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CONTROLLING *PSEUDOMONAS AERUGINOSA* IN CONTAMINATED WATER AND MILK VIA SPECIFIC BACTERIOPHAGES

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ABSTRACT

In this study *Pseudomonas aeruginosa* was isolated from a sewage water sample collected from treatment plant of El-kawther City, Sohag, Egypt. Sensitivity test of *Pseudomonas aeruginosa* to 19 antibiotics indicated that two (ciprofloxacin and meropenem**)** out of 19 antibiotics tested exhibited inhibitory effect against *P. aeruginosa*. Ciprofloxacin was the most efficient antibiotic followed by meropenem, they exhibited inebriation zones of 30 and 25 mm. in diameters, respectively. *P. aeruginosa* was found to be tolerant to salinity stress. It has the ability to grow in 9% NaCl. In addition, *P. aeruginosa* was tolerant to pH levels ranging from 4 -10.

Bacteriophages specific to *P. aeruginosa* were successfully isolated from the same sewage water sample. Bacteriophages were applied to water and milk contaminated with *P. aeruginosa* at 4 and 37ºC. Due to application of phages number of bacteria was gradually decreased in both water and milk and no bacterial cells were detected after 24 hr. at 4 and 37ºC. Whereas, number of phage particles increased and the highest number was recorded after 24hr.

Therefore**,** application of specific phages to foodstuffs is highly recommended to avoid contamination or to get rid of this pathogenic bacterium.

Key words: Bacteriophage**,** *Pseudomonas aeruginosa***,** sewage**,** salinity**.**

INTRODUCTION

The genus *Pseudomonas* is the most heterogeneous and ecologically significant group of known bacteria. Owing to the fact that the nutritional requirements of *Pseudomonas* spp.

are very simple, representatives of the genus have been detected in virtually all natural habitats (*e.g.*, soil, house dust, fresh water and clouds), and have also been isolated from clinical instruments, aseptic solutions, cosmetics and medical products (Franzetti and Scarpellini, 2007)

Milk has been referred as the most perfect food. Unhygienic methods of production and high ambient temperatures along with the lack of prompt cooling after milking and unsatisfactory washed milking equipment are the main reasons influencing the bacteriological quality of raw milk (Sharaf, *et al.,* 1989).

P. aeruginosa can get into water distribution systems from source waters. These organisms have the ability to regrow in such distribution systems. The importance of these organisms as water-borne pathogens is based primarily on their ability to live in biofilms in water distribution systems, where they can act as a continuous source of contamination **(**Szewzyk *et al*., 2000).

Mcphee and Griffiths (2011) found that the average counts of psychrotrophic aerobic bacteria in milk silos at several dairies in southwest Scotland were reported to be 1.3×10^5 cfu mL^{-1}. The majority of the bacteria present were *Pseudomonads* (70.2%), but Enterobacteriaceae (7.7%), Gram-positive bacteria (6.9%), and other Gram-negative, rod-shaped organisms were also isolated. Following storage for a further 48 h at 6 °C, the psychrotrophic counts increased by two log cycles to 1.3×10^7 cfu mL^{-1}.

The interest in applied bacteriophage research has increased during recent years mainly due to positive results obtained with phage therapy applied to animals (Atterbury *et al.,* 2003 and Wagenaar *et al.,* 2005). Also phage application to certain meat products was allowed, since August 2006, by the United States Food and Drug Administration (FDA) in order to control *Listeria monocytogenes.* (FDA, 2006). Moreover, there is an increase in the number of patents of application of phages to control pathogenic bacteria in industrial environments and foodstuffs (Loessner and Carlton 2005 and Sulakvelidze. *et al.,* 2004). The idea in this area is to either keep the pathogen propagation limited by the phages over long times, by insertion of phages in surface layers (Curtin and Donlan 2006). or to apply phages at different stages of production and processing to reduce food product contamination at that point or to protect against contaminations at subsequent points, which can be performed also in combination of sterilizing chemical agents as long as these agents do not reduce the biological activity of the phages (Sulakvelidze *et al*., 2004).

The aim of this study is to isolate bacteriophages specific to *P. aeruginosa* to be used as bio-controlling agent against this pathogenic microorganism in contaminated water and milk.

