Research Article

Cocktail Phage Therapy for Bacteria Contaminating Meat in Egypt

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Abstract | Bacteriophages have crucial advantages over conventional methods of controlling pathogenic bacteria in which they have a high ability to self-reproduce, host specificity, and develop with their bacterial hosts. The most common pathogenic bacteria were isolated from fresh meat and identified using the VITEK II automated system. Phages specific to *Escherichia coli, Staphylococcus aureus,* and *Klebsiella pneumoniae* were isolated and mixed in a cocktail, then applied for the treatment of the collected meat samples. At room temperature (24°C), the number falls over time until reaching 72 hours for all bacterial species. The results obtained showed a significant reduction in the bacterial count of *E. coli*, *Staph. aureus*, *and K. pneumoniae,* indicating that the cocktail phage therapy has a potential application for replacing antibiotics and other means used for meat sterilization.

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Introduction

Meat is a superior source of protein for humans, but it is also very susceptible to microbial contamination, which results in human spoilage and foodborne illnesses and causes monetary and health costs (Komba *et al*., 2012). Foodborne diseases caused by bacterial pathogens such as *Staphylococcus aereus*, *Escherichia coli* and *Listeria* have continuously increased. Food is exposed to these pathogens during production, processing, storage, and packaging. Physical treatments including UV radiation, high pressure, dry heat, and steam are workable methods to reduce pathogenic germs in raw materials. These strategies have been taken into consideration because the use of antibiotics has been reduced over time. This is because it is possible for antibiotic-resistant bacteria to penetrate the human food chain and negatively impact human antimicrobial care. However, it is well recognized that physical methods of microbial load reduction in raw foods have a negative impact on the goods organoleptic qualities, which lowers their acceptability (García *et al*., 2008).

Bacteriophages are extremely effective and precise against their hosts, and as a result, there is a growing need for novel methods to eliminate bacterial infections in foods while still meeting consumer demand for minimally processed foods with low quantities of chemical preservatives. As bacterial infection rises, a low concentration of bacteriophages will reduce the essential pathogen because phages

self-replicate. Bacteriophage realization is relatively easy and quite resistant to preservation under various external factors (Hagens and Loessner, 2010). The clinical application of phages for the avoidance of pathogenic bacterial infections is known as phage treatment (Sun *et al*., 2019). Until the first significant discovery of the antibiotic penicillin in 1944, it was developed and widely used throughout the 1920s and 1940s (Ofir and Sorek, 2018). After that, although some regions of the world still employ phage therapy, most phage therapy research ceased until a significant rise in the prevalence of antibiotic-resistant bacteria (ARBs) in the environment (Chen *et al*., 2018; Qiao *et al*., 2017). In our study, the predominant pathogenic bacterial contaminants in meat were detected and their sensitivity to antibiotics was studied. Specific bacteriophages were used in a cocktail to control the bacteria in the contaminated fresh meat.

Materials and Methods

Meat samples collection and bacterial isolation

A total of 20 fresh meat samples were collected from January to June (2018) from different butcher's shops in Fayoum Governorate, Egypt. The collected samples were transported to the laboratory and analyzed immediately. Ten grams of each sample in 90 mL of buffered peptone water were mixed and then filtered. One mL of this filtrate was subjected to a ten-fold dilution series of homogenate samples inoculated onto mannitol salt agar (MSA) for *Staphylococcus aureus* isolation, eosin methylene blue (EMB) agar for *E. coli* isolation, and xylose lysine desoxycholate (XLD) agar medium was used in the isolation of *Klebsiella* sp. These pathogenic bacteria were isolated according to Cheesbrough (1985) on different selective media that were mentioned above to observe the colony (morphology, shape, size, surface texture, edge, elevation, color, and opacity).

Identification the selected bacterial isolates

It was done using VITEK II automated system (Funke *et al*., 1998).

Antibiotic sensitivity test

The single disc diffusion method (Bauer *et al*., 1966) was used. Twelve antibiotics were used to examine three bacterial isolates' susceptibility to antibiotics. Antibiotic impregnated discs were applied to the surface of the inoculated plates with sterile forceps. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. The plates were inverted and incubated at 37°C. After 16 to 18 hours of incubation, the plates were examined, and the diameters of the inhibition zones were measured in mm.

