**Survival of Shiga-toxin- Producing *Escherichia coli* (STEC) during storage of Karish cheese**

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 ABSTRACT

 Non O157:H7Shiga-toxin- Producing *Escherichia coli* (STEC)is a major foodborne pathogen that causes severe disease in humans ranged from mild gastroenteritis to critical illness and death. This study investigated survival of (STEC)during storage of experimentally manufactured kareish cheese. Cheese were prepared using pasteurized milk inoculated with 2 STEC strains  (O26& O111) at (9 log10 CFU/g) with and without yogurt starter culture (*Lactobacillus. bulgaricus and Streptococcus*. *thermophilus*) & 3% NaCl, then expulsion of whey and stored at refrigerator. The obtained results revealed that mean STEC counts (log10 CFU/g) were 6.46±5.66 , 6.06 ± 5.75 at zero time then reached 8.01±7.69, 8.07 ± 7.76 ( their maximum count) on 12 th day of storage, finally decreased to 4.63±4.55, 4.34 ± 4.08(at 21th day) in (O26&O111) treated groups with starter culture. Concerning (O26&O111) untreated groups, the mean STEC counts were 6.67±5.32, 6.50±5.98 at zero time then reached to 9.08±8.65, 9.11±8.90 on 12 th day, finally decreased to5.53±4.85, 5.61±4.82 at 15 th day of storage at refrigerator. Effect of starter culture on survival of STEC were highly significant (P< 0.05).In conclusion presence of starter culture in manufactured Karish cheese significantly enhanced reduction of Non O157:H7 STEC counts over its storage time.

**Key Words: Kariesh cheese; STEC; Lactic acid bacteria; NaCl.**

1. **INTRODUCTION**

Kariesh is the most popular soft cheese in Egyptian cities and Arabian countries, similar to Domiati **(Brown , 2004; Abd-Ehamid , 2012; Hegazy etal., 2012).** It is an acid coagulated fresh cheese, made from skimmed buffalo's or cow's milk or a mixture of both with soft composition, white curd and slightly salty **(Francois et al., 2004; Abou-Donia , 2008 )**, but mostly produced from skimmed buffaloes' milk and its manufacturing depends on acid coagulation by the action of rennet & lactic acid bacteria **(Blassy et al.,2003).**

Lactic acid bacteria (LAB) (Lactobacilli, Streptococci, Lactococci, and Bifidobacteria) are the primary commercially available microbes **(Ranadheera et al., 2010).** They produce various metabolites, such as organic acids, hydrogen peroxide, diacetyl, antifungal compounds and phenyllactic acid, which protect the products from spoilage-causing microorganisms and pathogens **(Ryan et al., 2011; Ogueke et al., 2014; Anjum et al., 2014)**. Among them *Streptococcus thermophilus and Lactobacillus bulgaricus* are important starter microorganisms required for the manufacture of fermented dairy foods such as yogurt and certain cheese varieties **(Heller, 2001; Lourens-Hattingh, & Viljoen, (2001)**.

Sodium chloride is an important ingredient for cheese manufacture which exerts a major influence on its composition, microflora, ripening, texture, flavor and quality **(Salem and Abeid,1997).**

Shiga toxin–producing *E. coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC) or enterohaemorrhagic *E. coli* (EHEC), have been known as a group of highly pathogenic *E.coli* strains producing one or more Shiga toxins **(Monaghan *et al*., 2011).**

STEC represent a hazardous public health problem worldwide causing various human gastrointestinal tract diseases, including watery or bloody diarrhea and might develop a life-threatening diseases, such as haemorrhagic colitis (HC), Thrombotic Thrombocytopenic Purpura (TTP) and Haemolytic Uraemic Syndrome (HUS) **( Pennington, 2010).**

STEC strains produce two powerful phage-encoded cytotoxins causing tissue damage in humans and animals, called Shiga toxins or verotoxins (Stx1/VT1 and Stx2/VT2), which are the common feature and main virulence factors of STEC and are directly correlated with human pathogenicity **(Lindgren *et al*., 1993)**. Stx2 is the most powerful toxin, and the toxin producing strains are usually associated with more severe infections **(Muniesa *et al*., 2004 and Gyles, 2007).**

STEC serotypes including O157:H7 STEC and non-O157. The O157:H7 STEC strain is considered one of the most important of all known food-borne pathogens because of the severity of associated illnesses produced by it and also, the apparent low infective dose ( of less than 10 cells) **(Bach *et al*., 2002, Blanco *et al*., 2003).** The most common, non-O157 strains associated with disease in humans including O26, O91, O103, O111, O128, O113 and O145 are responsible for up to 20 to 50% of all STEC infections **(Johnson *et al*., 2006)**.

There is a lack of information concerning the stress responses of many non‐O157:H7 STEC causing human illness under stress conditions as most of these studies only focused on O157:H7 **(Allen *et al*.**[**2008**](https://onlinelibrary.wiley.com/doi/full/10.1111/lam.12023#lam12023-bib-0001)**; Dong and Schellhorn**[**2009**](https://onlinelibrary.wiley.com/doi/full/10.1111/lam.12023#lam12023-bib-0010)**; Vanaja *et al*.**[**2010**](https://onlinelibrary.wiley.com/doi/full/10.1111/lam.12023#lam12023-bib-0038)**).**

This study was under taken to investigate the effect of starter culture on Survival of non‐O157:H7 Shiga-toxin- Producing *Escherichia coli* (STEC) during storage of Karish cheese under refrigeration temperature.

