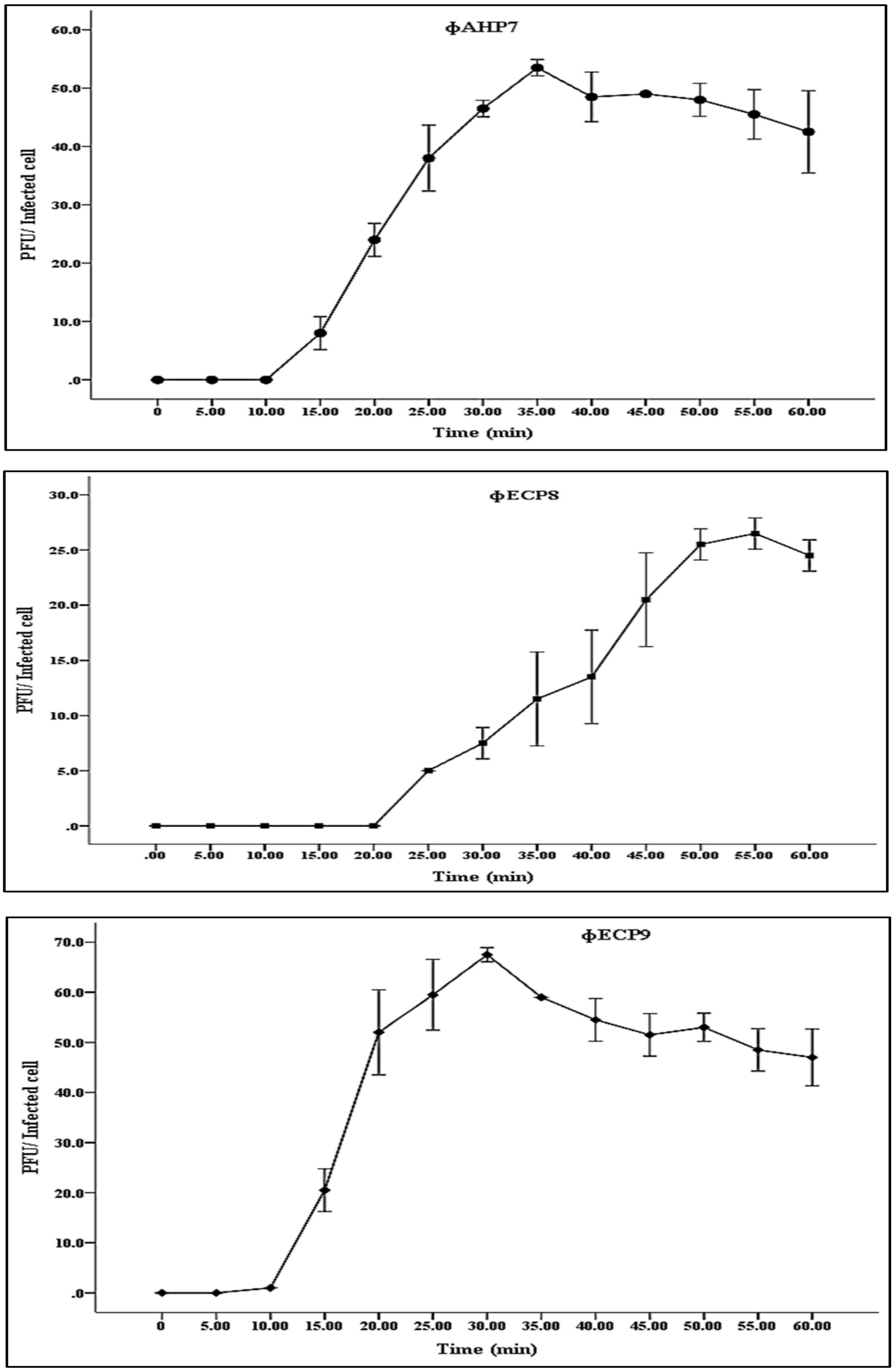

Fig. 3. One-step growth curves of three phages ΦAHP7, ΦECP8 and ΦECP9 [Bacteriophages showed the latent period of about 10, 20 and 10min and burst sizes 53.5 ±0.5, 26.5 ±0.5 and 67.5 ±0.5 virions per bacterial cell, respectively, error bars represent ± the standard deviation].

Bacteriophages stability

In order to evaluate the stability of phages in various conditions, different temperatures, pH values and NaCl concentrations were subjected. Phage ΦAHP7 was shown to be active at ranging of the temperature between 25°C to 45°C and it was inactivated at temperatures 55°C. Phages ΦECP8 and ΦECP9 were shown to be active over the temperatures tested at 25°C, 37°C, 45°C and 55°C. They were inactivated at temperatures 65°C (Fig. 4). According to phage titer, the optimum pH value for the three phages was 7 after overnight incubation at 28°C; phage ΦAHP7 was shown to be stable at ranging of pH value from 7 to 9 after 60min of incubation. On the other hand, phage ΦECP9 showed pH stability at ranging from 5 to 9, after 30min at 28°C and it retained its lytic ability up to 12hrs at pH ranging from 7 to 9. While ΦECP8 phage was stable at pH value ranging from 7 to 9 after 60min of incubation only (Fig. 5). Phages titer was relatively stable when phages ΦAHP7, ΦECP8, and ΦECP9 were incubated for 30 min in solutions containing 0.1, 0.25, 0.75, 1 and 2M NaCl (Fig. 6).