MATERIALS AND METHODS

Source of *pseudomonas aeruginosa* bacteria: A sewage water sample was collected from the sewage

treatment station of El-kawther City, Sohage, Egypt and identified by vitec 2 systems in Sohag University.

Isolation of bacteria: Serial dilutions of the collected sewage water sample were prepared. Drops from each dilution were spread on MacConkey Agar medium plates, then plates were incubated at 30°C for 24 – 48 h. One single colony was transferred onto slant surface of MacConkey in a test tube. The slant was incubated at 30°C for 48 h. The isolate was streaked for purification on MacConkey medium plate and then a single colony was selected and transferred onto slant surface of MacConkey in a test tube. The tube was incubated at 30°C for 48 h., then stored at 4°C.

Morphological studies were carried out on the bacterial isolate. A smear was prepared on a glass slide and Gram stained. The prepared smear was microscopically examined. Cell morphology and Gram reaction were recorded.

The bacterial isolate was identified in Sohag University, Egypt using Biolog D. B. (2013**)** and was found to be *Pseudomonas. aeruginosa*.

Antibiotic sensitivity test: Sensitivity of the isolated bacterium (*P.aeruginosa*) to 19 different antibiotics was tested. The tested antibiotics were Ceftazidime, Ceftoxim calvulinic acid, Cefazolin, Czc, Ciprofloxacin, Streptomycin, Doxycylin, Cefoprazole sulbactum, Cefotax, Tobramycine, Ceftriaxone, Etp, Augmentin, Ampicillin, Meropenem, Nitrofuranotine, Tazobactam piperacillin, Cefoperazone and Cefoxitine. Liquid culture of the tested bacterium was spread on plates containing nutrient agar medium (Allen, 1959**)**. After about 20 min. antibiotic discs containing 30 µg antibiotic/ disc were placed on the surface of the plates. Plates were incubated at 30ºC, until the bacterial growth was sufficiently dense after about 24 h. plates were inspected for inhibition zones around the discs.

Effect of salinity stress on *P. aeruginosa*:

Erlenmayer flasks (100 ml) containing nutrient broth medium (50 ml/flask). The media were salinized with NaCl at concentrations of 0%, 1%, 2% , 3%, 4%, 5%, 6%, 7%, 8% and 9%. The prepared flasks were inoculated with *P. aeruginosa* isolate by transferring 1ml of 48 h. old liquid culture (10^7 cfu/ml) to every flask. Flasks were incubated at 37°C. The growth was estimated at intervals of 24 h upto 6 days as optical density, at wave length of 621 nm.

Source of Bacteriophages: The same sample of sewage water was used as a source of bacteriophages.

Isolation of Bacteriophages: The liquid enrichment technique of Adams (1966) was used to isolate phages specific to *P.aeruginosa* from the collected sewage water sample as described by Barnet (1972).

Detection of bacteriophages: The spot test was used for detection of bacteriophages of *P. aeruginosa* as described by Adams (1966).

Purification of bacteriophage isolates: The single plaque isolation technique was used to obtain pure single phage isolates as described by Kiraly *et al*. (1970).

Preparation of high titer phage suspension: Agar double layer plates method described by Maniatis *et al.* (1982) was used to prepare the high titer phage suspension for each single phage isolate as described by Hammad and Dora (1993)

Titer Estimation: Titer was estimated using the method described by Kiraly *et al.* (1970). From the phage suspension, a series of tenfold dilution was prepared in sterile eppendorf vials. The dilutions were prepared by measuring 90 µl of SM medium **(**Maniatis *et al.,* 1982) into each vial. Ten µl of phage suspension were added to the first vial and mixed, then 10 µl from the first vial were transferred into the second one and so on, until the last vial. After dilution, 200 µl of indicator bacterial suspension were placed in each vial. The contents of each tube were shaken and transferred to a sterile test tube containing 3 ml of melted nutrient agar semi-solid medium (0.7% ager), which had been prepared before and kept at 50-55 °C. Each tube was shaken separately, and the contents were poured onto previously prepared solid medium plates, then they were incubated at 30-33°C for 24 h. The formed plaques were counted and the titer was calculated and expressed as plaque forming unit (pfu)/ml.

