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Viability of The Isolated *Yersinia enterocolitica* Strains from Damietta Cheese and Ice Cream at Different Refrigeration and Freezing Temperatures

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ABSTRACT

Yersinia *enterocolitica* is considered one of the most prevalent pathogens transmitted through milk and milk products. Therefore, we aimed to detect the prevalence of these bacteria in cheese and ice cream and study the influence of refrigeration and freezing on its growth patterns. A total of 80 samples of cheese and ice cream were collected from Assiut city, Egypt (40 samples each). The collected samples were examined for the isolation of *Y. enterocolitica* by the classical culture method and improved by the PCR technique. The incidence of *Y. enterocolitica was* 17.5 and 25.0 % in the examined cheese and ice cream samples by culture method, respectively, its prevalence in the tested cheese and ice cream samples basing on PCR were 7.5 and 15% since 42.8 and 60.0% of the isolated *Y. enterocolotica* were confirmed positive. Bio- typing and serotyping of the isolated strains revealed that 8 out of the confirmed strains were pathogenic ; *Y. enterocolitica* serotype O: 3 was the most prevalent strain, and all of the pathogenic strains carried the virulent *ail* gene. Nearly similar growth patterns of *Y. enterocolitica* were recorded during storage of cheese at 4 ± 2°C and 30 ± 2ºC and, a significant difference was observed in the 3rd week, the organism found to be survived for 18 weeks with a mean value of 7.4 ± 0.5 and 6.9 ± 0.16 log cfu/ g for cheese stored at 4 ± 2°C and 30 ± 2°C respectively. In the case of ice cream, there was a significant difference between the behavior of *Y. enterocolitica* during freezing storage at -6 ± 2°C and -18± 2ºC in the 2nd week, the mean value for the organism count on the 16th week was 5.3 ± 0.26 and 5.6 ± 0.39 log cfu/ g, respectively.

Keywords: Cheese, Ice cream, PCR, Virulence genes, *Y. enterocolitica.*

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INTRODUCTION

Microbial safety is considered the focal theme in the current food industry scenario, so more advanced methods for detecting microorganisms and their pathogenicity have been evolved over the years **(Severgnini, *et al.*, 2011).** Recently, food-borne outbreaks are associated with the consumption of milk and milk products, which have been contaminated with pathogenic bacteria like *Listeria, salmonella, campylobacter and Yersinia spp.* **(Gram *et al.*, 2002).**

Yersinia *enterocolitica* is a well-known food-borne infectious agent that cause enteric disease in human with abundant gastrointestinal disorders ranging from mild diarrhea, inflammation of the mesenteric glands, appendicitis, septicemia in children to underlying disease. Yersiniosis is recorded in the third place among food-borne illness after campylobacteriosis and salmonellosis (**Bottone, 1997; Bolton *et al.*, 2013**).

The Genus Yersinia is a member of the Enterobacteriaceae family and comprises 17 gram-negative bacteria, many of which are non-pathogenic,

but *Y. enterocolitica*, *Y. pestis* and *Y. pseudotuberculosis* are considered pathogenic for human. *Y. enterocolitica* includes a very heterogeneous group of strains which are classified biochemically to 6 biovars (1A, 1B, 2, 3, 4 and 5). The strains of biovars1B, 2, 3, 4 and 5 are considered pathogenic due to their possession of the virulence plasmid (pYV) and the chromosomal genes ail, ystA, myfA and hreP however the strains that belong to biotype 1A are non-pathogenic because they have not pYV plasmid and the chromosomally encoded genes **(Bahgat and Virdi, 2007; Shurnik and Toivonen, 2011; Anna and Jordi, 2012).** Serologically, more than 70 serotypes were detected and the most frequent serotypes that cause human infection are O: 3, O: 5, 27, O: 8 and O: 9 **(Vazlerova and steinhauserova, 2006)**.

Yersinia *enterocolitica* can contaminate milk, meat, eggs, seafood and vegetables primary or secondary during food processing. This pathogen is ubiquitously found in the environment which gets contaminated with feces of infected humans and animals. Humans get the infection through ingested contaminated food **(Nusrat *et al.*, 2009; Fredriksson-Ahomaa, *et al.*, 2010)**.

Yersinia *enterocolitic*a has become of particular public health concern because of its nature as a psychrotrophic bacterium, where the pathogenic strains of *Y. enterocolitica* have the ability to survive and multiply at a temperature as low as -5°C without inducing apparent signs of food spoilage. Its ability to grow at low temperatures is owing to the nature and structure of the plasma membrane which can undergo a phase of transition from a liquid crystalline state to a rigid gel when exposed to low temperature. In addition to the relatively resistance of its enzymatic systems to freezing and remain active even at -30°C **(Iliev and Najdenski, 2008 and Rakesh, 2007)**.

There is no need to prove that cheese and ice cream are the most common milk products stored by refrigeration and freezing process which are consumed by a wide range of people all over the world and these milk products may be contaminated with *Y. enterocolitica* from milk used in manufacturing or during processing and storage.

Because of the wide consuming cheese and ice cream and the psychotropic nature of *Y. enterocolitica*, this investigation aimed to detect the prevalence of *Y. enterocolitica* in these products and confirm the pathogenic strains' behavioral patterns during refrigeration and freezing as different storage aspects of cheese and ice cream.

MATERIALS AND METHODS

1.Samples

A total of 8o random samples were collected from supermarkets in Assiut city, Egypt including 40 samples for Damietta cheese and 40 samples for non-industrial ice cream. The samples were delivered to the laboratory immediately in an icebox.

2. Isolation of *Y. enterocolitica*

Twenty-five grams of the sample was aseptically added to phosphate-buffered saline (PBS) in a stomacher bag and homogenized for 2 min; then, the homogenate was incubated 2 min at 25°C. Twenty ml of the homogenate was added to 80 ml trypticase soya broth (TSB) and incubated at 25°C for 24 h, and then, the enrichment broth was treated with KOH (0.5% KOH in 0.5% saline) to inhibit other flora in the broth.