Isolation and propagation of bacteriophages

According to Adam's (1959) technique, biologically stock phage lysates were prepared utilizing the single plaque separation method based on morphological characteristics such as diameter, halo, turbidity, and shape. By using the liquid culture propagative approach, a significant volume of high titer phage stock was produced (Adam's, 1959). With the use of the alternative differential centrifugation technique, the propagated phages were purified and concentrated (Figrski and Christensen, 1974).

Microscopic examination of S. aureus, E. coli, and klebsiella phages

The morphology of phage particles of *S. aureus, E. coli* and *klebsiella* was examined by TEM microscopy (Othman, 1997). On a 200-mesh carbon-coated copper grid, a drop of concentrated phage sample was applied, and it was left to adsorb for roughly 20 minutes. Filter paper was used to remove the extra liquid. The grids were stained negatively for 90 seconds with 2% Uranyl acetate (pH 4.5) before being allowed to dry. The preparations were examined using a JOEL-JEM 1010 electron microscope (Electron Microscope Unit, Regional Center for Mycology and Biotechnology (Al-Azhar Univ., Cairo).

Longevity in vitro

The infectivity of *S. aureus, E. coli*, and *klebsiella* phages was examined according to Yoshida *et al*. (2006) by employing the spot test approach, incubating phage stocks at temperatures of 4 °C, -20 °C, and room temperature for 63 days spotting phage lysates every week.

Phage cocktail application at room temperature

Grinded fresh meat was taken from the butchers' shops and transported directly to the laboratory. Grinded fresh meat was left without any treatment after enumeration of the *S. aureus, E. coli, and K. pneumoniae* bacteria on it. Grinded fresh meat was treated with a phage cocktail. Three replicates were used for each treatment. Phages were mixed with an equal 10^{10} PFU/mL for each phage to make a phage cocktail which was applied to non-sterilized meat in the presence of their respective hosts at room temperature.

Statistical analysis

Duncan multiple range tests were used to compare between means (Duncan, 1953). Level of the significance in all tests was P ≤ 0.05. The results were introduced as means of three replicates ± standard error (SE).

Results and Discussion

Bacterial characterization and identification

The most common bacterial isolates were characterized morphologically by their growth on solid selective media and investigated microscopically using gram staining. *S. aureus* isolate was gram positive cocci and gave yellow colonies on MSA medium, *K. pneumoniae* isolate was gram negative rods and gave yellow colonies on XLD agar, and *E. coli* isolate was gram negative rods and gave metallic green colonies on EMB agar. VITEK II automated system confirmed with probability 99% that they were *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Escherichia coli* (Tables 1, 2 and 3).

Table 1: *Biochemical details for Staphylococcus aureus.*

Bio number								**42004601673231**							
Organism								*Staphylococcus aureus*							
Probability								99%							
Confidence								Excellent identification							
2	AMY	-	4	PIPLC	-	5	dXYL	+	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	-
20	LeuA	-	23	ProA	-	24	BGURr	+	25	AGAL	-	26	PyrA	+	27	BGUR	+
28	AIeA	-	29	TyrA	-	30	dSOR	-	31	URE	+	32	POLYB	-	37	dGAL	-
36	dRIB	-	39	ILATK	+	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Table 2: *Biochemical details for Klebsiella pneumoniae pneumoniae.*

Bio number								**6605734373564010**							
Organism								*Klebsiella pneumoniae*							
Probability								99%							
Confidence								Excellent identification							
2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GCT	-	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAIap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	-
33	SAC	+	34	dTAG	+	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATK	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GIyA	-	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GCAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Table 3: *Biochemical details for Escherichia coli.*

Bio number								**0405610550466610**							
Organism								*Escherichia coli*							
Probability								99%							
Confidence								Excellent identification							
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GCT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAIap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATK	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GIyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GCAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Antibiotic sensitivity test

The inhibition zone diameters of the used thirteen antibiotics showed that *S. aureus* was a sensitive isolate that showed sensitivity to all antibiotics except ampicillin and penicillin. *E. coli* was moderately resistant isolate that showed sensitivity to ceftriaxone, tigecycline, gentamicin, rifampicin, cefoxitin, and ciprofloxacin, but was resistant to ampicillin, penicillin, erythromycin, fusidic acid, vancomycin, tetracycline, and clindamycin. *K. pneumoniae* was a resistant isolate that showed sensitivity to gentamicin, tetracycline, and cefoxitin but was resistant to ampicillin, tigecycline, penicillin, erythromycin, fusidic acid, vancomycin, rifampicin, ceftriaxone, ciprofloxacin, and clindamycin (Table 4).

