Fate of enterohaemorrhagic Escherichia Coli O157:H7 in buffalo's milk and some of its manufacturing products

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Summary

The present study was conducted to trace the survivability of *E. coli* O157:H7 in raw and pasteurized buffaloes' milks, yoghurt and Damietta cheese. The results revealed that the organism populations in raw milk of 8.34-$\log_{10}$ cfu/ml initial inoculum stored at 22±2°C increased slightly through the first day, then decreased gradually from 9.5-$\log_{10}$ cfu/ml at the 2nd day to 2.35- $\log_{10}$ cfu/ml at the 10th day. It could survive at higher counts in the refrigerated raw milk achieving 5.74-$\log_{10}$ cfu/ml by the end of the same holding period. In the pasteurized milk, the tested organism undergone continuous regular decrease from 7.86-$\log_{10}$ cfu/ml at 0 time to 1.4-$\log_{10}$ cfu/ml by the end of the 30 days holding period. In yoghurt, the pathogen survived for 8 days at refrigerated

storage following its preparation. However, its populations reduced from 8.23-$\log_{10}$ cfu/ml at inoculation time to 1.8-$\log_{10}$ cfu/ml at the 8th day. The pH of the product was 4.12 and reached 4.06 through the corresponding period. Concerning the three manufactured cheese varieties (LS, MS and HS), the organism populations decreased sharply during the curd formation, started to increase during the draining period and through the first 4 days of storage at 22±2°C and returned to decrease again through the rest of storage term achieving 2.76-, 3.4 and 0.0-$\log_{10}$ cfu/ml in LS, MS and HS cheeses, respectively. For cheeses held at 4±1°C, the organism persisted at considerably higher counts achieving 7.11-, 4.08 and 4.15-$\log_{10}$ cfu/ml in the 3 cheese varieties, respectively. The inhibitory effect of the high salt percentage on EHEC was more pronounced than that arising from the lowered pH values in all the 3 cheeses. The public health significance of the tested pathogen as well as suggestions for its exposure avoidance via consumption of foods, particularly dairy products, were discussed.

Introduction

Since 1920s, *Escherichia coli (E. coli*) has been isolated from dairy products with variable incidences and counts. Some of its serotypes have been involved in outbreaks of human gastroenteritis (Aureli et al., 1992). As the organism is commonly present in gastrointestinal tract of animals and man, many types of foods including meat products, fish, milk and dairy products, vegetables, baked products, and water would be hazardous if exposed to direct or indirect contamination with faecal materials carrying it, along with improper storage temperature and inadequate heat treatment. As a result, these foods have been associated with gastroenteritis of *E. coli* origin in many countries (Kornacki and Marth, 1982 and Garvani, 1987). The term enteropathogenic *E. coli* has been used rather indiscriminately in the past for strains of *E. coli* which cause infantile diarrhea by different mechanisms (Moon et al., 1979). More recently, it was shown that diarrheogenic *E. coli* strains are now classified on the basis of clinical symptoms, mechanisms of pathogenesis and in some instances, biochemical and serological markers into five categories: enterotoxigenic (ETEC), enteroinvasive EIEC), enterohaemorrhagic (EHEC), enteropathogenic (EPEC) and enteroaggregative-diffuse adherent (EAgg-DAEC) (Donnenberg and Karper, 1992; Gomez et al., 1989 and Giron et al., 1991).

EHEC (O157:H7) has received recently a considerable attention, as it was implicated in several outbreaks of gastroenteritis, with several cases developing haemolytic ureamic syndrome (HUS), haemorrhagic colitis (HC) and thrombotic thrombocytopenic purpura (TTP). Raw milk (Martin et al., 1986), yoghurt (Morgan et al., 1993) and acidic foods such as mayonnaise (Keene et al., 1994) were among the food vehicles incriminated in the EHEC outbreaks. The organism can affect all ages, requires a low inocula (50 viable cells) and can cause death (Neil, 1994). The virulence factor of EHEC is the production of shiga-like toxins (SLTs) or verotoxins (VTs) which have two types, the SLT-I and SLT-II (Konowalchuck et al., 1977 and 1978a & 1978b). The organism and its verotoxins are destroyed by adequate cooking or pasteurization (Verman and Evans, 1991).