The bacterial growth reduction assay

The lytic activities *in vitro* of the three phages against multidrug-resistant *A. hydrophila* D2007 and *E. coli* W102 isolates were evaluated by measuring the optical density (OD_{600}) of liquid medium during the growth of host bacteria at 37°C and MOI 0.001 (optimal MOI). Similar patterns of inhibition were observed when *A. hydrophila* D2007 and *E. coli* W102 have been used as hosts with individually isolated phages ΦAHP7, ΦECP8 and ΦECP9. A noticeable gradual reduction in OD_{600} was observed where the reduction was about 85.29% for *A. hydrophila* ΦAHP7 after 48hrs of incubation when compared to the untreated control. While the reductions were 85.88 and 88.23% for *E. coli* W102 treated with ΦECP8, ΦECP9 phages respectively, when compared to the untreated control, which reached 1.7 OD_{600} at 48hrs (Fig. 7).

Application of bacteriophages in artificially contaminated fresh vegetables

Treatment of the experimentally contaminated red cabbage with ΦAHP7 reduced the number of viable *A. hydrophila* D2007 organisms by 1.55 log CFU/g (P< 0.01) after 30min of incubation and by 1.61 log CFU/g (P< 0.01) after 24hrs of incubation at room temperature. Reductions of viable *E. coli* W102 counts were 1.68 log CFU/g (P< 0.01) and 1.76 log CFU/g) (P< 0.01); after 30min and 24hrs of the storage period or incubation period respectively with phage ΦECP8, while reductions counts were 2.28 log CFU/g (P< 0.01) after 30min and 2 log CFU/g (P< 0.01) after 24hrs of the storage period or incubation period with phage ΦECP9 (Table 4).

Treating the contaminated tomato slices with *A. hydrophila* D2007 preparations containing phage ΦAHP7 at a concentration (10^6 PFU/mL) produced reductions by 1.48 log CFU/g (P< 0.01) and 1.39 log CFU/g (P< 0.01) after 30min and 24hrs of incubation at room temperature respectively. Reductions of viable *E. coli* W102 counts were 1.38 log CFU/g (P< 0.01) after 30min and 1.52 log CFU/g (P< 0.01); after 24hrs of the storage period or incubation period with phage ΦECP8 while, reductions counts were 1.68 log CFU/g (P< 0.01) after 30 min and 1.53 log CFU/g P< 0.01) after 24hrs of the storage period or incubation period with phage ΦECP9 (Table 5).

Phages ΦAHP7, ΦECP8, and ΦECP9 rapidly reduced multidrug-resistant (MDR) *A. hydrophila* D2007 and *E. coli* W102 viable counts on red cabbage and slices of tomato after 30min followed by a stable antibacterial effect 24hrs of storage at room temperature.

Discussion

Food-borne pathogens are managed using various natural or chemical food preservatives. Weak and limited antimicrobial activities were shown by natural preservatives such as organic acids and lactoferrin (Juneja et al., 2012). Nonetheless, because of their known side effects consumers do not usually prefer chemical preservatives (Pawlowska et al., 2012). In addition, although antibiotics are robust and strongly antimicrobial, are not suitable for food applications, and many foodborne pathogens are resistant to several antibiotics. Therefore, lytic phages possess ideal properties to serve as an antibacterial biocontrol agent. In this study, antibiotic-resistant *Aeromonas hydrophila* D2007 and *Escherichia coli* W102 were isolated from leafy herb (Dill) and drinking water samples respectively in Benha City of Egypt (Denis et al., 2016; Chen et al., 2017; Tanner et al., 2019). The rising incidence of pathogenic antibiotic-resistant bacteria has sparked a renewed interest in phage bicontrol agents. In this regard three newly isolated bacteriophages ΦAHP7, ΦECP8, and ΦECP9 have been characterized. Whereas, ΦAHP7 infecting *A. hydrophila* D2007 while ΦECP8 and ΦECP9 infecting *E. coli* W102 and they were isolated from sewage samples (Peng & Yuan, 2018) to reduce health hazards for fresh food and water consumers.