Table 4: *Antibiotic susceptibility test for the three bacterial isolates by measuring the inhibition zone (mm).*

Inhibition zone (mm) using the disc diffusion method			
Type of the antibiotic	***S. aureus***	***E. coli***	***K. pneumoniae***
Ampicillin (10µg)	0	0	0
Penicillin (10µg)	0	0	0
Ceftriaxone (30µg)	11	18	0
Erythromycin (15µg)	12	0	0
Fusidic acid (10 µg)	16	0	0
Tigecycline (15 µg)	22	14	0
Gentamicin (10 µg)	25	10	15
Vancomycin (30 µg)	24	0	0
Tetracycline (30 µg)	21	0	15
Rifampicin (5 µg)	20	8	0
Clindamycin (2 µg)	15	0	0
Cefoxitin (30 µg)	20	10	16
Ciprofloxacin (5µg)	27	15	0

Detection the S. aureus, E. coli, and K. pneumoniae phages

Two *E. coli* phages and only one phage for *K. pneumoniae* and *S. aureus* were isolated from different collected sewage water samples. Single plaque isolation was repeated three times to obtain biologically purified phages. *S. aureus, E. coli* and *K. pneumoniae* phages were isolated depending upon the plaque morphology (Figure 1).

Phage titer of lysates resulting after single plaque isolation

Concentration of *S. aureus* phage lysate was 2.1×10^{6} PFU/mL, while *E. coli* phage 1 was 6×10^{7}PFU/mL, *E. coli* phage 2 was 1.5×10^{7} PFU/mL, and *K. pneumoniae* phage was 1.5×10^{6} PFU/mL.