In the last decade, efforts were conducted by several investigators on the detection of EHEC in dairy products. In USA, Padhye and Doyle (1991) and Chapman and Wright (1993) similarly isolated EHEC O157: H7 from 10% of the examined raw milk samples. In Germany, Montag (1994); Bockemuehl and Karch (1996) and Perlberg (1996) found that 2.63%, 4% and 3.95% of the examined raw milk samples, respectively, contained the pathogen. On the other hand, Knappstein et al. (1996) detected *E. coli* O157: H- in 9.2% and 59% of the examined heated and raw milk

cheeses, respectively. Abdel-Hakiem et al. (1998) isolated the pathogen of SLT-I and SLT-II producer from a yoghurt sample representing 0.81% of the examined dairy products. Literature on the behaviour of EHEC (O157:H7) in dairy products are nearly still lacking. However, Sharpe et al. (1995) found the organism survived well in refrigerated raw milk and dairy products, as well as low pH products. Reitsma and Henning (1996) reported that the pathogen survived in Cheddar cheese along the manufacuring course and remained viable for 102 days thereafter.

In Egypt, in some instances, milk produced by individual owners in small farms that lack of proper sanitation. In addition, some dairy products as soft cheeses, cream and butter are manufactured under local conditions from raw milk without addition of lactic acid starters or colourant. Such products, if consumed fresh or after being preserved in refrigerated conditions, represent a major source of foodborne illness with any of the virulent *E. coli* serotypes. Therefore, the present investigation is conducted to trace the pathway and fate of EHEC (O157:H7) when being inoculated in raw buffalo's milk, laboratory pasteurized milk, and the milk used in manufacturing of yoghurt and Damietta cheese.

Material and Methods

Preparation of EHEC culture

EHEC (O157:H7) strain of SLT-I and SLT-II producer was obtained from Dr. Aman, I. M, Faculty of Veterinary Medicine, Tanta University to be used in this investigation. The strain was subcultured overnight in trypticase soya broth at 30°C, centrifuged at 1200 rpm for 10 min at 4°C and the cells were washed with sterile saline 2-3 times with centrifugation. Finally, the gathered cells were suspended in sterile saline to be used for inoculation.

Viability of EHEC in raw milk

Two liters of freshly drawn buffaloes' milk were obtained from the dairy farm of the Faculty of Veterinary Medicine, Suez Canal University. Before being inoculated, the milk was analyzed for naturally occurring *E. coli* by streaking 0.1 ml from the prepared tenth fold dilutions over sorbitol MacConkey agar (SMA) plates as described in A.P.H.A. (1992). The milk was then inoculated with the prepared cultures of the chosen organisms to obtain an initial inocula of ca 10^8 cells/ml. The inoculated milk was distributed aseptically after thorough mixing into two sterile stoppered-bottles. The first was placed in refrigerator (4±1°C), while the second was left at room temperature (22±2°C). Then, they were examined at 0 time and daily thereafter for EHEC counts, total bacterial counts (background microflora) and pH values.

Viability of EHEC in pasteurized milk

The laboratory pasteurized buffaloes' milk (63°C for 30 min followed by immediate cooling to <10°C within 5 min) was inoculated with the prepared cultures of the chosen organisms to provide an initial inocula of ca 10^8 cells/ml. The inoculated milk was held in refrigerator (4±1°C) and examined for EHEC counts, total bacterial counts (background microflora) and pH values during the 30 days storage period. Examination was performed daily during the first 5 days, every other day until the 15^{th} day and every third day until the 24^{th} day.