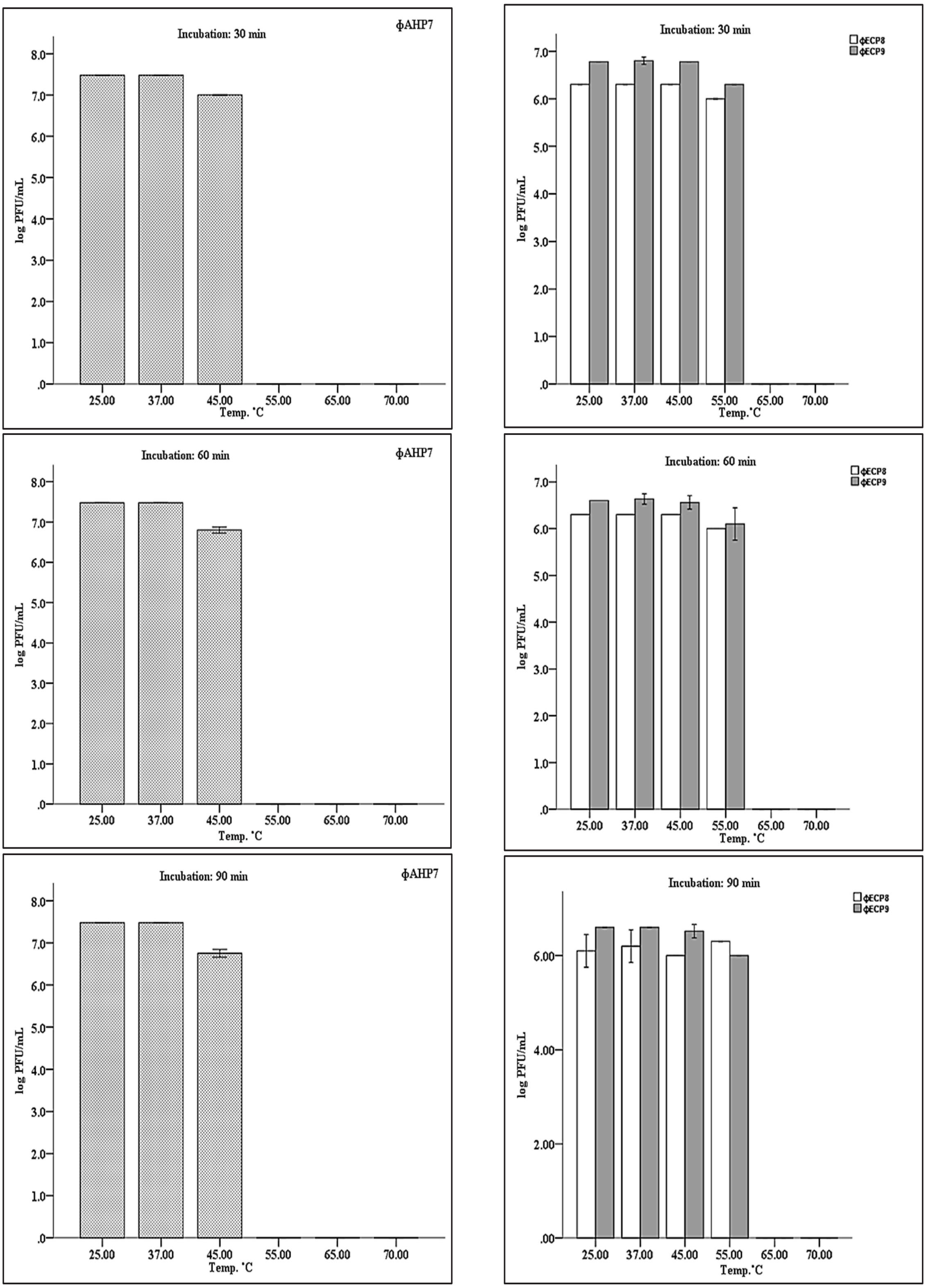

Fig. 4. Thermo stability of *A. hydrophila* D2007 phage (ΦAHP7) and *E. coli* W102 phages (ΦECP8 and ΦECP9) [Phages were incubated at different temperatures at incubation periods 30, 60 and 90min, error bars represent standard deviation (SD) of the mean of three replicates].

