



## The application of bacteriophage ZCEC5 to control multi-drug resistant *E. coli* in dairy products

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### Abstract

*Escherichia coli* are one of the most important causes of foodborne diseases worldwide, and it can be transmitted via the fecal-oral route. Antibiotics are considered the main treatment to control *E. coli*, and due to the abuse and misuse of antibiotics, multidrug-resistant bacteria have emerged and become a challenge to public health worldwide. Bacteriophages are a group of viruses that can infect and kill bacteria.

Dairy samples from different locations in Cairo were collected to isolate *E. coli* strains and their specific bacteriophages. Isolated *E. coli* strains were subjected to an antibiotic sensitivity test using the disk diffusion method. In addition, isolates were identified by polymerase chain reaction (PCR) and 16S rRNA. Bacteriophage was isolated, purified, amplified, and tested against the isolated *E. coli* strains. Consequently, the morphology of the isolated phage was studied using the transmission electron microscope (TEM), and the phage genome size was determined by pulsed-field gel electrophoresis (PFGE). The stability of phage at different pH and temperature values, in addition to UV exposure, was also studied. In addition, the replication dynamics of isolated phage were investigated in milk.

Thirty-two dairy samples were collected, and only twenty-seven isolates were *E. coli* positive. The 16S rRNA results of the selected isolate showed that the strain had a 99.57% sequence identity to the *E. coli* strains. The antibiotic sensitivity profile of the *E. coli* strain, according to CLSI, confirmed that it is a multi-drug-resistant bacterium. One bacteriophage was isolated from Karish cheese, and its morphology confirmed that it belongs to the Siphovirus family with an icosahedral capsid containing double strands of DNA and a non-enveloped phage with a long and non-contractile tail and a genome size of approximately 70 kb. The phage showed stability at different temperatures up to 70 °C and at different pH values between 4 and 12. In addition, the phage withstands UV exposure for one hour. The phage exhibited lytic activity against *E. coli* growth in milk at an MOI of 2 after 3 hours of incubation at 37 °C.

The results showed the high activity and ability of the isolated phage to control *E. coli* growth in milk. Thus, phage(s) can be used as a biocontrol agent to control pathogenic bacteria in milk and extend its shelf life.

**Keywords:** Bacteriophage, bio-control, pathogenic bacteria, *E. coli*, milk.

### 1. Introduction

Several millions of people around the world are consuming milk and dairy products as part of their diet since they are rich in calcium, protein, phosphorus, and other micronutrients that might improve the health status of the skeletal, muscular, and neurological systems [1]. In addition, milk and milk products may protect against some chronic

diseases such as bladder cancer [2]. Moreover, some fermented dairy products, such as cheese and yogurt are associated with a reduction in type 2 diabetes [3]. The quality of raw milk can be reduced if it is contaminated with certain species of pathogenic and undesirable bacteria, especially those that produce enzymes and toxins that can tolerate pasteurization and consequently cause serious health problems [4, 5]. *Escherichia coli* (*E. coli*) are one of the most common bacteria associated with foodborne diseases

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worldwide. Milk and dairy products are considered one of the main sources of human infection with *E.*

*coli* [6-8]. *E. coli* belongs to the *Enterobacteriaceae* family and is a Gram-negative, non-spore-forming, flagellated, facultative anaerobic, and rod-shaped bacterium [8,9]. *E. coli* can cause intestinal and extra-intestinal infections and it is easy to transmit by the fecal-oral route due to its ability to colonize the gut of humans and animals. In humans, these bacteria have been identified as one of the main causes of urinary tract infections in addition to their ability to infect other cells in the human body. It can be associated with gastrointestinal infections, skin abscesses, appendicitis, bloodstream infections, pneumonia, meningitis, endocarditis, intra-amniotic, and puerperal infections in pregnant women [10,11]. In animals, during early lactation, *E. coli* may cause acute clinical mastitis in dairy cows [12,13].

In the United States, multidrug-resistant (MDR) Gram-negative bacteria are spreading and causing diseases according to the Centers for Disease Control and Prevention (CDC) [10], and approximately 25000 people die every year from (MDR) infection in Europe [14]. The development of antibiotic resistance is increasing globally and in developing countries, such as India [15, 16], which may be due to the misuse of antibiotics without a prescription from the concerned doctors. Furthermore, many people do not know the appropriate dose of antibiotics, even though there is a high incidence of infectious diseases these days, due to a lack of healthcare systems and a lack of public health awareness among people [17]. Antibiotic resistance may also occur due to the presence of mutations that can be transferred to the offspring by vertical gene transfer or to another bacterium by horizontal gene transfer such as transduction, conjugation, or [18]. *E. coli* showed resistance to different antibiotics in the past two decades such as quinolones,  $\beta$ -lactams, sulfonamides, fosfomycin, and aminoglycosides, and unfortunately, showed resistance to the most effective antibiotics such as carbapenems and polymyxins that can be used to control *E. coli* strains [19,20,11]. But because of the abuse of antibiotics, MDR bacteria have emerged and caused a great challenge to the medical sector in the 21st century [21].