Loopfull from the inoculated broth was streaked on cefsulodin irgasan novobiocin agar (CIN) with *Yersinia* selective supplement (Merck – Germany) and incubated at 25°C for 48 h for the appearance of the suspected typical "bull's eye" colonies. After that, the colonies were streaked on tryptone soya agar plates for purification. The purified colonies were examined for gram staining, utilization of Simmons citrate, kliglers iron agar reaction and urease activity **(MacFaddin 2000; FDA, 2007)**.

3.Confirmation of the isolated strains by using PCR

Yersinia *enterocolitica* strains that were previously isolated and identified were confirmed using the PCR technique in the Reference Laboratory for Veterinary Quality Control on Poultry Production in Animal Health Research Institute, Dokki, Giza, Egypt according to **Wannet *et al.*, (2001).**

DNA extraction

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer

Primers used were supplied from Metabion (Germany) are listed in Table (1). PCR, Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The PCR products were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products were loaded in each gel slot. Generuler 100 bp DNA Ladder (Fermentas, Thermo, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed through computer software.

4. Bio- typing of *Y. enterocolitica* strains

The confirmed *Y. enterocolitica* strains by PCR were bio- typed biochemically by Esculin hydrolysis, Indole production, Voges Proskuaer, Nitrate reduction, Xylose fermentation, Trehalose fermentation, Salicin fermentation and β- Glucosidase tests according to **Schriefer and Petersen (2011).**

5. Serotyping of the isolates

The commercial antisera (O: 3, O: 4, O: 5, O: 8, O: 9 & O: 14) were applied for the demonstration of various serotypes of *Y. enterocolitica*. Briefly, the suspected culture was suspended in normal saline and one drop of the definite antiserum was individually added to one drop of the suspension. The positive result was attained by agglutination according to **Tennat *et al.,* (2003).**

6. Detection of the virulence genes

The previously bio- typed pathogenic strains of *Y. enterocolitica* were sent to the Reference Laboratory for Veterinary Quality Control on Poultry Production in Animal Health Research Institute, Dokki, Giza, Egypt for detection of the chromosomal virulence ail and yst genes according to **Wannet *et al.*, (2001); Koua *et al.,* (2014).**

7. Behavioral patterns of *Y. enterocolitica* in Damietta cheese and ice cream:

Culture Preparation:

Strains of *Y. enterocolitica* O: 3 biotypes 2 used in the experiment were the previously isolated strains from cheese and ice cream. They were propagated in tryptone soya broth at 25°C for 48 h and tenfold serial dilution was carried out and matched to McFarland tubes to determine the density of the organism in the broth.

7.1. Effect of refrigeration temperature (4 ± 2°C) on the survival of *Y. enterocolitica* in Damietta cheese

Preparation of Cheese

Four liters of pasteurized condensed milk were obtained from Animal Production Research Institute and examined for *Y. enterocolitica* before used to make sure it was free from the organism; then, sodium chloride was added in a concentration of 3% and warmed to 40°C. Thereafter, rennet was added according to the manufacturer, and two portions were taken before inoculation as two control negative samples. The rest was inoculated with *Y. enterocolitica* serotype O: 3 isolated from the examined cheese to yield 5×10^6 cfu/ g and divided into six portions that were poured in the containers and were left at room temperature till curdling. Samples from each portion were taken to detect the initial count before keeping three portions at 30 ± 2°C and the second three portions and control negative samples were kept at refrigeration temperature (4 ± 2°C) for 18 weeks. The prepared cheese portions were examined to determine the count of *Y. enterocolitica* on the 1st, 5th days and every week in the first month. Then, the count was determined every two weeks till the 14th week and finally in the 18th weeks of storage.

7.2. Effect of freezing at –6 ± 2°C and -18 ± 2°C on the survival of *Y. enterocolitica* in ice cream

Preparation of ice cream

The ice cream was prepared from ice cream powder packets in the laboratory according to the manufacturer and two control portions were taken before inoculation. The rest was inoculated with *Y. enterocolitica* serotype O: 3 isolated from the examined ice cream to yield a count of 5×10^6 cfu/ g and divided into six portions; three portions were stored at -6 ± 2°C and the other three portions at -18 ± 2°C. Samples from two controls and six portions were examined to determine the initial count of the organism before freezing storage, and the count after freezing was detected on 1st, 3rd, 5th days and in the 1st and 2nd weeks, then, the microbial load was determined every two weeks till the end of the experiment.

8. Statistical analysis

The statistical analysis was performed using IBM SPSS (IBM CO. Version), and data statistics analysis was transforming after $\log_{10}$ values. The mean value of compared using T-test at $\alpha < 0.05$.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension
				Secondary denaturation	Annealing	Extension	
Y. enterocolitica 16S rRNA	AAT ACC GCA TAA CGT CTT CG CTT CTT CTG CGA GTA ACG TC	330	94˚C 5 min.	94˚C 30 sec.	62˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.
Ail	TAATGTGTACGCTGCGAG GACGTCTTACTTGCACTG	351	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.
Yst	AATGCTGTCTTCATTTGGAGC ATCCCAATCACTACTGACTTC	145	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.

RESULTS

Yersinia *enterocolitica* could be isolated from 17.5 and 2.5% of the examined cheese and ice cream samples. At the same time, *Y. frenderikenii* could be detected in 2.5% of the examined cheese and ice cream samples, respectively. On the other hand, *Y. intermedia* was found on 2.5% of the tested ice cream samples and was not isolated from cheese samples (table, 2).