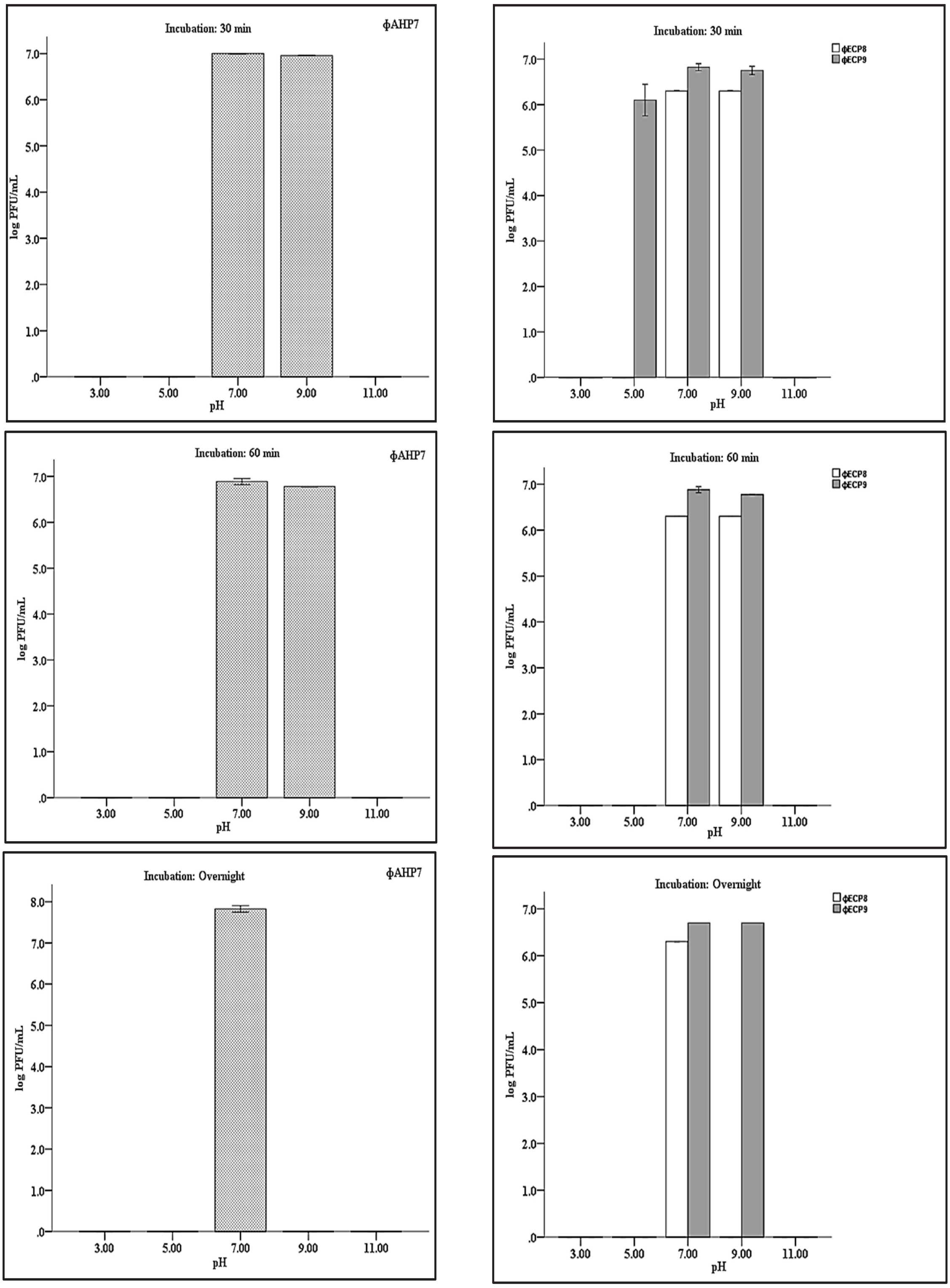

Fig. 5. Stability of *A. hydrophila* D2007 phage (ΦAHP7) and *E. coli* W102 phages (ΦECP8 and ΦECP9) treated with different pH values at incubation 30, 60min and overnight at 28°C [Error bars represent standard deviation (SD) of the mean of three replicates].

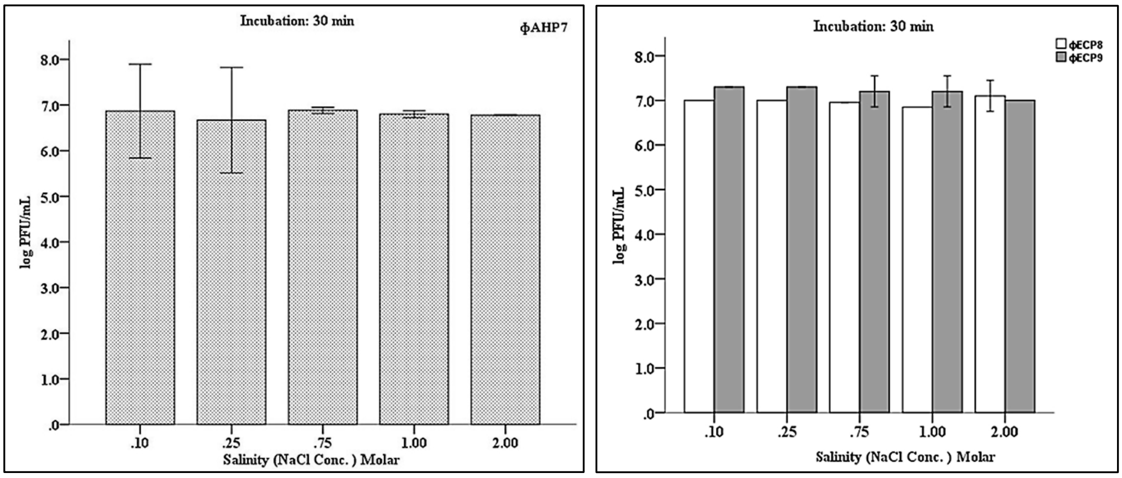

Fig. 6. Stability of *A. hydrophila* D2007 phage (ΦAHP7) and *E. coli* W102 phages (ΦECP8 and ΦECP9) at different salinity values (Molar NaCl Conc.) after 30min of incubation at 28°C [Error bars represent standard deviation (SD) of the mean of three replicates].