Bacteriophages (phages in short) are a group of viruses that can infect and kill bacteria [22]. They are the most abundant microorganisms found on the earth and can be isolated from their bacterial host in soil,

seawater, animal and human tissues, oceanic surfaces, and also in extreme environments [23]. They are very specific toward their bacterial host and can sometimes infect a few strains within a species [24]. The classification of bacteriophages is based on morphological characteristics, the site where they can mostly be found, bacterial species that they can infect, and their biological cycle [25].

Previous studies indicated that foodborne pathogenic bacteria such as *E. coli* O157:H7 have been controlled in ground beef by applying bacteriophage [26, 27]. Phages have also been used as a cocktail ( $\Phi$ 565.2;  $\Phi$ 565.1;  $\Phi$ 13;  $\Phi$ 3) to reduce the growth of *E. coli* at the beginning of the fermentation process in milk without any change in microbiological and physicochemical parameters. During the first steps of cheese manufacturing, the phage cocktail's effect was dose-related and led to a notable decrease in the number of cheese eyes and the region filled by gas holes [28]. Therefore, bacteriophages can be used as biocontrol agents in any stage of dairy processing. Thus, this study aimed to isolate and identify the antibiotic-resistant *E. coli* bacterium from conventional dairy products, as well as to isolate and characterize a phage strain that can lyse *E. coli* in milk.

## 2. Materials and methods:

### 2.1. Collection of samples:

Thirty-two samples of milk and dairy products were collected from different locations in Great-Cairo Governorate in October (2019) as follows: eleven samples of K.arish cheese, eight raw milk samples, four cream samples, three yogurt samples, and six soft cheese samples. All samples were taken aseptically, put in sterile containers, kept cold (4°C), and then brought to the lab for bacteriological analysis and *E. coli* isolation. All samples were examined for the presence of *E. coli* under aseptic conditions.

### 2.2. Isolation, identification, and cultural characteristics of *E. coli*:

Enumeration of *E. coli* in all samples was carried out by using selective media by spreading 0.1 ml of each sufficient dilution onto plates of Sorbitol MacConkey agar medium (Oxoid, UK) and the plates were incubated at 35° C incubation for 24 hrs. The growth of *E. coli* on MacConkey agar with sorbitol shows colorless colonies. Three to five colonies that

had the characteristic *E. coli* morphology (colorless) were chosen from each plate and cultured on blood Agar (BA). Every selected suspicious colony was streaked onto plates containing Eosin Methylene Blue agar (EMB, Merck, Germany) [29, 30], and incubated overnight at 37°C. After 24 hr. of incubation, further confirmation was applied by using biochemical tests including conventional indole reduction, methyl red, Voges Proskauer, citrate utilization, and lysine decarboxylase tests. The strains were stocked in tryptone soya broth (TSB) containing glycerol (20% (w/v) [31] and stored at -80°C until needed.

To identify the bacterial strain, further confirmation by polymerase chain reaction (PCR) was performed using specific primers (FP: AAGAAGCACCGGCTAACTCC and RP: CGCTTCTCTTTGTATGCGCC) followed by sequencing its 16S–23S rRNA internal transcribed spacer. Conserved primers (FP; 5'-ATTTGAAGAGGTTGCAAACGAT -3' and RP: 5'-TTCACCTCTGAAGTTTTCTTGTGTTC -3') were synthesized by LGC Biosearch Technologies, UK, and run using PCR [32]. The DNA size was confirmed by gel electrophoresis, extracted from the agarose gel, and sequenced at Macrogen, Korea. The obtained sequence was checked by BLASTn against the NCBI database.

### 2.3. Antibiotic Susceptibility Testing:

The antibiotic susceptible test was done using the disc diffusion method on 10 *E. coli* isolates according to National Committee for Clinical Standards using eight antibiotic agents (Oxoid, England): Penicillin G (P; 10µg), Levofloxacin (LEV; 5µg), Tetracycline (TE; 30µg), Cefazidime (CAZ; 30µg), Chloramphenicol (C; 30µg), Methicillin (ME; 5µg), Norfloxacin (NOR; 10µg) and Ciprofloxacin (CIP; 5µg). In brief, a single bacterial colony from a TSA plate was grown in 1 ml of TSB, to prepare an overnight culture, and then incubated overnight at 37°C [33]. After that, 100 µl of the overnight culture was spread on the surface of the plate and the antibiotic disks were then placed on the surface of the plates. Plates were incubated at 37°C for 24 hrs. After the incubation period, the diameters of inhibition zones were measured [34, 35].