Phages as bio-controlling agent:

An experiment was carried out as an attempt to get rid of *P. aeruginosa* contaminating milk and water. Four Erlenmeyer flasks each containing 100 ml of milk and four flasks containing tap water (100 ml/flask) were prepared and autoclaved at 121ºC for 20 min. Ten µl of *P. aeruginosa* liquid culture (10^5 cfu/ml) were added to each flask. Phage suspension (Mixture of phage isolates 10^8 pfu/ml) was added to two flasks containing *P. aeruginosa* contaminated milk and to two flasks containing *P. aeruginosa* contaminated water (50 µl/flask). A flask containing contaminated milk and a flask containing contaminated water were incubated at 37ºC and other ones incubated at 7ºC. The flasks containing contaminated milk and water plus phages were incubated at 37 ºC and 7ºC.

Numbers of *P. aeruginosa* in all treatments were estimated after 1, 4, 18 and 24 h. The phage titer was estimated in the treatments which received phage suspension at zero time and after 24 h.

RESULTS AND DISCUSSION

The isolated bacteria

A bacterial isolate was picked from sewage water sample collected from treatment station of El-kawther City, Sohage, Egypt.

A smear of the isolated bacterium was prepared on a glass slide and Gram stained. As shown in figure (1) this isolate was found to be a rod-shaped Gram-negative aerobic bacterium. The isolated bacterium was identified in Sohag University,

Egypt, using Biolog D. B. (2013) and was found to be *Pseudomonas .aeruginosa*. Similarly, Filali *et al*. (2000) isolated *P. aeruginosa* from Sewage water of Casablanca city in Morocco. Moreover, Shah **(**2017) isolated *P. aeruginosa* from azo dye (Remazol Black B) contaminated water which was collected from an area of industrial zone.

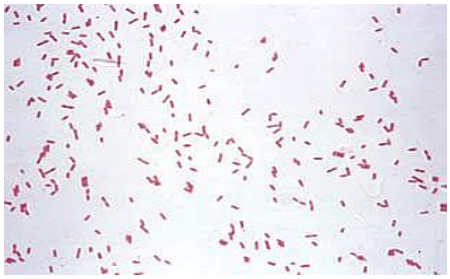

Figure (1): Light micrograph of a Gram stained *Pseudomonas aeruginosa*.

Antibiotic sensitivity test

Sensitivity of *Pseudomonas aeruginosa* to 19 antibiotics was tested. Data presented in figure (2) and Table (1) indicated that two out of 19 antibiotics tested exhibited inhibitory effect against *P. aeruginosa*. these two antibiotics were ciprofloxacin and meropenem. According to the obtained results, ciprofloxacin was the most efficient antibiotic followed by meropenem, they exhibited inebriation zones of 30 and 25 mm. in diameters, respectively (Table 1). Such results may indicate that *P. aeruginosa* is highly resistant to most antibiotics tested. Filali *et al*. (2000) isolated different strains of *P. aeruginosa* from sewage water. These strains showed high resistance to heavy metals, antibiotics and aromatic hydrocarbons. Therefore, they suggested that these strains could be good candidates for remediation of some heavy metals and aromatic compounds in heavily polluted sites. Benie *et al.* (2017) reported that all strains of *Pseudomonas aeruginosa* isolated from bovine meat, fresh and smoked fish expressed resistance to almost all antibiotics.

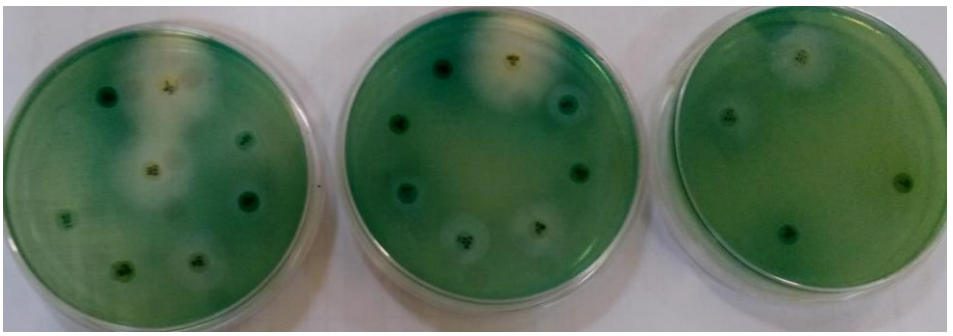

Figure (2): Antibiotics sensitivity test of *P. aeruginosa***.** The inhibition zone around some discs can be clearly seen.