Viability of EHEC in yoghurt

Yoghurt was prepared according to Lampert (1975) from previously heated (90°C) buffaloes' milk. After being cooled to 42°C, the milk was inoculated at a rate of ca 10^8 cells/ml with the prepared cultures of EHEC. Then the starter culture (fresh commercial product made from pasteurized milk having a minimum of 15 days shelf-life) was added at a rate of ca 0.2%. The inoculated milk was distributed in sterile, stoppered glass bottles (150 ml capacity) and incubated at 42°C until the formation of the desired curd. The prepared yoghurt was store in the refrigerator (4±1°C). Samples were taken before the addition of the starter and EHEC cultures, after their addition, after curd formation, daily until the 9^{th} day and every other day until the end of the 15 days investigation period to be examined for EHEC counts and pH values.

Viability of EHEC in Damietta cheese

Three lots of Damietta cheese were prepared from partially raw and heated (90°C) buffaloes' milk according to the traditional method of making such type of cheese in Egypt; the procedure described by Fahmi and Sharara (1950). The milks were inoculated with the test organism at a rate of ca 10^8 cfu/ml. Sodium chloride was added at concentrations of 1% (light salted "LS"), 3% (medium salted "MS") and 5% (high salted "HS"). Rennet extract was added according to the manufacturer's direction at a rate of 0.2%, and the formed curd was left to drain its whey for 24 h at room temperature. Each lot of prepared cheese, with its whey, was divided into two equal portions, one of which was left at room temperature (22±2°C) while the other was kept in refrigerator (4±1°C). Samples to be examined for EHEC and pH values were taken before inoculating the test organism, immediately after inocultion, after setting and curd formation, after draining the whey and periodically through the storage period of every type of the prepared cheeses.

Enumeration of EHEC and total bacterial count (TBC)

A quantities of 0.1 ml of tenth-fold serial dilutions of milk and milk products were streaked on sorbitol MacConkey agar (SMA) and incubated at 32±1°C for 24 hours. When the counting procedure fail to find the characteristic colonies of EHEC from the dilutions as well as from the original sample, isolation trials using trypticase soya broth (TSB) overnight cultures were performed (A.P.H.A., 1992).

Total bacterial counts (TBC) of raw and pasteurized milk were determined according to A.P.H.A. (1992).

Measurement of pH

The pH of the tested samples was measured using Jenway 3051 pH meter supplied with standard combination glass electrode. The apparatus was calibrated before each measure using standard buffer solutions pH 4.00 and pH 7.00 at 25°C.

Results and Discussion

Viability of EHEC in raw milk

Populations of the EHEC (O157:H7) test strain in the raw buffaloes' milk held at room temperature (22±2°C) and in refrigerator (4±1°C) are shown in Fig. 1. For milk left at room temperature (Fig. 1/A), it is very obvious that, EHEC O157:H7 populations remained unchanged during the first 5 days of incubation although the milk is curdled at the second day (pH 4.55). At the end of the holding period (10 days), the counts showed ca 5-$\log_{10}$ decrease. Such decrease can be assumed to be due to both competitive microbial growth, evidenced by the very high counts of background microflora achieving ca 14-$\log_{10}$ cfu/ml at the end of the 8th day, and the very low pH; reached 3.96 by the end of the holding period.

Concerning the refrigerated milk, it is easy to declare from Fig. 1/B that there was a somewhat regular slow reduction in the numbers of the inoculated organism along the term of incubation. However, the decline rate was greater during the first day, achieving ca 1.8-$\log_{10}$. Such decrease in EHEC populations was accompanied by a corresponding regular increase in the background bacteria from 9.9-$\log_{10}$ cfu/ml at 0 time to 12.5-$\log_{10}$ cfu/ml by the end of the holding period. These findings lie in a very close agreement with those reported by Sharpe et al. (1995) and Heuvelink et al. (1998) upon testing the survivability of *E. coli* O157:H7 in raw cow's milk. Unfortunately, from such obtained results one can expect that raw milk, whatever its type, may play a significant role in disseminating *E. coli* O157:H7 among consumers. This is particularly true within the circumference of underdeveloped countries where the tradition of consuming milk at its raw state still common.