Multiple Antibiotic Resistance Index (MAR) is calculated for a single isolate according to the method described by Osundiya et al. [36] as shown in the following equation: (MAR) = (the number of

antimicrobial drugs that show resistance / the total number of antimicrobial drugs used in the test). Consequently, additional sixteen antibiotic discs were tested on the EC5 isolate, including Amoxicillin (AMX;10µg), Ampicillin (AMP;10µg), Gentamicin (GMN;10µg), Meropenem (MEM;10µg), Erythromycin (ERY;15,30µg), Cefotaxime (CTX;30µg), Cefoxitin(Cephoxitin) (CX;30µg), Cefepime (FEP;30µg), Clindamycin (CMN;2µg), Oxacillin (OXA;5µg), Azithromycin (AZM;15µg), Rifampicin (RAM;30µg), Trimethoprim-sulfamethoxazole (SXT;25µg), Amoxicillin+clavulanic acid (AMC;30µg), Nitrofurantoin (FTN;300µg) and Tobramycin (TOB;10 µg).

### 2.4. Phage Isolation, Selection, Purification, and Amplification

Phages were isolated from environmental dairy samples collected from some markets in Cairo, Egypt (four raw milk and four karish cheese samples) in November (2020). One gram of each sample was mixed with 5 mL of TSB in a Falcon tube containing *E. coli* as a bacterial host strain and then incubated for 4 hr. at 37 °C. After an incubation period, the sample was centrifuged for 20 min at 5000 rpm at 4 °C. Ten percent volume per volume (v/v) chloroform (CHCl<sub>3</sub>) was added for additional lyses of the infected bacterial cells [37]. The double agar overlay plaque technique was used to detect the presence of phage, subsequently, isolate a single plaque by sterile micropipette tip to obtain a single phage after serial dilution using a 96-well plate. To confirm that a single plaque was isolated and this step was repeated 4 times in the SM buffer [38]. In the amplification process, the phage was added to the *E. coli* strain with an MOI of 1 and left for around 4 h. The chloroform was added for 30 min before centrifuging the lysates for another 20 min at 7000 rpm at 4 °C. The phage titer was determined using standard double-agar overlay plaque assays and stored in the fridge at 4 °C for further use [39]. By using a 96-well plate the titer of phage suspension was enumerated, and each lane containing 180 µL of TSB media and 20 µL of phage suspension was serially diluted in 10-fold [40]. Only 5 µL aliquots were spotted in triplicate onto bacterial lawns incubated overnight at 37°C, and then the number of plaques was estimated [41].

### 2.5. Transmission Electron Microscopy Examination of Phage Morphology (TEM)

The morphology of the isolated phage was imaged using TEM as reported by Ackermann, [42] at the National Research Center (Cairo, Egypt). Ten µL of

phage (109 PFU/mL) were placed on a grid fixed with 2.5% glutaraldehyde and stained with 3% phosphotungstic acid. The stained phage was observed using a high-resolution-transmission electron microscope (HR-TEM) (JEM-2100, JEOL, Tokyo, Japan) and images were captured at different magnifications.

### 2.6. Pulsed-Field Gel Electrophoresis (PFGE)

The Bacteriophage genome was determined by Pulsed-Field Gel Electrophoresis (PFGE) as follows: Phage plugs were prepared, by suspending 200  $\mu$ L of phage in 200  $\mu$ L agarose gel prepared from 1 $\times$  TE buffer (Tris-HCl, Loba Chemie, Mumbai, India, EDTA, Fisher chemical, Pittsburgh, PA, USA, pH 8) and 1.4% agarose (Lonza, Basel, Switzerland), and left to dry for around 15 min according to Senczek et al., [43], then 5 mL of lysis buffer (proteinase K, Biologics 2021, 1 172 Fisher Scientific, USA, Tris-HCl, EDTA, pH 8) was added to the plugs and incubated for 18 h at 55 °C for digestion. Following the incubation period, plugs were transferred to 5 mL washing buffer and incubated for 1 h at 37 °C, and another two washing steps were performed for 20 min in 1 mL washing buffer. Then, 1% agarose gel was prepared using 0.5 $\times$  TBE buffer (Tris-HCl, boric acid, Fisher Chemical, USA, EDTA, pH 8), and 2700 mL of 0.5 TBE buffer was prepared for the run. The plugs were then inserted into the gel, and the gel was run for 18 h at 6 V, with a switch time of 5 s initial and 13 s final, and the cooling module was set at 14 °C. The size of the genome was determined by comparison to standard concatenated lambda DNA markers (Sigma Aldrich, Gillingham, UK). After incubation, the gel was visualized using Ethidium bromide.