Table 2: Prevalence of Yersinia species in the examined samples based on culture method

Yersinia spp.	Damietta cheese		Ice Cream	
	No.	%	No.	%
Y. enterocolitica	7/40	17.5	10/40	25.0
Y. Frenderikenii	1/40	2.5	1/40	2.5
Y. intermedia	-	-	1/40	2.5
Total	8/40	20.0	12/40	30.0

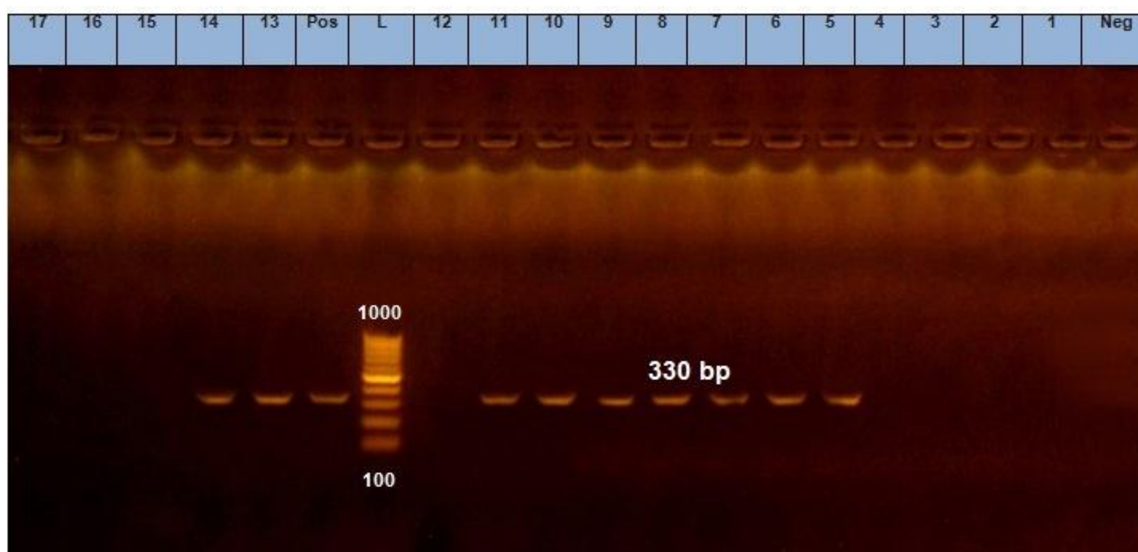

Fig. 1: Confirmation of the isolated *Y. enterocolitica* strains

Molecular confirmation of the isolated strains from cheese and ice cream revealed that 3 and 6 out of the identified strains were confirmed positive for *Y. enterocolitica* with a 42.8 and 60% percentage, respectively. Consequently, the incidence of *Y. enterocolitica* in the tested cheese and ice cream samples was 7.5 and 15.0%, respectively (table, 3).

Table 3: Results of molecular conformation of the isolated *Y. enterocolitica* strains by using PCR technique

Samples	Prevalence of *Y. enterocolitica* in the isolated strains		Incidence of *Y. enterocolitia* in the examined samples	
	No.	%	No.	%
Damietta cheese	3/ 7	42.8	3/40	7.5
Ice cream	6/ 10	60.0	6/40	15.0

The results of bio- typing and serotyping revealed that, 8 out of the confirmed strains have belonged to bio- type 2, 4 and 5 and one strain was bio- typed 1A. On the other hand, 66.66% and 50% of the isolated strains from cheese and ice cream were belonged to O: 3. In addition, serotype O: 8 was found in a frequency of 33.3% in cheese, while, serotypes O: 8, O: 4 and O: 5 were found in a percentage of 16.66% of the isolated strains from ice cream (table, 4).

Table 4: Bio- typing and serotyping of the confirmed *Y. enterocolotica* strains.

Strain	Damietta cheese		Ice cream	
	No.	%	No.	%
O:3/ biotype 2	1	33.3	2	33.33
O:3/ biotype 4	1	33.3	1	16.66
O:8/ biotype 2	1	33.3	-	-
O:8/ biotype 4	-	-	1	16.66
O:4/ biotype 1A	-	-	1	16.66
O:5/ biotype 5	-	-	1	16.66
Total	3	100	6	100

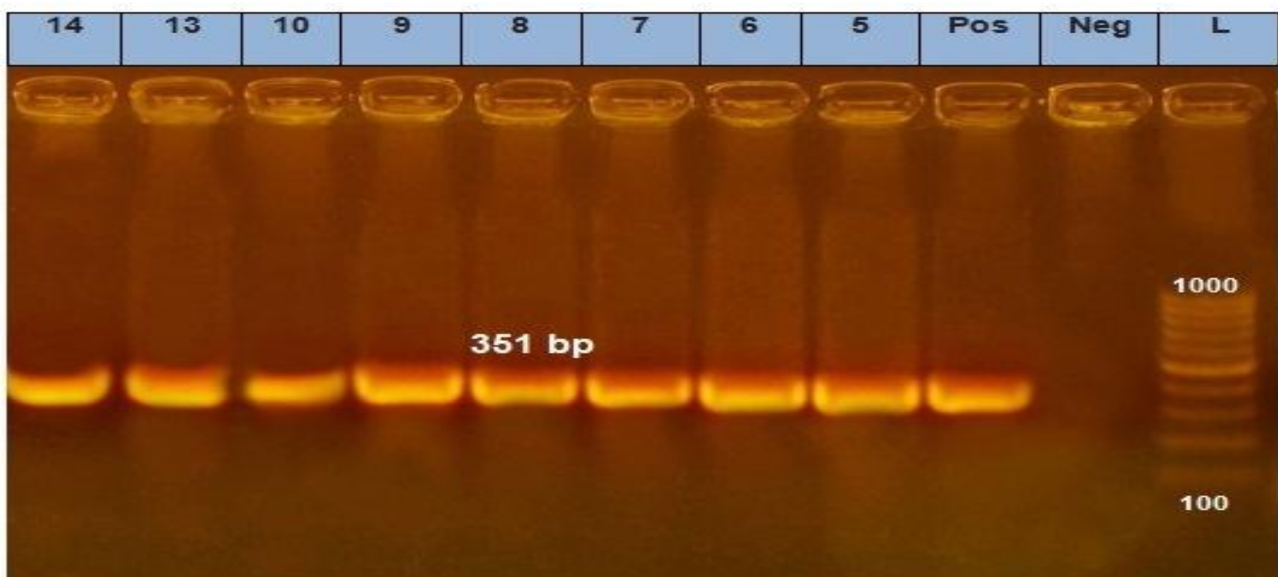

Fig. 2: Detection of the virulent ail gene.