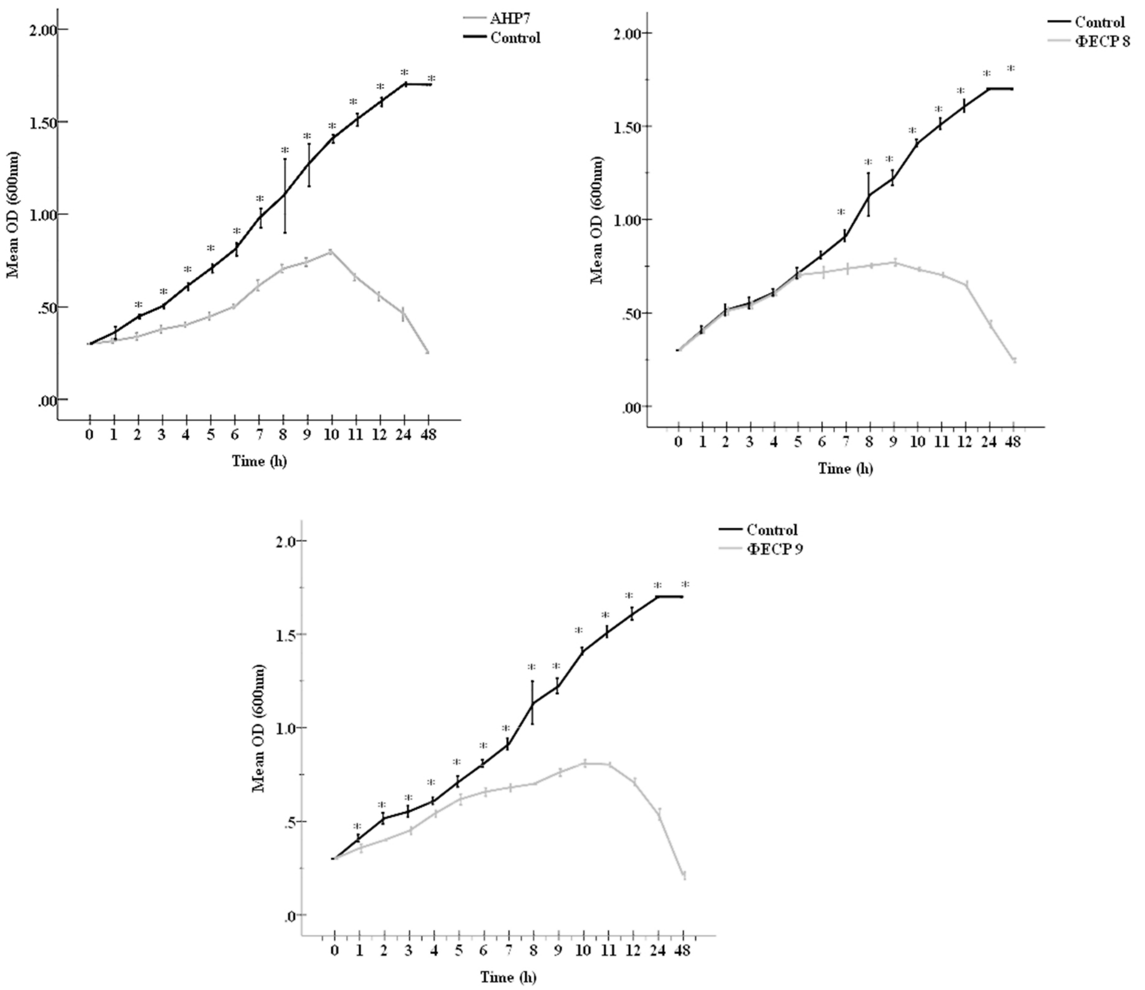

Fig. 7. Growth of *A. hydrophila* D2007 and *E. coli* W102 in LB broth, treated with individual ΦAHP7, ΦECP8 and ΦECP9 phages at optimum MOI (0.001) and without phages (control) [Error bars represent standard deviation of the mean of three replicates, asterisk (*) indicates statistically significant difference (P< 0.05)]. .

TABLE 4. Treatment results of red cabbage.