Table (1): Sensitivity test of 19 different antibiotics against *P. aeruginosa*

Antibiotic	Code	Sensitivity	
		Diameter of inhibition Zone (mm.)	
Ceftazidime	CAZ	15	I
Ceftoxim calvulinic acid	CTC	R	R
Cefazolin	KZ	R	R
Colistin	Czc	R	R
Ciprofloxacin	CIP	30	S
Streptomycin	S	R	R
Doxycylin	DO	10	I
Cefoprazole, sulbactum	CES	20	I
Cefotax	CTX	R	R
Tobramycine	TOB	15	I
Ceftriaxone	CRO	15	I
Eertapenem	ETP	8	I
Augmentin	AMC	R	R
Ampicillin	AM	R	R
Meropenem	MEM	25	S
Nitrofuranotine	F	R	R
Tazobactam, piperacillin	TZP	20	I
Cefoperazone	CFP	10	I
Cefoxitine	FOX	R	R

R= Resistant I = Intermediate sensitive S = Sensitive

Effect of different concentrations of NaCl on growth of *P. aeruginosa.*

Data presented in Table (2) indicated that *P. aeruginosa* was found to be tolerant to salinity stress. At any of the tested concentrations of NaCl the growth of *P. aeruginosa* tended to increase with increasing the incubation period. The highest growth at any NaCl concentration was recorded at the 6th day of incubation at 37ºC.

Moreover, among the different NaCl concentrations tested, the highest growth of *P. aeruginosa* was recorded at 1% NaCl. Viktória *et al.* (2008) observed that high salt impairs *P. aeruginosa* motility and inhibits growth, this may be attributed to hypertonic saline. Moreover, Michon *et al.* (2014) reported that the growth of all *P. aeruginosa* isolates from 34 patients was inhibited by 6% NaCl solution. A 10% concentration had a bactericidal activity on 90% of the isolates.

Effect of different pH levels on growth* of *P. aeruginosa.*

As one of the major surrounding conditions, pH plays the significant role in bacteria growth. Growth of *P. aeruginosa* at different pH levels (pH 4 upto 10) was tested. As shown in Table (3) *P. aeruginosa* was found to be tolerant to all tested pH levels. At any pH level the growth tended to increase with increasing the incubation period. The highest growth

value was recorded at pH 6 after incubation for 6 days.

Table (2): Effect of different NaCl concentrations on growth* of *P. aeruginosa.*

NaCl concentration	Incubation period (day)					
	1	2	3	4	5	6
1%	0.036	0.240	0.391	0.818	0.965	0.984
2%	0.034	0.270	0.470	0.751	0.882	0.886
3%	0.025	0.252	0.349	0.621	0.629	0.631
4%	0.036	0.141	0.243	0.452	0.457	0.511
5%	0.029	0.081	0.171	0.300	0.352	0.380
6%	0.030	0.067	0.172	0.227	0.288	0.295
7%	0.031	0.066	0.139	0.210	0.242	0.253
8%	0.035	0.029	0.096	0.138	0.154	0.159
9%	0.032	0.027	0.113	0.096	0.144	0.169

* Growth was measured as optical density.

Table (3): Effect of different pH levels on growth* of *P. aeruginosa.*

pH level	Incubation period (day)					
	1	2	3	4	5	6
4	0.038	0.050	0.227	0.701	0.845	0.871
5	0.029	0.311	0.449	0.882	0.918	0.946
6	0.031	0.378	0.493	0.898	0.918	1.028
7	0.031	0.212	0.502	0.799	0.974	0.952
8	0.028	0.281	0.401	0.733	0.814	0.848
9	0.025	0.275	0.485	0.869	0.914	0.857
10	0.029	0.038	0.346	0.500	0.582	0.658

* Growth was measured as optical density.

Russell and Wilson (1996) reported that low pH (high H+ concentration) would cause the destruction of small pH gradient balance across the cell membrane, which led to an intracellular accumulation of volatile fatty acid anions. In addition, low pH also may lead to the bacterial DNA damage (Cotter and Hill, 2003). The lower concentrations decrease of both polysaccharide and protein was because of inactive bacterium due to low pH (3-5). However, the concentration variation trends of polysaccharide and protein with high pH (8-13) were gentler than those with low pH (3-5). It indicated that alkali-resistance of *P.aeruginosa* biofilm formation was stronger than that of acid-resistance.