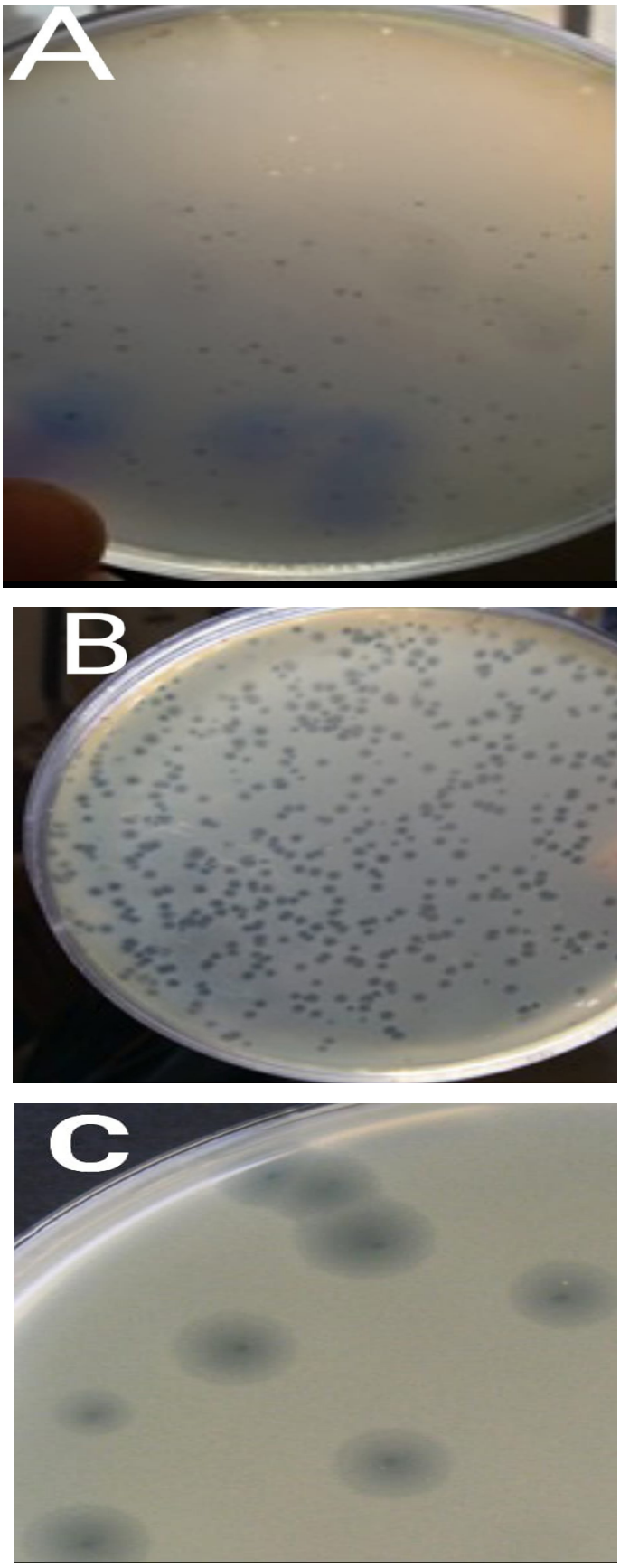

Figure 1: *Plate photos showing A: S. aureus phage plaques, B: E. coli phages plaques and C: K. pneumoniae phage plaques. (A) The plaque is circular, clear, without center, without halo, and its size is 1.5mm. (B) E. coli phage 1: plaque zone Circular, clear, without center, without halo, and its diameter is 1mm. E. coli phage 2: Circular, turbid, without center, with Halo, and its diameter is 1.5 mm. (C) Circular, clear with center, with large halo, and its diameter is 6 mm.*

Propagation, purification and concentration of phages

The biologically purified *S. aureus, E. coli, and K. pneumoniae* phage lysates were propagated by the liquid culture method to obtain a high titer phage suspension before ultracentrifugation. The precipitates obtained after ultracentrifugation were resuspended in a small amount of the buffer to concentrate the bacteriophages. The concentrated phages were assayed quantitatively and it was found that *S. aureus* phage titer became 2.6×10^9 PFU/mL, *E. coli* phage 1 became 3×10^{10} PFU/mL, *E. coli* phage 2 became 1.4×10^{10} PFU/mL, and *K. pneumoniae* phage became 2×10^9 PFU/mL.

Microscopic examination of isolated phages

Pure concentrated phages were negatively stained and investigated using transmission electron microscopy (TEM). Electron micrographs (Figure 2) showed four phages belong to three families. *S. aureus* phage belongs to the *Myoviridae* family, *E. coli* phages 1and2 belong to *Siphoviridae*, and *K. pneumoniae* phage belongs to *Podoviridae* (Table 5).