### 2.7. Temperature, UV, and pH Stability of Phage.

Phage stability by using different pH was studied. To determine the pH effect, 30 $\mu$ L of phage (109 PFU/mL) was added to 2970 mL of sterile water at different pH levels (ranging from 2 to 13) (adjusted by using NaOH or HCl and incubated at 4 °C for 24 h), phage stability and tier was detected by plaque assay technique [39].

To investigate the effect of temperature, 100  $\mu$ L of phage (109 PFU/mL) was added to 900  $\mu$ L of TSB buffer (pH 7) and incubated at different temperatures (−20 °C, 4 °C, 37 °C, 50 °C, 60 °C, 70 °C and 80 °C)

for 1 h. Also, a plaque assay technique was conducted to detect the reduction in phage titer and noticed phage stability [35]. According to Tom et al., [44], phage exposure to UV was studied at 15, 30, 45, and 60 min. 100  $\mu$ L phage added to 900  $\mu$ L tryptone soya broth, then incubated for 1hr. Phage titer was detected by the plaque assay method.

### 2.8. Determination of the Phage Stability and Lytic Activity in Milk:

In raw milk, the activity of isolated phage was investigated after sterilizing the raw milk at a high temperature of 110 °C for 20 min to ensure that the sample is free from any source of contamination. A day culture of *E. coli* (4.3 $\times$ 10<sup>6</sup> CFU/mL) was mixed with 5 mL of milk in sterile falcon tubes. Then, the sample tube was treated with phage at MOI = 2 but the control without phage treatment was incubated at 37 °C for 3 h with a shaking incubator. To calculate the *E. coli* count (CFU), 100  $\mu$ L of aliquots were withdrawn from each tube and serially diluted after 0, 90, and 180 min. In addition, Phage titer (PFU/mL) was enumerated before and after the incubation period [39].

## 3. Results and Discussion

### 3.1. Isolation and genotyping identification of *E. coli*:

A total of 27 isolates were identified as *E. coli* by using selective media and confirmed by different biochemical tests. All 8 samples of milk (100%) and 4 samples of cream (100%) were positive for the presence of *E. coli*, while about 91% of Karish cheese, 33.3% of yoghurt and 66.6% of soft cheese samples were positive for *E. coli* (Table 1).

Table 1 *E. coli* presence in milk and dairy products samples.

Type of sample	Number of samples	Positive samples	Incidence percentage (%)
Karish cheese	11	10	91 %
Raw milk	8	8	100 %
Cream	4	4	100 %
Yogurt	3	1	33.3 %
Soft cheese	6	4	66.6 %
Total	32	27	84%

Out of the 32 different dairy samples, 27 isolates were *E. coli* and all of them were Gram-negative rods, positive catalase, and negative oxidase [12], and confirmed as *E. coli* by different biochemical tests.

The highest incidence of *E. coli* was observed in Raw milk and Cream (100%) while, yogurt was recorded as the lowest contaminated product (33.3%). On the other hand, other products such as Karish cheese and soft cheese were found to be more contaminated with *E. coli* than yogurt since they were positive for the presence of *E. coli* by 91 and 66.6% respectively. The fact that *E. coli* was present in 100% of the raw milk may indicate the high rate of cross-contamination during the milking process. Similar results were observed before when 120 samples of kariesh and Rascheese (Hard cheese) were tested positive for the presence of *E. coli* by 100% and 51.67% respectively [45] (Nosir *et al.*, 2014). In another study, Abd El-Atty and Meshref [46] screened different dairy products for the presence of *E. coli* O157 and found that *E. coli* was detected in 2% of Kareish cheese samples. On the other hand, Daood [47] reported a total of 167 isolates of *E. coli* O157 in raw bovine milk, some dairy products.