Viability of EHEC in pasteurized milk

Populations of the *E. coli* O157:H7 test strain in the inoculated pasteurized buffaloes' milk held in refrigerator (4±1°C) are shown in Fig. 2. It is very clear from the data assembled in the figure that, *E. coli* O157:H7 populations survived for the whole test period (30 days). They showed gradual regular decrease from the beginning (7.82-$\log_{10}$ cfu/ml) until the end of the holding period (1.4-$\log_{10}$ cfu/ml). The pH of the milk did not altered significantly through whole storage term as it was 6.6 at 0 h and achieved 6.28 at the 30th day. The background microflora increased at first achieving 9.32-$\log_{10}$ cfu/ml through the 3rd day after which they undergone a marked decrease along the rest of the investigation period. This decline in both *E. Coli* O157:H7 and total bacterial counts is, of course, due to the unfavourable low temperature of incubation. At such temperature the majority of microorganisms loss their ability to grow and only what is called "psychrotrophs" can survive. Indeed, in order to produce pasteurized milk free from the pathogen in concern, properly pasteurized product protected from any sort of post pasteurization contamination is an essential requirement.

Viability of EHEC during preparation and storage of yoghurt

Careful inspection of the results shown in Fig. 3 reveals that *E. coli* O157:H7 populations have undergone considerable reduction (ca 2 $\log_{10}$) by the end of yoghurt preparation. Such reduction occurred during the 3 hours incubation at 42°C. Simultaneously, the pH of the product has decreased sharply from 6.16 upon adding the starter culture to 4.12 by the end of curd formation, explaining why did such decline in EHEC counts has taken place. There was continuous regular decrease in the numbers of the pathogen from day to day for upto the 9th day, after which 3 successive, every other day, isolation trials have failed to find the organism. This was certainly because it can no longer persist in the product. The pH of the yoghurt continued to de-

crease during its refrigerated storage, but to a much lesser extent than before. By the end of the investigation period, the pH of yoghurt has reached 3.96. These findings support what has been reported by Hudson et al. (1997), Abdel-Ghany and Hosny (1998) and El-Hawary and Aman (1998). A faster disappearance of *E. coli* O157:H7, occurring just after the curd formation of yoghurt, was recorded by Dineen et al. (1998). Such difference could be attributed to the smaller initial inoculum (10^5 cfu/ml milk) added upon processing the yoghurt and/or variability in the virulence among the tested strains. However, the same authors established that *E. coli* O157:H7 was recovered at <10 cfu/g at 12 days from commercial yoghurt (pH 4.0) inoculated by the same organism at a rate of 10^3 cfu/g. So, they concluded the presence of *E. coli* O157:H7 cells in yoghurt is more likely to reflect post processing contamination than survival of the organism through the yoghurt fermentation process. Indeed, the obtained findings suggest that both ways can lead to its presence in yoghurt during the few days of its refrigerated storage following processing. The persistence of the pathogen in yoghurt at low pH for such period confirmed the implication of yoghurt in an outbreak of HC in London as reported by Morgan et al. (1993).