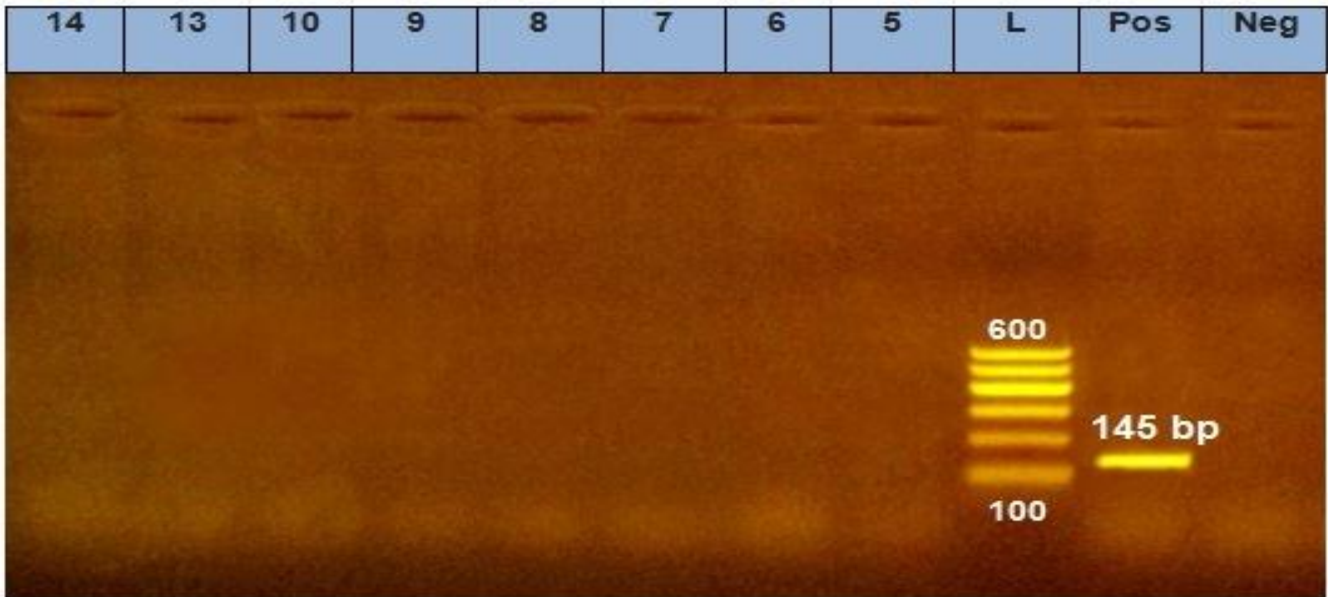

Fig.3: Detection of the virulent yst gene

The results of PCR detection of the virulent genes revealed that all the confirmed pathogenic *Y. enterocolitica* strains carried the ail gene, while, yst gene was failed to be detected, as observed in figures (2&3).

The initial count of the organism was 5.9, 6.5, and 5.0 with a mean value of 5.8 ± 0.75 logs cfu/ g and at the 1st day, its count was 6.2, 7.0 and 6.5 log cfu/ g and the mean value was 6.6 ± 0.40 log cfu/ g. and returned to decrease on the 5th day to 4.9 ± 0.92 logs cfu/ g. A gradual increase in the microbial load from the 1st to the 10th week was recorded, where the mean value was increased from 5.1 ± 0.34 to 10.2 ± 0.74 log cfu/ g. The mean value of the count in the 14th week was 9.4 ± 0.35 and the count was 8.0, 6.2 and 7.0 log cfu/g at the end of the experiment and the mean value was 7.4 ± 0.50 log cfu/ g (table, 5).

Table 5: Survival of *Y. enterocolitica in* cheese during refrigeration storage

Storage time	Trial 1 (log cfu/ g)	Trail 2 (log cfu/ g)	Trail 3 (log cfu/ g)	Mean (log cfu/ g)
Time zero	5.9	6.5	5.0	5.8 ± 0.75
1st day	6.9	7.0	6.5	6..6 ± 0.40
5th day	5.0	4.1	5.9	4.9 ± 0.92
1st week	5.1	4.8	5.5	5.1 ± 0.34
2nd week	7.1	6.9	8.1	7.4 ± 0.63
3rd week	6.9	6.5	7.0	6.8 ± 0.25
4th week	7.5	8.0	8.0	7.8 ± 0.27
6th week	7.6	7.9	8.1	7.8 ± 0.16
8th week	10.0	9.7	10.0	9.9 ± 0.16
10th week	10.5	9.4	10.3	10.1 ± 0.74
14th week	9.7	9.0	9.5	9.4 ± 0.35
18th week	8.0	7.2	7.0	7.4 ± 0.50

There was a low decreasing percentage in the count of *Y. enterocolitica* on the first day and the microbial load was 5.6, 6.3 and 5.4 log cfu/ g with a mean value of 5.8 ± 0.48 log cfu/ g. Then the count was increased on the 5th day. The mean value for the bacterial cells count was 6.3 ± 0.46 log cfu/ g, and a continuous increase in the count was reported till the number of the organism reach its highest value in the 10th week and the count was 11.0, 10.9 and 10.5 log cfu/ g with a mean value of 10. 8 ± 0.27 log cfu/ g. The count was rapidly decreased in the 14th and 18th weeks with a mean value of 9.2 ± 0.65 and 6.9 ± 0.16 log cfu/ g, respectively (table, 6).