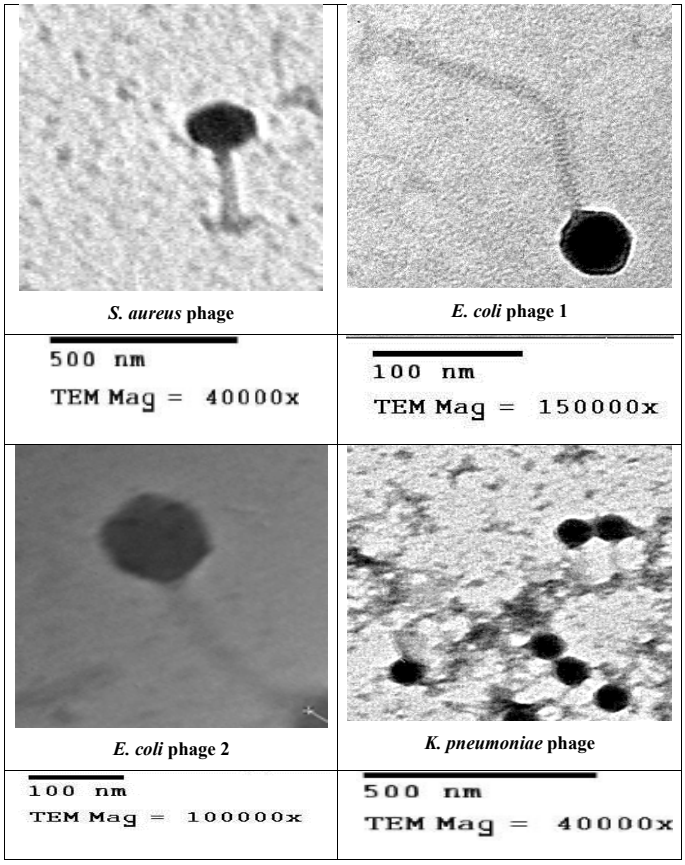

Figure 2: *Electron micrographs of the four isolated phages. The magnification bar corresponds to 100 and 500 nm with high voltage equal to 80 KV.*

Longevity in vitro

Phages were preserved at three different temperatures and tested weekly for their infectivity using the plaque assay test. Results indicated that phages could maintain their infectivity for more than two months. The titer of phages was reduced gradually by time but with very low values. The results showed that - 20 °C was the best degree for long preservation for phages. 4°C was also a good temperature for preservation, and the sample that was preserved at room temperature showed the least phage concentration after nine weeks of preservation (Figure 3).

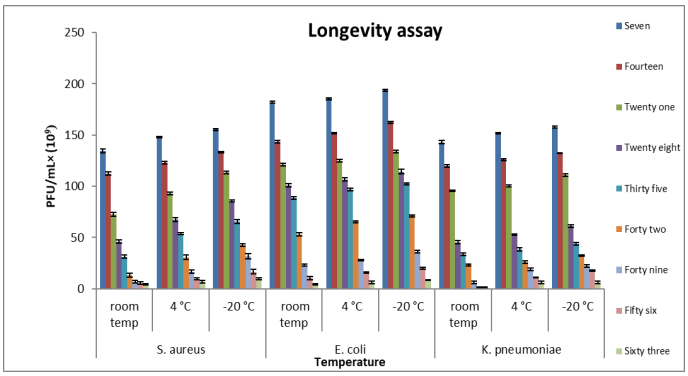

Figure 3: *Histogram showing longevity of S. aureus, E. coli, and K. pneumoniae phages at different temperatures. Data are the means of the three replicates and error bars represent the standard errors of the means.*

Phage cocktail application at room temperature

S. aureus, *E. coli*, and *K. pneumoniae* from non-sterilized grinded fresh meat were controlled with phage cocktail as follow:

Bacterial numbers in non-phage treated fresh meat were increased by long preservation time. It was found that *S. aureus* bacterial number at zero time was 4.67 log CFU/g, after 12 h, it was 5.21 log CFU/g, after 24h, it was 7.37 log CFU/g, after 48h, it was 9.45 log CFU/g, and 10.90 log CFU/g after 72 h as well as *E. coli* bacterial number at zero time was 4.365 log CFU/g, after 12 h, it was 5.09 log CFU/g, after 24 h, it was 6.31 log CFU/g, after 48 h, it was 7.90 log CFU/g, and 9.61 log CFU/g after 72 h, and *K. pneumoniae* bacterial number at zero time was 4.13 log CFU/g, after 12 h, it was 5.92 log CFU/g, after 24 h, it was 6.17 log CFU/g, after 48 h, it was 7.35 log CFU/g, and 8.90 log CFU/g after 72 h. As showed in Table 6, after adding phage cocktail to minimize bacterial contamination, it was observed that the count of *S. aureus* bacteria was reduced after 12 h from the addition by 0.44 log CFU/g, by 1.4 log CFU/g after 24 h, and by 2.53 log after 48 h CFU/g, and after 72 h, the reduction was 3.19 log CFU/g. *E. coli* bacteria were reduced after 12 h from the addition by 0.62 log CFU/g, after 24 h by 2.14 log CFU/g, after 48 h by

Table 5: *Morphological properties and prospected family of phage isolates by TEM.*

Phages	Head (nm)		Tail (nm)		Prospected family
	Size	Shape	Size	Shape	
S. aureus	38	Isometric	153	contractile	Myoviridae
E. coli 1	47	Oval	184	Non contractile	Siphoviridae
E. coli 2	83	Hexagonal	183.5	Non contractile	Siphoviridae
K. pneumoniae	69.2	Icosahedral	---	----	Podoviridae