Viability of EHEC during manufacturing and storage of Damiette cheese

With regard to the cheeses stored at room temperature assembled in Fig. 4/A, one can recognize that *E. coli* O157:H7 is considerably inhibited as the salt content of the cheese increased. In the LS cheese, populations of the inoculated pathogen remained unchanged for as long as 28 days storage. The organism appeared as if unaffected by the lowered pH (3.59) of the cheese at that time. Populations of *E. coli* O157:H7 showed a marked decrease during the rest of the storage term from 7.08-$\log_{10}$ cfu/g at the 28th day to 2.76-$\log_{10}$ cfu/g at the 70th day. In MS cheese, the organism behaved more or less similarly as in LS one. Concerning HS cheese, the effect of the high salt content on the test organism was very pronounced from the first moment. It was reflected by a ca 3.5-$\log_{10}$ cfu/g decrease in comparison with the former ones (LS & MS) during course of manufacturing and storage. The test organism could not survive until the end of its storage term. Actually, we have to point out here that there were a marked fungal growth (unpresented data) in all the 3 types during the last stages of storage (40th -70th day). Perhaps, this phenomenon (competitive growth) was the reason of the higher survivability of *E. coli* O157:H7 in LS and MS cheeses than in HS one, because it was more pronounced in the former than in the later two cheeses.

Concerning the refrigerated cheeses (Fig. 1/B), the organism populations in the LS cheese showed a regular gradual slight increase during the first 4 weeks after which they started to decrease. There were 8.5-$\log_{10}$ cfu/g at the beginning of the refrigerated storage, 9.7- $\log_{10}$ cfu/g at the 28th day and reached to 7.1- $\log_{10}$ cfu/g by the end of the storage term. In correspondance, the pH has undergone a steady gradual decrease from ca 5.5 at the first day to ca 4.5 by the end of the storage term. A somewhat lower EHEC counts and higher pH values were registered through the whole refrigerated storage term of the MS type of cheese. Therefore, the inhibitory effect of the higher salt concentration upon EHEC in the MS cheese overcomed that of the lower pH values in the LS one. In the HS cheese, EHEC populations undergone a significant decrease through the manufacturing course, but remained a more or less unchanged during the refrigerated storage term. In comparison with the other 2 cheese types, HS cheese appeared to be the most inhibitory one to the tested strain, although its pH was relatively higher. In general, such obtained results revealed that the higher the salt content in the cheese, the higher the degree of inhibition of EHEC regardless of the product pH. These findings to have some degrees of similarity with

those reported by Reitsma and Henning (1996) and Ramsaran et al. (1998) upon their tested cheese varieties. However, Glass et al. (1998) recorded a comparably lower *E. coli* O157:H7 in the inoculated processed cheese slices. The obtained results also revealed that the storage temperature had a marked effect upon the survival rate of the tested strain. If the temperature is high, it favours the growth of lactic acid producing bacteria resulting in high death rate of the organism.

In conclusion, these findings gave us a clear idea about the high possibility with which dairy products contaminated by such powerful pathogen be hazardous to consumers due to its longevity therein. The most important factor in the prevention of gastroenteritis in human by pathogenic *E. coli* is to prevent contamination of food and water, directly or indirectly, by faecal matters. This can be achieved by developing effective sanitation in water supplies, and treatment and disposal of sewage. Another factor to be considered is the prevention of contamination of foods due to poor personal hygiene by people who are shedding the pathogen. Here, in Egypt, we are in great need to stop the manufacturing of dairy products from raw milk or inadequately heat treated one. Finally, we have to recognize that if the pathogen is present in very small initial numbers in a food, temperature abuse can facilitate multiplication of cells to high levels necessary for disease symptoms. Thus foods, including, of course, milk and various types of dairy products should be refrigerated or eaten quickly (Kornacki and Marth, 1982 and Garvani, 1987).