### 3.2. Antibiotics susceptibility testing:

Antibiotic susceptibility tests for all antibiotics that are in use to treat human infections with *E. coli* are screened (Table 2). Exactly, eight different antibiotics were used, and the results were interpreted according to the guidelines of CLSI [48]. The results demonstrated that 10 *E. coli* isolates showed different sensitivities to the antibiotics used. The bacteria are classified into 3 different categories; sensitive, intermediate, and resistant, according to their sensitivity to the antibiotic used. According to the data, eight isolates showed resistance to Methicillin, six isolates were resistant to Ciprofloxacin and five isolates were resistant to Penicillin G and Cefazidime. There was only one resistant isolate to Levofloxacin. On the contrary, there were no resistant isolates to Tetracycline, Chloramphenicol, and Norfloxacin. According to the published data, most isolated *E. coli* from different sources are multidrug-resistant bacterial strains (MDR) because of the misuse and abuse of antibiotics in humans or animals, which may cause death [49, 50]. *E. coli* is considered one of the most common bacteria that is associated with foodborne diseases worldwide and milk and dairy products are considered the main sources of human infection with *E. coli* [12, 11, 8]. It was found that *EC5* isolate was the most resistant strain to 15 different antibiotics (93.7%) of all antibiotics used (24) including; Nitrofurantoin, Meropenem, Erythromycin, Cefotaxime, Cefoxitin,

Cefepime, Clindamycin, Oxacillin, Azithromycin, Amoxicillin, Gentamicin, Penicillin G, Cefazidime, Methicillin, and Ciprofloxacin. Ebrahim *et al.*, [51] described that all *E. coli* O157:H7 isolated from different dairy products were sensitive to chloramphenicol, and this agrees with our study. It is known that *E. coli* is one of the most common pathogens that cause Urinary Tract Infections (UTIs) and the antibiotic Nitrofurantoin is commonly used to treat uncomplicated UTIs [52]. However, our results showed that *EC5* was resistant to Nitrofurantoin.

Usually, multiple antibiotic resistance (MAR) in any bacteria is associated with the presence of plasmids in which one or more resistance genes are present, each encoding for a single antibiotic resistance phenotype [36, 53]. The MAR index of the *EC5* isolate was 0.68, and this value indicates a high level of environmental contamination. It was clear that *E. coli* isolates *EC5* is the most resistant bacteria since it resists half of the different antibiotics tested and is used as the primary host. Consequently, additional antibiotics were tested on *EC5* as shown in Table 3. The results showed that the strain is resistant to eleven out of sixteen antibiotics including Nitrofurantoin, Meropenem, Erythromycin, Cefotaxime, Cefoxitin, Cefepime, Clindamycin, Oxacillin, Azithromycin, Amoxicillin and Gentamicin with a 0.68 MAR Index.

In this study, four antibiotic-resistance genes named blaCTX, tetA, blaSHV, and blaTEM and two virulence genes fimH and traT were tested for their presence in *E. coli EC5* isolate using PCR and the results showed that fimH gene was present, while the other genes were absent. FimH is encoded by the fimH gene and it directly affects the management and regulation of fimbriae as indicated in previous studies [54- 56]. Uropathogenic *E. coli* (UPEC) associated with urinary tract infections (UTIs) showed the presence of special virulence factors, including type 1 fimbriae that can result in the worsening of UTIs. The pathogenicity of *E. coli* is increased when it binds to host cells by the fim H gene [57, 58] and causes the severity of that bacterium because this gene helps bacteria to stick to the walls of the inner bowel cells and facilitates their colonization to cause urinary tract infections.



Table 2  
Antibiotic sensitivity profile of *E. coli* isolates and MAR index.

Antibiotic	Symbol	EC 10	EC 15	EC 3	EC 5	EC 8	EC 1	EC 4	EC 7	EC 20	EC 6	Total R (%)
Penicillin G Class: Natural Penicillins	<b>P 10</b>	20	27	11	R	27	R	R	16	20	7	<b>50%</b>
	S	S	R	R	S	R	R	I	S	R		
Tetracycline Class: Tetracycline	<b>TE 30</b>	23	29	23	17.5	23	19	17	22	30	16	<b>0%</b>
	S	S	S	S	S	S	S	S	S	S	S	
Levofloxacin Class: Fluoroquinolones	<b>LEV 5</b>	20	23	25	20	16	22	20	17	20	20	<b>10%</b>
	I	S	S	I	R	S	I	I	I	I		
Cefazidime Class: Third generation cephalosporins	<b>CAZ 30</b>	R	27	25	13.5	R	20	22	R	R	21	<b>50%</b>
	R	S	S	R	R	I	S	R	R	S		
Methicillin Class: $\beta$ -lactam antibiotic of the penicillins	<b>ME 5</b>	R	11	R	R	-	R	16	R	R	8	<b>88.8%</b>
	R	R	R	R	-	R	I	R	R	R		
Chloramphenicol Class: Phenicol	<b>C 30</b>	23	27	28	20	23	22	20	23	23	29	<b>0%</b>
	S	S	S	S	S	S	S	S	S	S	S	
Norfloxacin Class: Fluoroquinolone	<b>NOR 10</b>	19	31	20	16	16	18	18	15	20	17	<b>0%</b>
	S	S	S	I	I	S	S	I	S	I		
Ciprofloxacin Class:quinolone	<b>CIP 5</b>	23	24	24	15.5	20	18	17	23	20	18	<b>60%</b>
	I	I	I	R	R	R	R	I	R	R		
MAR Index		<b>0.2</b>	<b>0.1</b>	<b>0.2</b>	<b>0.5</b>	<b>0.4</b>	<b>0.3</b>	<b>0.2</b>	<b>0.2</b>	<b>0.3</b>	<b>0.3</b>	