Incubation period	Bacterial counts (Log CFU per g ± SD)							
	A. hydrophila D2007	*A. hydrophila* D2007 + phage (ΦAHP7)	Reduction	*E. coli* W102	*E. coli* W102 + phage (ΦECP8)	Reduction	*E. coli* W102 + phage (ΦECP9)	Reduction
30min	8.46±0.01	6.91±0.01	1.55±0.02	8.47±0.00	6.78±0.07	1.68±0.07	6.18±0.04	2.28±0.04
60min	8.45±0.01	6.88±0.03	1.57±0.02	8.44±0.01	6.48±0.02	1.97±0.01	6.31±0.06	2.13±0.05
90min	8.41±0.01	6.80±0.02	1.61±0.01	8.43±0.00	6.57±0.07	1.86±0.06	6.40±0.05	2.02±0.03
24hrs	8.39±0.00	6.78±0.02	1.61±0.01	8.41±0.03	6.65±0.01	1.76±0.03	6.43±0.07	2.00±0.07

± SD represents the standard deviation

TABLE 5. Treatment results of tomato slices.

Incubation period	Bacterial counts (Log CFU per g ± SD)							
	A. hydrophila 2007D	*A. hydrophila* 2007D + phage (ΦAHP7)	Reduction	*E. coli* 102W	*E. coli* 102W + phage (ΦECP8)	Reduction	*E. coli* 102W + phage (ΦECP9)	Reduction
30min	8.44±0.00	6.96±0.00	1.48±0.00	8.22±0.00	6.84±0.01	1.38±0.00	6.52±0.04	1.68 ±0.03
60min	8.36±0.01	6.94±0.01	1.41 ±0.00	8.17±0.01	6.72±0.04	1.45±0.03	6.63±0.04	1.53± 0.02
90min	8.31±0.01	6.87±0.02	1.43± 0.01	8.14±0.02	6.76±0.03	1.39±0.00	6.69±0.03	1.45±0.02
24hrs	8.25±0.05	6.85±0.01	1.39±0.03	8.30±0.02	6.78±0.04	1.52±0.02	6.73±0.03	1.53±0.04

± SD represents the standard deviation.

Plaque morphology is important criteria for detection, identification, and classification of phages (Haq et al., 2012). Phages with clear plaque may demonstrate that they are phages of lytic (virulent) type. Different sizes of each phages formed plaque are caused by different phages types. A small plaque will be formed by phages from the Myoviridae family, while the Siphoviridae and Podoviridae families shall produce a larger plaque (Haq et al., 2012). Typically, Phage ΦAHP7 produced small transparent plaques as indicated by Hoang et al. (2019). Phages ΦECP8 and ΦECP9 produced clear large plaques 4 and 6 mm in diameter respectively while in another study, *E. coli* phage (vB-EcoS-95) formed clear plaques 2.5 ± 0.5mm in diameter, with a halo, on *E. coli* lawn (Topka et al., 2019).

Different studies have shown that more than 90 percent of isolated lytic phages and characterized with TEM belong to the Caudovirales order (Ackermann, 2003). In the current study, identified phages belong to the Caudovirales which they were morphologically characterized by TEM whereas ΦAHP7 belongs to Myoviridae, and these results in accordance to Le et al. (2018) who successfully isolated *A. hydrophila* phages (Φ2 and Φ5) which belonging to the Myoviridae family. While *E. coli* phages (ΦECP8 and ΦECP9) belong to Siphoviridae and Podoviridae families respectively as reported by Peng & Yuan (2018).

The optimal multiplicity of infection (MOI) value of ΦAHP7, ΦECP8 and ΦECP9 phages was 0.001. Some researchers reported the MOI value of *A. hydrophila* bacteriophage ranged from 0.1 to 0.0001(Richards, 2014; Wang et al., 2016; Hoang et al., 2019). Phage Bp7 MOI value to infect *E. coli* for higher yields was about 0.001(Zhang et al., 2013). Li et al. (2019) reported that the optimal MOI for *E. coli* phages (IME281, IME338, IME339, IME340, and IME341) was 0.001. A low MOI is an advantage in the large-scale application, phage production and can thus effectively reduce the overall cost of a commercial phage preparation (Wong et al., 2014; Bao et al., 2015; Yildirim et al., 2019).

Characterization of the three phages was achieved by determining their host range, ΦAHP7 infected the three *A. hydrophila* isolates in addition to *Acinetobacter baumanni* complex 15 and *Klebsiella pneumoniae* spp. pneumoniae14 out of 22 bacterial isolates used in this study while in another study stated that *A. hydrophila* phages (Φ2 and Φ5) were found to inhibit the growth of all *A. hydrophila* strains tested only (Le et al., 2018). The two phages ΦECP8 and ΦECP9 showed high lytic activity against all multidrug-resistant *E. coli* isolates and Gram positive *Streptococcu*s spp. out of 22 tested bacterial isolates (Litt & Jaroni, 2017). On the other hand, the phage vB_EcoS_HSE2 could only infect 6 out of 11 tested *E. coli* strains (Peng and Yuan, 2018). A phage is known to be a very lytic phage when it has a short latent time and/or large burst size. The three phages derived from the survey showed relatively short latent time with burst sizes (26±0.5– 67.5±0.5 PFU/cell) indicating their applicability for a bacterial treatment scheme (Duc et al., 2018).