Bacteriophage specific to *P. aeruginosa* in the collected sewage water sample

Bacteriophages specific to *P. aeruginosa* were successfully isolated from the sewage water sample collected from sewage treatment plant of El-kawther City, Sohage, Egypt. The spot test was used for detection of phages in the collected sample. As shown in

Figure (3) the spot test indicates that phages of *P. aeruginosa* were found to be common in the collected sample.

Figure (3): A bacterial lawn of *pseudomonas aeruginosa*, spotted with drops of the prepared phage lysate and incubated for 24 h at 37 °C. The lysed spot is clearly seen.

Similarly**,** Kumari *et al.* (2009) and Alsaffar and Jarallah (2016) isolated bacteriophages specific to *P. aeruginosa* from sewage water.

Purification of phages

Since it is assumed that each plaque has originated from the progeny of a single phage particle **(**Kiraly, *et al.*,1970**)** and Elmaghraby *et al*., 2015). The single plaque isolation technique was used to obtain pure phage isolates of *p.aeruginosa* As shown in Fig. (4) the phages specific to *p.aeruginosa* formed single plaques of different morphologies. Twenty single plaques morphologically different were selected and kept as pure phage isolates. The isolated phages formed circular single plaques of 1 to 3 mm in diameter and clear in appearance.

The high titer phage suspensions: One hundred ml of high titer phage suspension were prepared for each phage isolate of *p. aeruginosa*. The titers of the prepared suspensions of the twenty phage isolates specific to *P. aeruginosa* were ranged from 43 $x10^8$ to 58 $x10^8$ pfu/ml. Such high concentrations of phages are not surprising, since a single plaque of 2 mm in diameter may contain between 107 and 109 recoverable phage particles (Fathy, 2008 and Elsharouny, 2007).

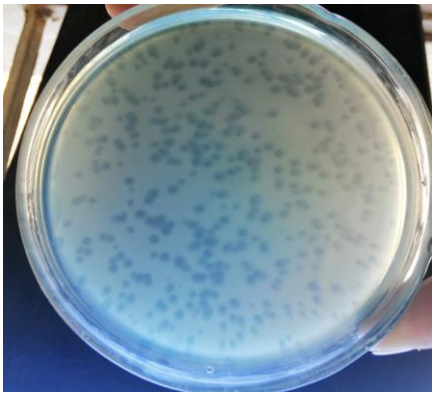

Figure (4) A plate containing single plaques of bacteriophages specific to *p.aeruginosa*. The differences in morphology of the single plaques can be clearly seen.

Phages as bio-controlling agent:

An experiment was carried out as an attempt to eliminate *P. aeruginosa* contamination in milk and water.

P. aeruginosa is known to be of poor nutritional needs, therefore, it can survive in different environments, such as atmospheric dust, vegetation, water, and soil. Additionally, these characteristics allow this bacterium to

survive on the utensils and equipment used in the dairy production chain, such as milking machines, pipelines, and bulk tanks **(**Simões *et al*., 2010).

Pseudomonas spp. are among the most common microorganisms involved in spoilage of milk and dairy products during their refrigerated storage because many strains are psychrotolerant **(**Martin *et al*., 2011**).** In addition, many of these strains also produce heat-stable extracellular lipases, proteases and lecithinases which can further contribute to spoilage of milk and dairy products **(**Zhang *et al*., 2015).

As shown in Table (4) the initial count of *P. aeruginosa* in contaminated milk and water was $35x10^3$. In absence of the specific phages gradual increase in number of *P. aeruginosa* was recorded with the laps of time in case of incubation at 37 and 7ºC. The highest numbers were recorded in both water and milk after 24 h.

On the other hand, with application of phages to the contaminated milk and water, gradual reduction in numbers of *P. aeruginosa* was recorded. After 24h. no bacterial cells were detected neither in milk nor water incubated at 37 or 7ºC.

Moreover, in contaminated milk and water which treated with the specific phages, numbers of phage particles were estimated at zero time and after incubation for 24h at 37 and 7ºC (Table 5). Number of phage particles at zero time was estimated to be $29x10^7$ pfu/ml. Whereas, high numbers of phage particles were detected in both milk and water by the end of the experiment (after 24 h).