[R=Resistance, I=Intermediate, S=Susceptible, (-) = Not tested, MAR Index = Multiple Antibiotic Resistance]





Table 3  
Antibiotic sensitivity profile of ZCEC5 isolate and MAR index.

Antibiotic	Symbol	EC 5
Amoxicillin Class: Beta-lactams, penicillins	AMX 10	8 R
Ampicillin Class: aminopenicillins	AMP 10	17 S
Tobramycin Class: Aminoglycosides	TOB 10	14 I
Gentamicin Class: Aminoglycosides	GMN 10	9 R
Nitrofurantoin Class: Antibacterials (antibiotics)	FTN 300	16 R
Meropenem Class: Carbapenem class	MEM 10	15.5 R
Erythromycin Class: Macrolides	ERY 15	R R
Cefotaxime Class: Third-generation cephalosporin	CTX 30 µg	16 R
Cefoxitin(Cephoxitin) Class: Second-generation cephalosporins	CX 30	11.5 R
Cefepime Class: Fourth-generation cephalosporins	FEP 30	18 R
Clindamycin Class: Lincosamide	CMN 2	R R
Oxacillin Class: Penicillins	OXA 5	R R
Azithromycin Class: Macrolides	AZM 15	R R
Rifampicin Class: Rifamycins	RAM 30	14.5 I
Trimethoprim-sulfamethoxazole Class: Sulfonamide	SXT 25	17 S
Amoxicillin+clavulanic acid Class: Amoxycillin and Clavulanic acid	AMC 30	15.5 I
<b>MAR Index</b>		<b>0.68</b>

[R=Resistance, I=Intermediate, S=Susceptible, (-)=Not tested, MAR Index= Multiple Antibiotic Resistance]

### 3.3. Identification of *E. coli* by partial sequencing of 16S rRNA:

The selected EC5 isolate was further confirmed by PCR using a specific primer (766 bp) and sequencing of the 16S–23S rRNA region. The PCR product size showed that the bacterial strain was *E. coli*, with a 130 bp band corresponding to the conserved region in the 16S-23S internal transcribed spacer region of *E. coli*. The partial-genome sequencing of an *E. coli* isolate was performed afterward (GenBank: Acc. No. OQ941593 from the NCBI database) and the BLASTN of the 16S rRNA sequence showed a 99.57% sequence identity to *Escherichia coli* strain ECIUP\_23 16S ribosomal RNA gene, partial sequence (GenBank Acc. No. MN094132). The surrounding environment and the milking process are important sources of contamination of milk by *E. coli* [59]. Several strategies have been used and developed to protect food products from contamination with multi-drug-resistant, as well as humans from infection with those pathogens.

Therefore, the need to discover new alternatives such as bacteriophages to deal with this emergency has increased [60, 27, 61, 50].

### 3.4. Isolation, purification, and amplification of bacteriophage:

Bacteriophages were isolated from kraish cheese against the multi-drug resistant strain as reported before [62]. Plaques of phage isolated were small in size and gave complete lysis. The purification of the phage was repeated five times to confirm the isolation of a single phage [63]. Then, the bacteriophage was amplified to reach a titer of 1010 PFU/ml.

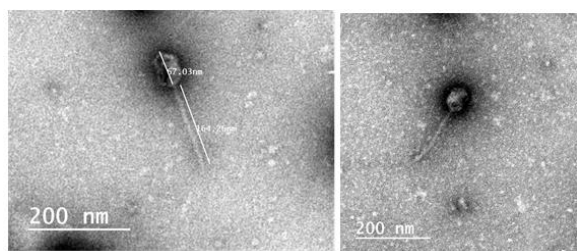
In the dairy industry, phages were added as a bio-control agent to combat many pathogens in different dairy products [64, 65] including raw bovine and ultrahigh-temperature (UHT) milk to control *E. coli* [59]. Different types of milk were treated with a cocktail of two phages ( $\Phi 5$  and  $\Phi 72$ ) to control *Staphylococcus aureus* contamination [66] in soft and curdle types of cheese and also to

control the growth of *Listeria monocytogenes* using the P100 phage [67]. Other studies showed that commercial phage (ShigaShield™) can be used to control *Shigella sonnei* in fermented products such as yogurt [68].