The survival and persistence of bacteriophages are affected by physico-chemical factors such as pH, ions, and temperature (Jonczyk et al., 2011). Thermal stability tests of phages ΦAHP7, ΦECP8 and ΦECP9 carried out to analyze the heat-resistant activity. The phages ΦAHP7, ΦECP8, and ΦECP9 showed stability over a wide temperature range. The lytic ability of ΦECP8 and ΦECP9 was not affected within a temperature range of 25–55°C, the infectivity of *Aeromonas hydrophila* phages ΦZH1 and ΦZH2 were highly sensitive to a temperature above 40°C (El-Araby et al., 2016) but in our results, the thermal stability of phage ΦAHP7 was sensitive to elevated temperature (55ºC to 70ºC) (Chen et al., 2018). In the same context, Akmal et al. (2020) reported that *Aeromonas* phage Akh-2 retained its infectivity from -80°C to 37°C after three days of incubation.

Phages ΦECP8 and ΦECP9 were more stable than phage ΦAHP7 which they retained its viability after exposure to 55ºC for 90 min, but they lost their infectivity after exposure to 65ºC for 90min. Samhan et al. (2016) reported that the coliphages ECP1, ECP2, and ECP3 were inactivated after exposure to 60ºC for 10min. However, the maximum lytic capacity of *E. coli* phage (P.E1) was reported to occur at 37°C (Bibi et al., 2016). The phage count was decreased gradually from 50–70°C. The stability of the phages with a wider pH range was essential to the preservation and application of phages in biocontrol applications (Goode et al., 2003). Phages were stable and survive at close to neutral pH values between 5 and 9. A reasonable lytic ability at pH 9 shows that bacteriophages ΦAHP7, ΦECP8, and ΦECP9 were resistant to alkaline conditions (Bibi et al., 2016; Chen et al., 2018). The viability of the phages tested decreased more quickly to acidity than alkalinity. Survival at alkaline pH could be used in processing facilities or food contact surfaces during a multi-hurdle approach (Tait et al., 2002; Srey et al., 2013). In this study, phages titer was relatively stable within different sodium chloride concentrations (0.1, 0.25, 0.75, 1 and 2 molar) after 30min of incubation (Mylon et al., 2010). Smolarska et al. (2018) stated that, phages ϕA38 or ϕA41 titers did not change within solutions containing different sodium chloride concentrations (0.05, 0.5 and 5.0M NaCl) after 24hrs of incubation. Stability results indicated that bacteriophages ΦAHP7, ΦECP8 and ΦECP9 have the potential to be used in diverse environments and different foodstuffs.

The current work has shown that all phages have decreased the growth of host bacteria *in vitro* as opposed to untreated controls. At a low MOI (0.001), a quick lysis of bacterial cells following Φ AHP7, ΦECP9 treatments was reported, while phage ΦECP8 started to lysis the host cells after 6 hours of treatment. This shows that the phages could to propagate, induce lysis, and eventually kill the host cells. Several studies were conducted to test phages ability to fight *A. hydrophila* and *E. coli* at Low MOI (Le et al., 2018; Hoang et al., 2019; El-Shibiny et al., 2017; Tanji et al., 2005; Ghasemian et al., 2017).

The obtained data show that separated treatments with *A. hydrophila* and *E. coli* specific lytic bacteriophages were an effective method for reducing (bacterial contamination) on fresh-cut foods (red cabbage- tomato), this is similar to the previous investigation with respect to *Salmonella* and *E. coli* O157:H7 specific phages which caused greater reduction than using chemical sanitizers (Abuladze et al., 2008). Our results showed *A. hydrophila* D2007 counts were rapidly reduced by 1.55 log CFU/g (P< 0.01) and 1.48 log CFU/g (P< 0.01) on red cabbage and slices of tomato respectively upon phage ΦAHP7 treatments at MOI of 100 after 30 min of application followed by a stable antibacterial effect 24hrs of storage at room temperature. As a phage therapy Le et al. (2018) demonstrated that phages (Φ2 and Φ5) are considered as potential biocontrol agents to combat *A. hydrophila* infections in fish farms. In the same direction, Kazimierczak et al. (2019) reported that, 6 new isolated phages could be used as a therapeutic cocktail giving the infected of