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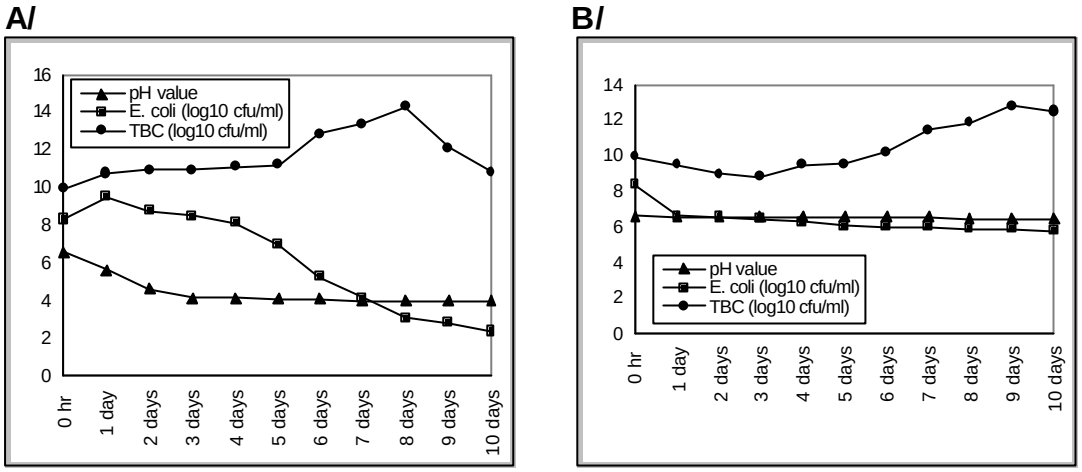

Fig.1 Survival period of EHEC in raw buffalo's milk held at:
A/ 22±2°C (room temperature)---B/ 4±1°C (refrigerator)

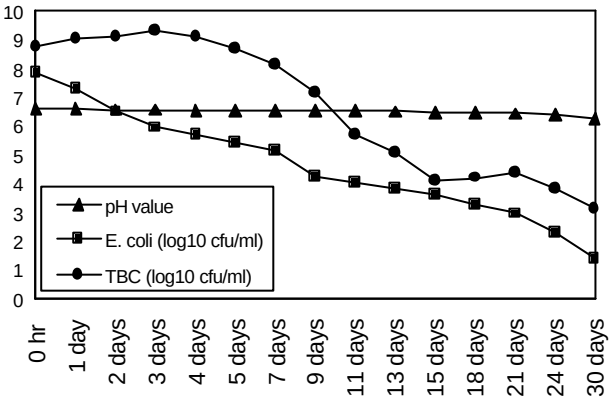

Fig.2 Survival period of EHEC in pasteurized milk held at (4±1°C).

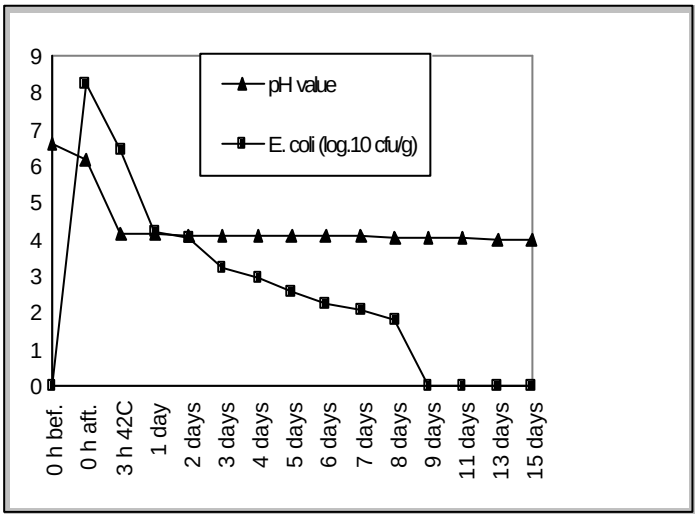

Fig.3 Survival period of EHEC during preparation and storage of yoghurt
0 h bef.= before inoculation of EHEC and starter
0 h aft.= after inoculation of EHEC and starter
3 h 42C= after 3 hours incubation at 42°C