Table 6:.*Increasing and reduction in Log numbers of S. aureus, E. coli, and K. pneumoniae after and before application of phage cocktail at room temperature.*

Bacterial isolate	*S. aureus*					*E. coli*					*K. pneumoniae*				
Preserved time/ Treatment	Zero	12 h	24 h	48 h	72 h	Zero	12 h	24 h	48 h	72 h	Zero	12 h	24 h	48 h	72 h
T_1	4.67± 0.007^{e}	5.21± 0.38^{d}	7.37± 0.13^{c}	9.45± 0.12^{b}	10.9± 0.3^{a}	4.3± 0.007^{e}	5.0± 0.23^{d}	6.31± 0.23^{c}	7.90± 0.12^{b}	9.61± 0.31^{a}	4.13± 0.029^{e}	5.92± 0.06^{d}	6.1± 0.68^{c}	7.3± 0.007^{b}	8.90± 0.17^{a}
T_2	4.67± 0.007^{e}	4.77± 0.06^{d}	5.97± 0.07^{c}	6.92± 0.41^{b}	7.71± 0.7^{a}	4.35± 0.007^{d}	4.47± 0.17^{c}	4.17± 0.03^{e}	4.61± 0.62^{b}	5.51± 0.09^{a}	4.13± 0.029^{e}	4.45± 0.29^{c}	4.37± 0.013^{d}	5.85± 0.3^{a}	5.62± 0.24^{b}

- (a, b, c) Average in the same row having different superscripts are differ significantly. T_1=untreated fresh meat; T_2=treated meat with phage cocktail.

3.29 log CFU/g, and by 4.1 log CFU/g after 72 h. The count of *K. pneumoniae* bacteria was reduced after 12 h from the addition by 1.47 log CFU/g, after 24 h by 1.8 log CFU/g, by 1.5 log CFU/g after 48 h, and after 72 h by 3.28 log CFU/g. In related to the phage cocktail, number of started phage was 3×10^{10} PFU/ml. After 24 h, it was 3.8×10^{10} PFU/ml. So, it slightly increasing suggesting that the phages reproduction, but after 72 h it was 1.7×10^{9} PFU/ml. So, it decreases with a small value.

Our results demonstrated the predominance of *S. aureus*, *E. coli*, and *Klebsiella pneumoniae* in fresh meat samples and that is in harmony with results of Olise *et al.* (2020) who reported that the microbiological quality of the most raw meat and meat products was significantly poor and their finding showed that raw meat and meat products which were collected from different market sources were contaminated with *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and other bacterial species and also indicated that the predominance of *Escherichia coli* (21.57%) in meat that sold on abattoir and *Staphylococcus aureus* (18.07%) in meat that sold on the open market but in our study the predominance of *Staphylococcus aureus* (39.88%), *Escherichia coli* (19.50%), and *Klebsiella pneumoniae* (29.98%) in all samples The susceptibility of *S. aureus*, *E. coli*, and *K. pneumoniae* against 13 different antibiotics (Ceftriaxone, Tigecycline, Gentamicin, Rifampicin, Cefoxitin, Ciprofloxacin Ampicillin, Penicillin, Erythromycin, Fusidic Acid, Vancomycin, Tetracycline and Clindamycin). *S. aureus* was sensitive for all antibiotics except for ampicillin and penicillin, *E. coli* and *K. pneumoniae* were resistant isolates. Resistance or sensitivity prevalence of the bacterial isolates varied by antimicrobial agent and by isolate source (Davis *et al.*, 2015). Data were supported by Olise *et al.* (2020) who reported that *S. aureus* isolates from meat and meat samples from abattoir and open market showed sensitivity to Gentamycin, Ciprofloxacin and Erythromycin. Results presented by Abdalrahman *et al.* (2015) and Pekana and Green (2018) showed that most *S. aureus* bacterium which isolated from beef samples resisted variety of antibiotics but most of the isolates in this study showed high sensitivity to several antibiotics. Our results in agreement with Apun *et al.* (2008) and Nthenge *et al.* (2008) who found that *Escherichia coli* was resistant to ampicillin, Kanamycin and nalidixic acid which was almost similar to our results. Davis *et al.* (2015) detected the resistance prevalence of *K. pneumoniae* that was greater among meat-source isolates than clinical isolates. Meat-source isolates showed resistance to ampicillin, ampicillin-sulbactam, cefazolin, cefoxitin, ceftriaxone, ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole. These results agree with our results in the cases of ampicillin, ceftriaxone, and ciprofloxacin. In our study, *S. aureus*, *E. coli and K. Pneumoniae* phages were isolated from different sources of the sewage influent (different healthy sewage stations at Fayoum Governate). Sewage