41% of the *Aeromonas* pathogenic environmental isolates. In this study, only 1.38 log CFU/g (P< 0.01) *E. coli* W102 viable count reduction was found with phage ΦECP8 treatment while 1.68 log CFU/g (P< 0.01) with phage ΦECP9 on tomato slices 30min of application at room temperature. An average of 1.68 log CFU/g (P< 0.01) *E. coli* W102 viable count reduction was found with phage ΦECP8 treatment while 2.28 log CFU/g (P< 0.01) with phage ΦECP9 on red cabbage 30min of application at 28°C. These results are in accordance with Ferguson et al. (2013) who reported that initial reduction in *E. coli* O157:H7 (~0.8–1.3 logs CFU/cm^2) counts upon phage mixture spraying were observed in lettuce. But dipping demanded that the lettuce was submerged for as long as 2min and there were no major initial reductions. After 1 day of storage at 4°C, *E. coli* was reduced by ~0.7 log CFU/cm^2 by dipping in the highest concentration of the phage cocktail. While *Salmonella* counts on lettuce decreased continuously from 1.9 to 2.7 log_{10} CFU/cm^2 of 3 to 5hrs after phage treatment (LPST10) at MOI of 100 (Huang et al., 2018). In addition, Bai et al. (2019) found that a phage cocktail treatment at MOI 1000 resulted in a significant decrease in *S.* Typhimurium counts on iceberg lettuce leaves from 1.1 to 1.9 log CFU/cm^2 after 4hrs of incubation at 25°C.

Nevertheless, the treatment of iceberg lettuce leaves with 20-200ppm of aqueous chlorine dioxide displayed less than 1 log inactivation (Keskinen et al., 2009). Therefore the use of bacteriophages to eliminate bacterial pathogens that contaminate fresh food is one of the most environmentally friendly and safe solutions to reducing the incidence of foodborne diseases.

Conclusions

In this study, Three specific phages were characterized, one for *A. hydrophila* D2007 and two phages for *E. coli* W102. Moreover, our findings demonstrate the efficacy of phages ΦAHP7, ΦECP8, and ΦECP9 for the rapid reduction of multidrug-resistant *A. hydrophila* D2007 and *E. coli* W102 *in vitro* and on red cabbage and slices of tomato after 30 min followed by a stable antibacterial effect 24 h of storage at room temperature. Phage ΦECP9 had high lytic activity against multi-drug resistant artificially contaminated *E. coli* W102 in fresh food than phage ΦECP8. In further studies, the combination of phages with high numbers of infections and strong natural preservatives will be made available to ensure the continued inactivation of these pathogens on food surfaces.

Conflict of interest: The authors reported no potential conflict of interest.

Authors contribution: M.A.N. and A.A.S. designed and performed the experiments. M.A.N. and A.A.S. analyzed the data. M.A.N. wrote the manuscript. All authors read and approved the manuscript.

Ethical approval: Not applicable.

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تقييم فاجات محلله جديدة تصيب ايروموناس هيدروفيلا و إيشيريشيا كولاى المعزولة من عينات مياه الصرف الصحي

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تظهرالأن البكتيريا المقاومه للعديد من الادوية لجميع المضادات الحيوية الحالية تقريبا. تم عزل بكتيريا ايروموناس هيدروفيلا(D2007) و إيشيريشيا كولاى (W102) من عينات الطعام الطازج ومياه الشرب وكانت مقاومة بنسبه 57.14% و 85.71% للعديد من المضادات الحيوية الشائعة المختبرة على التوالي. و تم عزل ثلاث فاجات متخصصة من عينات مياه الصرف الصحي. أكدت الاختبارات المورفولوجية أن فاج ΦAHP7 الذى يصيب ايروموناس هيدروفيلا(D2007) ينتمي إلى عائلة Myoviridae و الفاجات ΦECP8 و ΦECP9 القادرة على اصابة إيشيريشيا كولاى (W102) ينتمو إلى عائلات Siphoviridae و Podoviridae على التوالي. بالنسبة للفاجات الثلاثة ، تم حساب التعدد الأمثل للعدوى(MOI) ليكون 0.001. تم توصيف الفاجات بتحديد المدى العوائلى لهم وثباتهم فى قيم مختلفه من الأس الهيدروجيني ودرجات الحرارة والملوحة. كانت الفترات الكامنة للفاجات ΦAHP7وΦECP8 وΦECP9 هى 10 و 20 و 10 دقائق بمتوسط أحجام انفجار 53.5±0.5 و 26.5±0.5 و 67.5±0.5 فاجات لكل خلية مصابة على التوالي. خفضت الفاجات الثلاثة تدريجيًا OD600 وكانت قادرة على إيقاف نمو ايروموناس هيدروفيلا(D2007) و إيشيريشيا كولاى (W102) في المعمل عند (MOI) منخفضة قدرها 0.001. حققت المعاملات بالفاجات ΦAHP7 و ΦECP8 و ΦECP9انخفاض فى اعداد البكتيريا الحية في الكرنب الاحمر بمقدار 1.55 و 1.68 و 2.28 لوغاريتم CFU/g (P<0.01) و انخفاض بمقدار 1.48 و 1.38 و 1.68 لوغاريتم CFU/g (P<0.01) في الطماطم بعد 30 دقيقة في درجة حرارة الغرفة (28 درجة مئوية) على التوالي. ان تطبيقات الفاجات المحلله ΦAHP7 و ΦECP8 و ΦECP9 تؤدي إلى انخفاض سريع في أعداد البكتيريا الممرضه ايروموناس هيدروفيلا(D2007) و إيشيريشيا كولاى (W102) في الأغذية الطازجة للاستهلاك البشري.