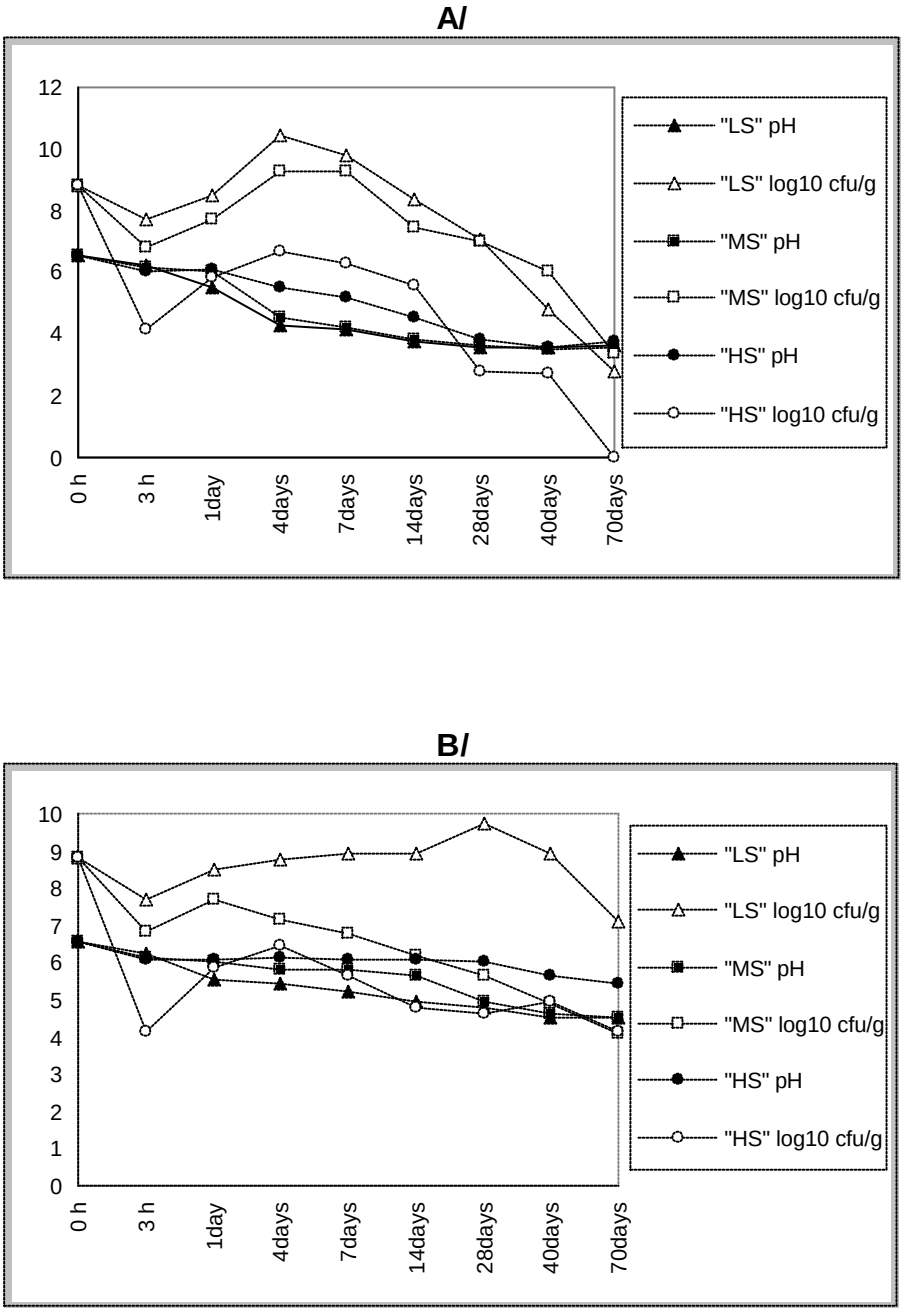

Fig.4 Survival period of EHEC during preparation and storage of Damietta cheese at: A/ 22±2°C (room temperature)---B/ 4±1°C (refrigerator)

0 h = from the milk just after adding the prepared culture

3 h = from the formed curd 3 hours later at 32°C

1 day = from the prepared cheese after draining the whey 24 hours later at room temperature.