influent was selected as the phage source because it has yielded a large variety of phages. Data agreed with Yoon *et al.* (2011), Karumidze *et al.* (2013), Kęsik-Szeloch *et al.* (2013), and Li *et al.* (2020) who isolated novel bacteriophages specific to *S. aureus*, *E. coli*, and *K. pneumoniae* from sewage samples. These phages exhibited potent lytic activity with clear plaques 2–8 mm in diameter and surrounded by a halo, indicating the production of large amounts of depolymerase enzymes. The preservation temperature of bacteriophage is a very critical factor which determines phage infectivity. *E. coli*, *S. aureus*, and *K. pneumoniae* phages were stored at three different temperatures 4 °C, room temperature, and - 20°C. One sample from each temperature was taken and tested for the presence of phages. The drop in the number of plaques of the first samples (stored at room temperature) was faster than the other two samples. At the end of the experiment, the number of plaques of the sample preserved at -20°C was higher than the sample preserved at 4°C for all phages, so -20°C was the best degree for long preservation. This result agrees with Ackermann *et al.* (2004). Drulis-Kawa *et al.* (2011) who studied the longevity of KP34 phage lysates which were stored at various temperatures and noted that there was no significant loss in plaque forming units of phage after storage for 3 months at all of the tested temperatures. Those findings agreed with our results. This stability leads us to use these bacteriophages as a safe sterilization technique. It was found that Phage cocktail was effective because it has a wide host range. After 72 h from the application of phage cocktail at room temperature. The numbers of *S. aureus*, *E. coli*, and *K. pneumoniae* on non-sterilized meat decreased by 3.19 log CFU/g, 4.1 log CFU/g and 3.28 log CFU/g, respectively. Data agreed with Bigwood *et al.* (2008) when the host density was high, the reductions in bacterial cell numbers were greater. The reduction percentage was high as using high MOI. The data ensure that high MOI (100) showed maximum control of bacterial pathogens. Leverentz *et al.* (2001) demonstrated that a mixture of four phages reduced the number of *S. enteritidis* on honeydew melon slices up to 3.5 log10 units at 5°C and 10°C and up to 2.5 log10 units at 20°C through seven days of incubation. The results of this trial agreed with us and with Leverentz *et al.* (2004) who mentioned that the phage application was most effective on honeydew pieces when applied between 0 to 1 h before contamination with *L. monocytogenes*, which suggests that it would have to be applied at the time of cutting or as soon as possible after cutting to be effective against potential contaminations occurring between the time of cutting and packaging. The correct timing of the phage application increases its effectiveness at the longer storage times as well. A low concentration of bacteriophages will reduce the essential pathogen because phages self-replicate. Bacteriophage realization is relatively easy and quite resistant to preservation under various external factors (Hagens and Loessner, 2010).

Conclusions and Recommendations

In a conclusion, the most common pathogenic bacterial contaminants in meat were identified in our study, and their susceptibility to antibiotics was investigated. To eradicate the bacteria in the contaminated fresh meat, a cocktail of specific bacteriophages is highly recommended.

Acknowledgement

Not applicable.

Novelty Statement

The potential application of cocktail phage therapy against pathogenic bacteria replacing antibiotics and other means used for meat sterilization.

Author's Contribution

Khalid El-Dougdoug, Reda Mohamed Taha, and Amany M. Reyad conceived, designed, and coordinated the study. Aya Maher Rabie carried out the experimental studies with the help of Amany M. Reyad. Amany M. Reyad wrote and organized the manuscript. Khalid El-Dougdoug and Reda Mohamed Taha revised and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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