Table 6: Survival of *Y. enterocolitica* in cheese during storage at room temperature

Storage time	Trial 1 (log cfu/ g)	Trail 2 (log cfu/ g)	Trail 3 (log cfu/ g)	Mean (log cfu/ g)
Time zero	5.9	6.6	5.6	6.1 ± 0.50
1st day	5.6	6.3	5.4	5.8 ± 0.48
5th day	6.5	6.6	5.8	6.3 ± 0.46
1st week	7.0	7.0	5.5	6.5 ± 0.85
2nd week	7.6	8.0	8.9	8.2 ± 0.68
3rd week	8.0	7.9	8.2	8.0 ± 0.15
4th week	8.8	8.1	9.0	8.6 ± 0.45
6th week	8.0	8.5	9.0	8.5 ± 0.47
8th week	9.1	9.8	9.80	9.6 ± 0.41
10th week	11.0	10.9	10.5	10.8 ± 0.27
14th week	8.6	9.9	9.0	9.2 ± 0.65
18th week	7.0	7.0	6.7	6.9 ± 0.16

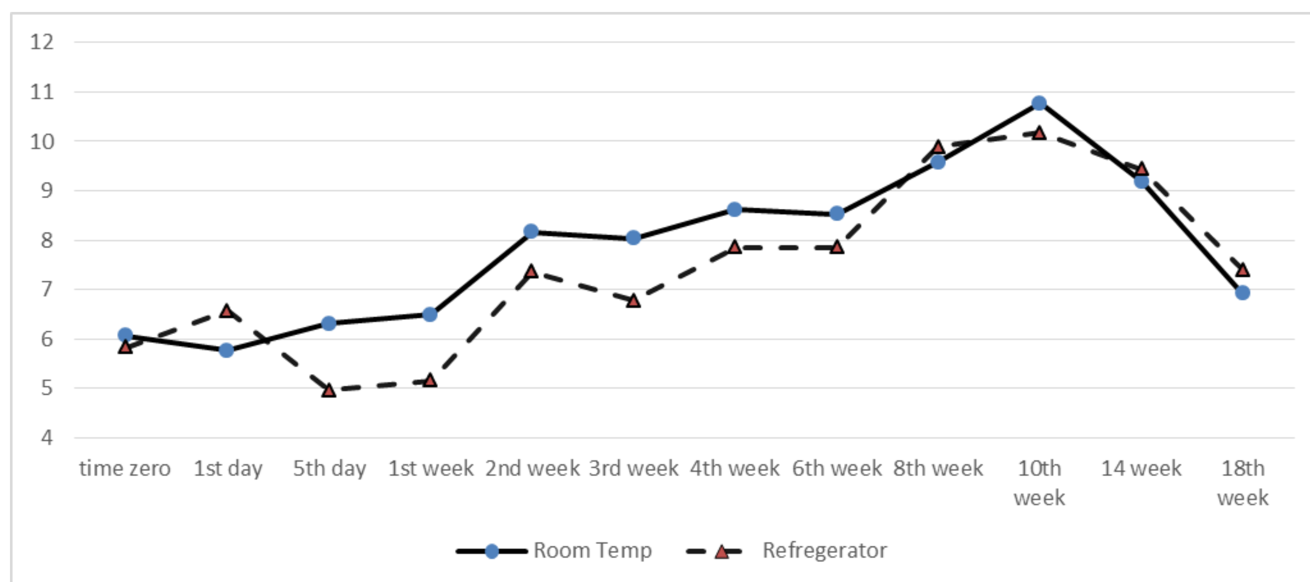

Fig.4: Survival of *Y. enterocolitica* in cheese

There are significant differences ($P < 0.05$) between means in the 3rd week.

During freezing of ice cream at -6 ± 2 ℃ there was a slight increase in the count of the organism and the count was 6.6, 6.2 and 7.0 log cfu/ g with a mean value of 6.6 ± 0.40 log cfu/ g on the first day and return to decrease on the 3rd and 5th days with a mean value of 6.2 ± 0.68 and 6.3 ± 0.14 log cfu/ g, then, the count was increased to 6.9 ± 0.16 log cfu/ g in the 1st week. In the 2nd week, the bacterial population declined to 6.5 ± 0.45 log cfu/ g. The highest increasing percentage was noted in the 4th week with a mean value of 8.2 ±0.46 log cfu/ g

and from this time to the end of the experiment, there was a decrease in the count, where the mean value of the organism was declined to 5.5, 5.2 and 5.0 log cfu/ g in the 16th week with a mean value of 5.3 ± 0.26 log cfu/ g (table, 7).

Table 7: Behavioral patterns of *Y. enterocolitica* in ice cream during freezing storage at -6 ± 2°C:

Storage time	Trial 1 (log cfu/ g)	Trail 2 (log cfu/ g)	Trail 3 (log cfu/ g)	Mean (log cfu/ g)
Time zero	6.5	6.0	6.4	6.3 ± 0.26
1st day	6.6	6.2	7.0	6.6 ± 0.40
3rd day	5.6	7.0	6.1	6.2 ± 0.68
5th day	6.4	6.4	6.2	6.3 ± 0.14
1st week	6.7	7.0	7.0	6.9 ± 0.16
2nd week	6.0	6.5	6.9	6.5 ± 0.45
4th week	8.7	8.1	7.8	8.2 ± 0.46
6th week	7.9	7.7	8.5	8.0 ± 0.37
8th week	6.0	6.8	5.8	6.2 ± 0.49
12th week	6.0	5.0	4.9	5.3 ± 0.58
16th week	5.5	5.2	5.0	5.3 ± 0.26

There was a reduction in the mean value of the bacterial population from 6.7 ± 0.43 log cfu/ g to 6.3 ± 0.52 and 5.3 ± 0.62 log cfu/ g on the 1st and 3rd days of ice cream storage at -18 ± 2ºC. On the contrary, there was a high, increasing percentage from the 3rd day to the 2nd week, where the recorded mean value was 8.4 ± 0.46 log cfu/ g in the 2nd week. Reduction in the count of *Y. enterocolitica* started from the 4th week to the end of this treatment with a mean value of 5.6 ± 0.39 log cfu/g (table, 8).