Similarly, Curtin and Donlan (2006) stated that there is an increase in the number of patents of application of phages to control pathogenic bacteria in industrial environments and foodstuffs. The idea in this area is to either keep the pathogen propagation limited by the phages over long times, e.g. by insertion of phages in surface layers or to apply phages at different stages of production and processing to reduce food product contamination at that point or to protect against contaminations at subsequent points, which can be performed also in combination of sterilizing chemical agents as long as these agents do not reduce the biological activity of the phages.

CONCLUSION

Generally, on the basis of the obtained results it can be concluded that *P. aeruginosa* was found to be of wide spread occurrence in sewage water. This microorganism is known to be resistant to most antibiotics, highly tolerant to wide range of pH levels and salinity stress. The use of bacteriophages specific to *P. aeruginosa* as bio-controlling agent was found to be effective to get rid of such pathogenic bacterium in contaminated water and milk. Therefore, application of specific phages to foodstuffs is highly recommended to avoid contamination or to get rid of this pathogenic bacterium.

Table (4): Effect of bacteriophages on the density of *P. aeruginosa* (cfu/ml) in contaminated milk and water incubated at 7 and 37ºC.

Sampling time (hour)	Milk				Water			
	Bacteria		Bacteria + phage		Bacteria		Bacteria+ phage	
	37° C	7° C	37° C	7° C	37° C	7° C	37° C	7° C
0	35 x10^3	35 x10^3	35 x10^3	35 x10^3	35 x10^3	35 x10^3	35 x10^3	35 x10^3
1	40 x10^3	26 x10^3	5.7 x10^3	6.5 x10^3	49 x10^3	39 x10^3	24 x10^3	24 x10^3
4	890 x10^3	370 x10^3	3.0 x10^3	4.0 x10^3	55 x 10^3	42 x10^3	20 x10^3	18 x10^3
18	1430 x10^3	230 x10^3	0.0013 x10^3	0.002 x 10^3	620 x10^3	480 x10^3	0.32 x10^3	0.6 x10^3
24	2780 x 10^3	300 x10^3	00	00	870x10^3	550 x10^3	0.00	0.00

Table (5): Titer of phages specific to *P. aeruginosa* (pfu/ml) in contaminated water and milk treated with phage suspension and incubated at 7 and 37 **ºC**

Sampling time (hour)	Milk		Water	
	37ºC	7 ºC	37 ºC	7 ºC
0	29 x10^7	29 x10^7	29 x10^7	29 x10^7
24	83 x 10^{12}	63 x10^{11}	17 x10^9	53 x10^8

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مقاومة بسيدوموناس ايروجينوزا فى المياه واللبن الملوث باستخدام الفيروسات البكتيرية المتخصصة

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في هذه الدراسة تم عزل بسيدوموناس ايروجينوزا من عينة مياه مجاري من محطة معالجة مياه مجاري مدينة الكوثر بسوهاج. تم دراسة حساسية بسيدوموناس ايروجينوزا لـ 19 مضاد حيوي وقد تبين ان اثنين فقط من بين 19 مضاد حيوى (سيبروفلوكساسين وميروبينيم) ذات تأثير قاتل على البكتيريا المعزولة. تبين من الدراسة أن بسيدوموناس ايروجينوزا أظهرت تحمل للملوحة حيث أمكنها النمو على 9% كلوريد صوديوم كما تبين انها تتحمل مدى واسع من الاس الهيدروجيني من pH 4- 10. تم عزل الفيروسات البكتيرية المتخصصة على بسيدوموناس ايروجينوزا من نفس عينة مياه المجاري. وبمعاملة الماء واللبن الملوث بـ بسيدوموناس ايروجينوزا وتحضينهما على درجة حرارة 4 و37 درجة مئوية انخفضت اعداد البكتيريا تدريجيا واختفت البكتيريا تماما بعد 24 ساعة في كل من العينات المحضنة على درجة حرارة 4 و37 درجة مئوية. بينما ازداد عدد الفيروسات البكتيرية حيث سجلت أعلى الأعداد بعد 24 ساعة. وبناء على هذا فإنه ينصح بمعاملة المواد الغذائية بالفاجات المتخصصة على بسيدوموناس ايروجينوزا للتخلص من هذه البكتيريا الممرضة.