### 3.5. Phage genome determination using PFGE and Morphology investigation by Transmission Electron Microscopy (TEM):

Pulsed-Field Gel Electrophoresis (PFGE) results showed that the genome size of the isolated phage is approximately < 97 kbp using the standard Lambda PFG ladder (ladder size range: 48.5 - 1,018 kb).

Transmission Electron Microscopy showed that the phage belongs to the Siphoviridae family with an icosahedral capsid containing a double strand of DNA and a nonenveloped phage with a long, flexible, and non-contractile tail like Phage  $\lambda$  [69]. The head size is 67.03nm while the length of the tail is 164.26 nm as shown in Figure 1.

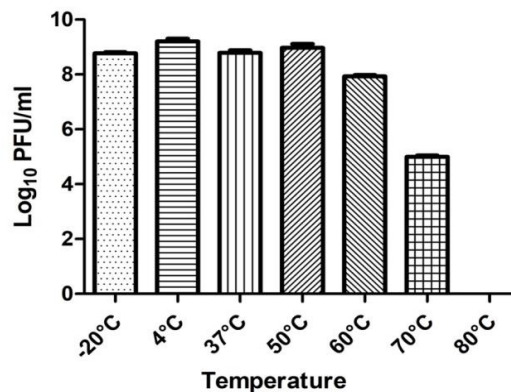


**Figure 1:** Transmission electron microscope (TEM) image of phage showing its morphology. Scale bar 200 nm

### 3.6. Phage stability

To use phages as a biocontrol agent, they should have the ability to withstand a wide range of temperatures and pH. The stability of the phage was investigated at different temperatures and pH values by enumerating the phage titer. The results indicated that the phage was stable at approximately  $10^8$  plaque-forming units (PFU)/mL after 1 h of incubation at 50 °C, while the titer was decreased at 60 °C and continued to decrease gradually at 70 °C and above to below  $10^8$  PFU /mL after 1 h of incubation, and the best stability was recorded at 4 °C ( $10^9$  PFU)/mL as indicated in Figure 2.

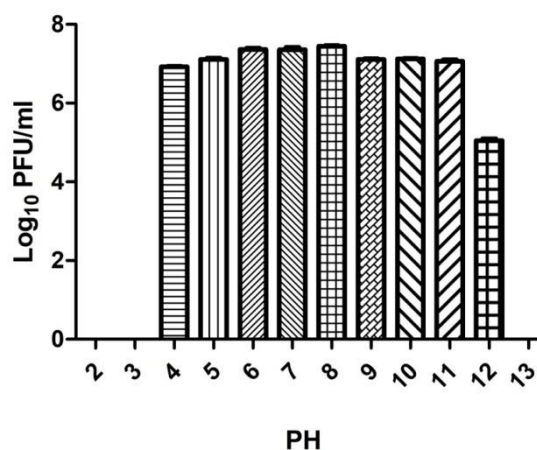
This good stability at a wide range of temperatures, from -20°C to 60°C with a slight decrease at 70°C, was reported before [70, 71, 72, 73, and 50]. However, several phages showed high stability to tolerate high temperatures [74, 50].



**Figure 2:** The stability of phage ZCEC5 at various temperature values, PFU stands for a plaque-forming unit.

The isolated phage was stable at a wide range of pH values, from 4 - 12 pH, but the optimum stability was observed at pH 7. A slight decrease in stability was observed at pH 12 with no ability to detect phages at pH 2, 3, and 13 (Figure 3).

The stability of our isolated phage at different values of pH indicated that the phage can withstand harsh conditions as indicated before [50, 75, 72, 76,77, 78] and this is considered an added value since the optimal minimum pH for most cheese manufacturing is in the range of (5.0 – 5.3) [79]. Such characteristics enable the use of phage in industrial applications since the stretching properties of cheese, melting, and texture can be influenced by many factors, including cheese composition, Ca content, salt content, protocols of manufacturing, and pH [80].



**Figure 3:** The stability of phage ZCEC5 at various pH values

The isolated phage showed excellent stability ( $10^8$  PFU/mL) when exposed to UV light for 20 min (Figure 4). The phage was still active ( $10^7$  PFU/mL) after 60 min of exposure and a similar stability was

observed with phage swi2 [81].