Table 8: Behavioral patterns of *Y. enterocolitica* during storage of ice cream at -18 ± 2ºC:

Storage time	Trial 1 (log cfu/ g)	Trail 2 (log cfu/ g)	Trail 3 (log cfu/ g)	Mean (log cfu/ g)
Time zero	6.4	6.8	5.9	6.7 ± 0.43
1st day	6.6	6.7	5.7	6.3 ± 0.52
3rd day	4.9	6.0	5.0	5.3 ± 0.62
5th day	6.6	7.0	7.5	7.0 ± 0.48
1st week	7.0	7.0	7.7	7.9 ± 0.48
2nd week	8.9	8.2	8.1	8.4 ± 0.46
4th week	7.7	7.4	8.0	7.7 ± 0.30
6th week	7.6	6.3	6.4	6.8 ± 0.72
8th week	6.0	5.9	5.6	5.8 ± 0.19
12th week	5.8	5.9	4.8	5.5 ± 0.62
16th week	5.8	5.9	5.1	5.6 ± 0.39

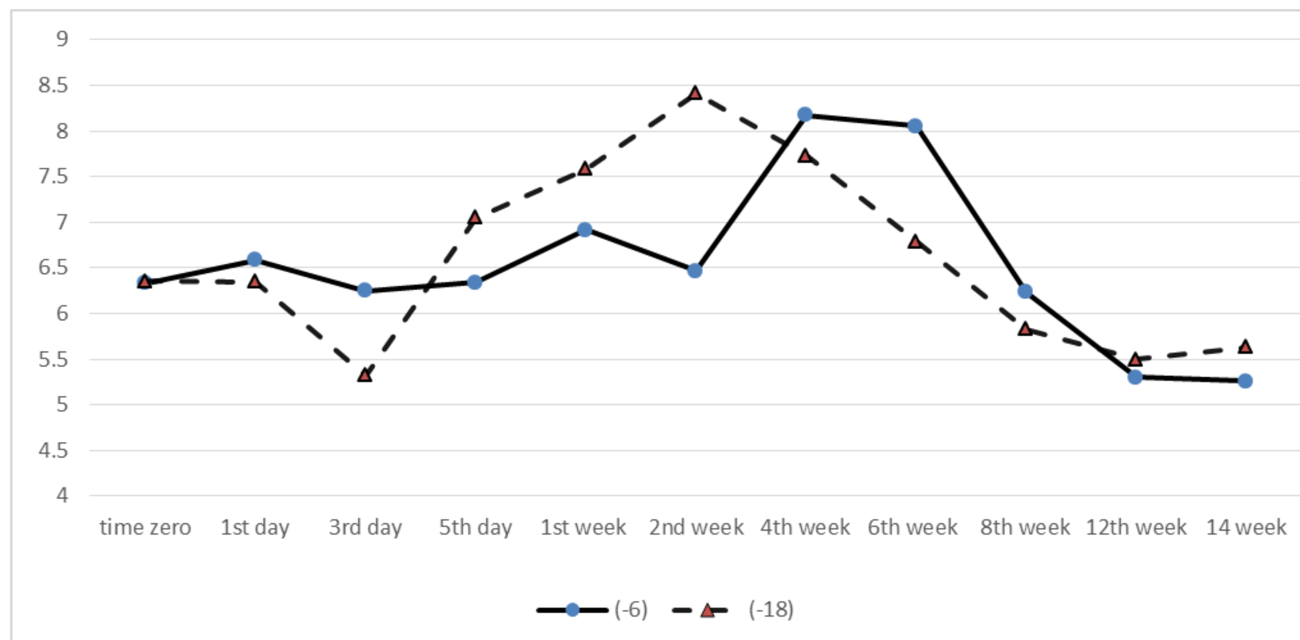

Fig 5: Survival of *Y. enterocolitica* in ice cream
There are significant differences ($P < 0.05$) between means in the 2nd week.

DISCUSSION

The presence of *Yersinia enterocolitica* in milk and its ability to survive at low temperatures during refrigeration and freezing storage is a public health concern in the dairy industry. **(Champagene *et al.* 2014)**. So, the need to detect the prevalence of *Yersinia enterocolitica* in cheese and ice cream becomes our study's goal.

According to the results of the culture method that were recorded in Table 2, the incidence of *Yersinia spp*. in the examined samples of cheese was 20%, this result was nearly similar to the results recorded by **Rahimi *et al*. (2014),** who could isolate *Yersinia spp.* from 23.3% of the tested cheese samples which was the highest incidence among all recorded results of the examined milk products. On the other hand, **Hamama *et al.* (1992)** could isolate *Yersinia spp*. from 14.3% of the analyzed traditional fresh cheese samples (Jben).

As recorded in Table 2, the prevalence of *Y. enterocolilica* in the examined cheese samples was 17.5%, 7 out of the isolated *Yersinia spp.* Strains were identified biochemically as *Y. enterocolitica*, and one strain was *Y. frenderikenii* (2.5%). This incidence of *Y. enterocolitica* in cheese was lower than the obtained result by **Salah *et al.* (2012)**, who could detect the organism in 24.4% of the tested samples of cheese, but lower results were recorded by **Abd El-Aal and Atta (2009); El-Malt (2009); Hanafian and Khani ((2012); Ali *et al.* (2015)** who could isolate the organism from 8, 6, 10. 5 and 4% of the examined cheese samples, respectively.

Regarding the examined ice cream samples, the incidence of *Yersinia spp.* in this study was 30%, which was higher than the reported results by **Rahimi *et al.* (2014),** who could detect *Yersinia spp* in a percentage of 5.7%. Ten out of the isolated strains of *Yersinia spp*. from ice cream were identified as *Y. enterocolitica* with a percentage of 25%, while *Y. frenderikenii and Y. intermedia* were found in 2.5% of the tested ice cream samples for each, as in Table 2.

All the biochemically identified *Y. enterocolitica* strains were subjected to molecular detection of the 16S rRNA gene of *Y. enterocolitica*. The result illustrated in Table 3, revealed that 42.8 and 60.0% of the isolated strains from cheese and ice cream were confirmed as *Y. enterocolitica*, respectively. Hence, the incidence of *Y. enterocolitia* in cheese and ice cream based on PCR result was 7.5 and 15%, respectively, according to the data represented in Table 3 and figure 1. **Rahimi *et al.* (2014) ; Ali and Al-Samari (2020)** demonstrated lower results for the detection of *Y. enterocolitica* by using PCR than the culture method's recorded result, and this is in agreement with our study. Still, they recorded lower incidence with a percentage of 7.77 and 7% for culture method and 6.6 and 4.5% for PCR respectively, this difference between the reported results by cultural methods and PCR may be due to that biochemical tests are complex and give unreliable results **(Neubauer *et al.,* 2001; Vishnubhatla *et al.,* 2001)** and some of Y. enterocolitica biochemical activities are temperature-dependent above and below 30°C, and so misidentifications can be found **(Manafi and Holzhammer,1994).**

Concerning the recovery rate of *Y. enterocolitica* from ice cream, the recorded results by **Elsherbini *et al.* (1999**) were in agreement with our results, where they could isolate *Y. enterocolitica* in a percentage of 24.5%, while, **Warke *et al.* (2000); Yucel and Ulusoy (2006)** recorded a higher prevalence of *Y. enterocolitica* and the rate of isolation was 40.3 and 35.7%. On the contrary, a lower incidence was recorded by **Norma and Ana (2000); Khalifa *et al.* (2007); Ye *et al.* (2014); Rahimi *et al.* (2014)** where, they could isolate *Y. enterocolitica* in a percentage of 2.5, 10, 5 and 2.85 % respectively.

It is worthwhile to state that, isolation of *Y. enterocolitica* from cheese and ice cream in high percentage to some extent may be attributed to using unpasteurized milk and processing of these products under unsanitary conditions, where contamination of the products may occur from human handlers or the environment. Also, the storage of these products at low temperatures supports the growth of the organism.

The results of bio- typing and serotyping as given in Table 4 revealed that, 8 out of the confirmed *Y. enterocolitica* strains proved to be pathogenic since they were belonged to biotypes 2, 4 and 5 and one strain was bio- typed 1A. On the other hand, O: 3 was the most prevalent serotype in cheese (66.66%) and ice cream (50%). In addition, serotype O: 8 was found in a frequency of 33.3% in cheese, while, serotypes O: 8, O: 4 and O: 5 were found in a percentage of 16.66% of the isolated strains from ice cream.

The isolated pathogenic strains detected by bio- typing and serotyping and belonged to biotype 2. 4 and 5 were examined to detect *ail* and *yst* genes by PCR technique. As observed in Figures 2&3, all the tested strains exhibited *ail* gene, but the *yst* gene failed to be detected. Obviously, detection of the pathogenic strains based on bio-typing was in agreement with the PCR assay result, where all detected pathogenic strains by bio- typing were positive for the targeting chromosomal *ail* gene.

The instability of the virulence plasmid gene (pYV), which can be lost under alkaline pH **(Thoerner *et al.* 2003; Myers *et al.* 2006)**, promoted us to choose the chromosomal genes for detecting the virulent strains. PCR assay can detect the pathogenic strains rapidly and with a higher degree of accuracy with the culture method, but this assay is very expensive when applied to the samples directly. Although the PCR technique is highly sensitive and effective in the case of pure isolates, when applied to the samples directly, its sensitivity is reduced due to the complexity of the sample composition and the low count of the target organism. So, enrichment of the sample before PCR is applied to increase the count and help in the detection of the bacterial cells **(Lantz *et al.*, 1998 and Fredriksoon- Ahomaa and Korkeala, 2003).**

Cheese is one of the most popular foods, and it is a part of the stable diet to the majority of the consumer in the world. The largest proportion of Damietta cheese is manufactured from raw milk. Hence, the numerous opportunities for cheese contamination with pathogenic *Y. enterocolitica* strain during manufacture. In addition, refrigeration storage of cheese supports the growth of this pathogen due to its psychrotrophic nature. Therefore, the growth patterns of *Y. enterocolitica* in Damietta cheese during storage were important in our study to determine the optimum condition that may be applied to ensure microbial safety.

The recorded data in Table 5 and 6 represented the effect of refrigeration storage on the viability of *Y. enterocolitica* in cheese during refrigeration storage, the generation time (GT) dependence of temperature for multiplication of *Y. enterocolitica* at room temperature (30 ± 2ºC) to refrigeration (4 ± 2ºC) required about 24 hours for growth from 5.8 ± 75 log cfu/ g to 6.6 ± 0.4 log cfu/ g, which is lesser than required by *Y. enterocolitica* isolates to reach 6.5 ± 0.85 log cfu/ g and these results was in agreement with that recorded by (**Amin and Drowghon 1978)** who reported 16.8 h to 25.9 h (GT) of *Y. enterocolitica* in skim milk stored at 4ºC during refrigeration storage. Then, there was an increase in the organism's count on the 2nd week of the experiment in refrigeration and room temperature storage to 7.4 ± 0.63 log cfu /g and 8.2 ± 0.68 log cfu/g, respectively. Then there was a state of lower growth and multiplication of *Y. enterocolitica* in refrigerating cheese samples, which was in agreement with **Champagene *et al.* (2014)**, who stated that at low temperature, the growth curve has a long lag phase and slower logarithmic phase, while, **Stern *et al.* 1980** recorded a four days lag phase followed by linear *Y. enterocolitica* population growth in milk held at 3ºC for 20 days.

An increase in the growth and survival of *Y. enterocolitica* was shown on the 8th week and the mean value of its count was 9.9 ± 0.16 log cfu/ g. and 9.6 ± 0.41 log cfu/ g in refrigeration and at room temperature storage, respectively. Then, gradual decline in the microbial density of *Y. enterocolitica* from the 14th week to the end of the experiment on the 18th week in which the mean value of the organism stored in refrigerator and room temperature was 7.4 ± 0.5 log cfu/ g. and 6.9 ± 0.16 log cfu/g., respectively. The significant difference was recorded at the 3rd week between cheese storage at 30 ± 2ºC and 4 ± 2ºC (Fig.4).

The behavior of *Y. enterocolitica* recorded by **Abdollahi and Hanafian 2015** showed an intensively increase in the count during the first day. The growth was consistent to some extent from the 1st to 5th days. Its population was declined from the 5th to the 60th day. The decreasing percentage during storage at 25ºC is higher than that reported for storage at 8ºC, which was recorded as more favorable for the organism's growth. On the other hand, **Otero *et al.* (2010)** found no significant changes in the count of *Y. enterocolitica* during the first two weeks of cheese storage at 4ºC; then, the count starts to decrease not be detected on the 45th day. While at 22ºC, the bacterial population was somewhat stable until the 5th day, the count declined and could not be isolated on the 45th day. **Hanafian and Khani** (**2012**) recorded survival of *Y. enterocolitica* for a long time) could not be detected in the 4th month. **Chenyang *et al.* (2020**) attributed the prolonged survival of *Y. enterocolitica* at refrigeration storage at 4ºC to the induction of cold-shock genes, demonstrating its ability to adapt to cold stress.