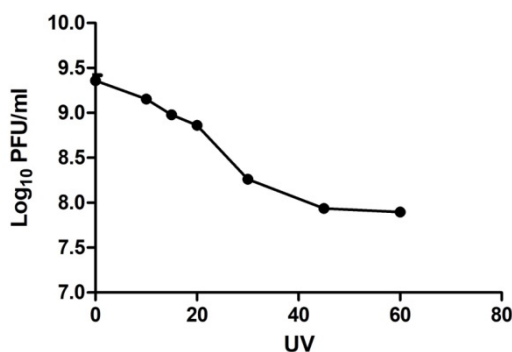


Figure 4: The stability of phage ZCEC5 under UV radiation ( $\lambda=253$  nm). PFU stands for a plaque-forming unit.

### 3.7. Control of *E. coli* growth in milk by using phage:

The isolated phage was examined for its ability to control the *E. coli* growth in milk for 3h at 37 °C at an MOI of about 2. After infection with phage, the viable bacterial count in milk showed that the phage reduced the numbers of *E. coli* to  $8.66 \times 10^3$  CFU/mL in comparison with the control which reached  $8.2 \times 10^9$  CFU/mL after 3 h of incubation. On the other hand, the titer of phage was increased significantly from  $2.4 \times 10^7$  PFU/mL to  $2.6 \times 10^9$  PFU/mL at 37 °C.

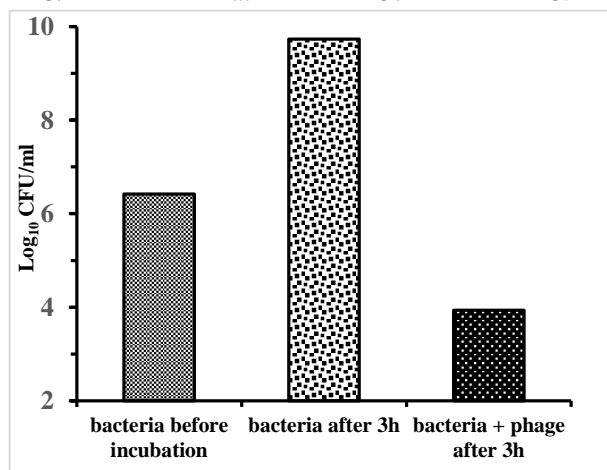


Figure 5: Reduction of *E. coli* growth in milk after 3 h of phage treatment at 37 °C.

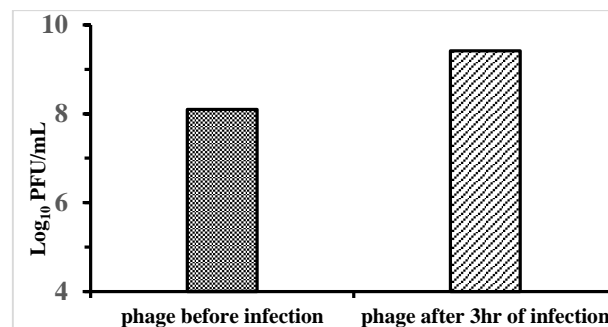


Figure 6: Phage enumeration in milk after 3 h of phage treatment at 37 °C.

In this study, the titer of *EC5* increased gradually after 3 h of incubation without phage but in the presence of phage, the bacterial count decreased gradually. Consequently, the titer of the phage was increased after incubation with bacteria in milk, showing the ability of the isolated phage to control the *E. coli* growth in a thick medium rich with minerals, proteins, and other macromolecules of milk as a previous study showed that phage ZCSE6 control the growth of *Salmonella* spp. in milk [39].

The results of this study showed that the isolated phage had a bactericidal effect against *EC5* isolate strain with a vital growth compared to the control. Other studies reported that phages can be used as a biocontrol agent against the bacterial hosts in milk including the eradication of *P. lactis* [82], *S. Typhimurium* [83], and *E. coli* O157: H7 [84]. Accordingly, bacteriophage can be used as a biocontrol agent as well as an alternative to antibiotics to control bacterial contamination in milk.

## 4. Conclusion

Milk and its products must be free from any pathogenic microorganisms that cause diseases in humans. Due to the increasing rate of antibiotic resistance, alternatives, such as bacteriophages, are needed to control these pathogenic microorganisms. In this study, a specific phage for antibiotic resistance isolated from *E. coli* was isolated and characterized. The isolated phage showed the ability to withstand different ranges of temperatures, pH values, and UV exposure, making it a good biocontrol agent to be used in food. In addition, it showed a promising biocontrol effect on *E. coli* in milk. More studies are required to investigate the ability of this phage to control bacterial growth in different dairy products.

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