The survival of *Y. enterocolitica* in our study both at 4 ±2ºC and at room temperature (30 ± 2ºC) revealed a potential health hazard for the consumers, especially in the 8th, 10th and 14th weeks where the count of *Y. enterocolitica* was higher than (10^9 cfu/ g.) the infective dose recorded by **Doyle (1990).** This may be attributed to the low percentage of salt (3%) used in cheese manufacture and no addition of probiotic bacteria during cheese manufacture in our research., So, an alternative method to decrease the population of *Y. enterocolitica* in cheese during storage other than the temperature factor may be used, where, the differences in the environmental factors of cheese in terms of salt percentage, pH, and lactic acid starter are the main factors affecting the viability of *Y. enterocolitica* **(Abdollahi and Hanafian, 2015).**

Preservation of food is used to prevent or retard microorganisms' growth to extend its shelf-life and prevent the risk of infection. Freezing is one of these methods that is used in food preservation, especially ice cream. Because ice cream may be contaminated during processing, handling, transportation, and storage, it is incriminated as a source of infection with a pathogenic bacteria that can multiply at low temperatures as *Y. enterocolitica*. So, we aimed to study the viability of *Y. enterocolitica* in ice cream during freezing storage at -6 ± 2°C and -18 ± 2ºC.

Comparing the behavioral patterns of *Y. enterocolitica* growth in ice cream during freezing storage at -6 ± 2 °C and -18 ± 2 ºC revealed that the generation time (GT) for *Y. enterocolitica* multiplication at 30 ºC after manufacturing to -6 ± 2°C storage required seven days to slight increase from a mean value of count 6.3 ± 0.26 log cfu/g. to 6.9 ± 0.16 log cfu/g. as recorded in Table 7. While GT. at 30°C to -18 ± 2 °C required only five days to reach a nearly similar count from 6.4 ± 0.43 to 7 ± 0.48 cfu/g (Table 8) and this reduction in replication during the first days of the experiment may be attributed to the presence of coliform bacteria inhibited the development of *Y. enterocolitica* in ice cream **(Slavchev, 1986).** As reported in Table 8, the highest increasing percentage was noted in the 4th week with a mean value of 8.2 ± 0.46 log cfu/ g. and from this time to the end of the experiment, there was a decrease in the count, where the mean value of the organism was declined to 5.5, 5.2 and 5 log cfu/g in the 16th week with a mean value of 5.3 ± 0.26 log cfu/g.

It is obvious from the previously noted data that the growth pattern of *Y.enterocolitica* was unevenly changed from increasing to decreasing, with active viability and survival of the organism in inoculated ice cream by *Y.enterocolitica* during freezing as recorded by **Slavchev, 1986**, who also stated that this survival recorded to be dependent on the count of the inoculated *Y. enterocolitica* to ice cream as inoculation of 100 cell/ cu cm remain viable up to the day 90 and the survival period and viability1000 cell/cu cm reach to the 8th month with showing a reduction in the count after the 6th month.

The data illustrated in Table 8 revealed a high increasing percentage from the 3rd day to the 2nd week. The reduction in the count of *Y. enterocolitica* started from the second week to the 8th week and still stable to the end of this treatment with a mean value of 5.6 ± 0.39 log cfu/g, which nearly agrees with the results recorded by **Norma and Ana (2000)** during studying the behavioral pattern of *Y. enterocolitica* at -18 ºC who recorded an increase in the bacterial density of the organism from time zero to the first month, then, the count return to decrease during the second month. The cell population remains stable to some extent for 16 months. Also, they attributed this to the low pH of ice cream to (4- 5), which decrease the viability of the organism, Since they failed to isolate the organism in the 5th month from ice cream with low pH, while **Abd El-Fatah *et al.* (2015)** reported low reduction rate in the count of *Y.enterocolitica* for two months during freezing storage at -20°C. Also, **Uzunlu *et al.* (2004)** recorded a similar decrease in *Y. enterocolitica* from 6.08 to 5.66 log cfu/g. During two months, when stored at -20°C with stable growth rate and low rate of decline in the organism viability.

The behavioral patterns of *Y. enterocolitica* during freezing storage of ice cream were unstable during the

first six weeks, but the significant differences between the effect of freezing -6± 2 ºC and -18± 2ºC was recorded in the 2nd week with P-value < 0.05 where the reported mean value of the bacterial density at -6 ± 2 ºC was lower than storage -18 ± 2 ºC by 1.94 log cfu/ gm and the rate of reduction in the number of viable cells at -18 ± 2ºC from the fourth week up to the end of the experiment was higher than that recorded at -6 ± 2ºC as represented in Fig. 2.

The viability of the organism in ice cream due to the existence of protein and fat, which have a protective effect on *Y. enterocolitica* **(Roora *et al*., 1992)** also the adjustment of fatty acids in the lipid bilayer membranes making shorter chain and involvement of unsaturated chains was the way to prevent fat solidification and breakage of the membrane of the microorganism. **(Jay 1996; Gounot 1991).**

CONCLUSION

It can be concluded that, the results of our study confirmed the probability of cheese and ice cream contamination with the pathogenic strains of *Y. enterocolitica* and this may be attributed to the unsanitary conditions during milking, processing and storage and using unpasteurized milk for manufacturing these products. On the other hand, its survival in cheese and ice cream for a long time during refrigeration and freezing temperature render it of special public health significance. Therefore, preventive measures must be applied during manufacture and during storage up to the consumption to decrease the chance of contamination. Also, studies and trials should be carried out in the future to affect its viability by adding different concentrations of salt and probiotics in cheese and decreasing the pH of ice cream.

Declaration of Competing interest

On behalf of all authors, I hereby declare that no conflict of interest may interfere with the publication of the manuscript.

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