**2. MATERIALS AND METHOD**

**2.1. Bacterial Cultures**

Lyophilized bacterial cells of *Lactobacillus.bulgaricus(LB) and Streptococcus. Thermophiles (ST)* (1:1) were obtained from Cairo-MIRCEN, (Microbiological Resource Center), Faculty of Agriculture, Ain- Shams University, Cairo, Egypt. Bacterial cultures were cultured in sterile MRS broth (de Man, Rogosa and Sharp) obtained from Biolife, Italy and were incubated anaerobically at 37 °C for 72 h in anaerobic jars and were transferred three times successively for their activation. Then the strain was sub cultured into sterile 11% reconstituted skimmed milk powder and were anaerobically incubated at 40 °C for 24 h before being used as starter culture( **Donkor *et al*., 2007).**

**2.2. Preparation of inocula**

Two different STEC strains were used in the present study O26: H11 andO111:H2 , For each inoculum preparation, STEC strains were grown individually in 10 ml tryptone soya broth (Oxoid) at 37°C for 18 h. Cells were sedimented at 3000 g for 20 min and then suspended in 10 ml of 0·1 mol /L phosphate‐buffered saline (PBS, pH 7·2). The optical density at spectrophotometer reading of 600 nm of the cell suspension was determined and adjusted to 0·5 (ca. 109CFU ml-1) using 0·1 mol /L PBS, pH 7·2. Inoculum concentration was determined by serial dilution in 0·1 mol /L PBS and spread plating the appropriate dilutions on tryptic soy agar (TSA, Difco), with incubation at 37°C for 24 h. **(Harrigan and MacCance,1976).**

**2.3. Manufacture of Karish cheese:**

Kariesh cheese was prepared as described by **Effat *et al*., (2001).** Buffalo’s milk was obtained from the herd of the faculty of Agriculture, Minoufiya University. Milk fat was separated using cream separator to obtain skimmed milk. Buffalo’s skimmed milk was pasteurized at 85°C for 30 min. and cooled to 30-38°c. Calcium chloride were added by (0.02%), Sodium chloride were added by (3 %) and mixed well and rennet powder was added at rate of (3g /100kg) milk. Then, the bulk volume of milk was divided into 2equal portions as follows: - The first portion was kept untreated (with activated starter culture) and sub divided into 3groups:

Group1 (G1): (without starter culture) served as control.

Group 2 (G2): Inoculated with (9 log 10CFU/ ml) of O26.

Group 3 (G3): Inoculated with (9 log 10CFU/ ml) of O111.

The second portion of milk (treated with activated starter culture which were added in ratio (2% w/w) ) and sub divided into 3 groups:

Group 4 (G4): 2%SC served as control.

Group 5 (G5): 2%SC + 9 log 10CFU/ ml of O26 inoculum.

Group 6 (G6): 2%SC + 9 log 10CFU/ ml of O111 inoculum.

 The prepared cheese mixtures were incubated at 40°C for 30-40 minutes till complete curd formation, drainage of whey then packaged in sterile cups(100 ml) and stored at refrigerator temperature. The cheese samples were examined microbiologically at zero time, 3,6,9,12,…….till signs of spoilage was detected. The experiment was repeated 3 times and the average results for each treatment was recorded.

**2.3.1. Microbiological examination of Cheese**

**2.3.2. Preparation of samples (BSI, 1984)**

Cheese samples were thoroughly homogenized with sodium citrate (2%) in mortar to obtain homogenous mixture.

**2.3.3. Preparation of serial dilution (APHA, 2004)**

Eleven gms of soft cheese were homogenized with 99 ml of sterile distilled water . One ml from cheese homogenate transferred to a separate sterile test tube containing 9ml of sterile distilled water, and then tenfold serial dilutions were prepared.

**2.3.4. Determination of STEC (shiga-toxin-producing Escherichia coli count ) (FDA, 2012).**

One ml of previously prepared serial dilution was taken and added into the centers of sterile petri dish, then sterile Eosin Methelyine Blue (EMB) was poured into each plate. Inoculated plates were mixed well and incubated at 37 °C for 24-48 hrs.

**2.3.5. Statistical analysis**

Statistical analyses were performed using the analysis of Difference in log cell numbers was analyzed for significance. Statistical comparisons were made by using one-way analysis of variance (ANOVA). The results were considered significantly different with p < 0.05 as described by **Clarke and Kempson (1997)**.

**3. RESULTS**

**Table (1) Survival of STEC serotypes (O26: H11) (log10 CFU/g) in different manufactured** **Karish cheese groups during** **refrigerated storage**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Storage days | G1 | G2 | G4 | G5 |
| Zero | ND | 6.67±5.32a | ND | 6.46±5.66b |
| 3 | ND | 6.56±6.20ab | ND | 6.28±6.05bc |
| 6 | ND | 7.73±6.84a | ND | 7.62±6.89ab |
| 9 | ND | 8.35±7.88a | ND | 7.76±6.84ab |
| 12 | ND | 9.08±8.65a | ND | 8.01±7.69b |
| 15 | S | 5.53±4.85ab | ND | 5.35±4.64bc |
| 18 |  | S | S | 5.07±4.05b |
| 21 |  |  |  | 4.63±4.55ab |
| 24 |  |  |  | S |

(G1): served as (control) ; (G2): 9log 10CFU/ ml of O26; (G4): 2% SC served as( control) ; (G5): 2% SC + 9log 10CFU/ ml of O26.

\***S**: Spoiled samples**.**

**\*ND:** not detected

\*values indicated are the mean ± S.E (n=3).

\*Values in the same column denoted by different letters (abc) differ significantly (p < 0.05) from each other. **)**

**Table (2): Survival of STEC serotypes (O111: H2) (log10 CFU/g) in different manufactured Kareish cheese groups during refrigerated storage**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Storage days | G1 | G3 | G4 | G6 |
| Zero | ND | 6.50±5.98ab | ND | 6.06 ± 5.75c |
| 3 | ND | 6.84±5.74a | ND | 6.62 ± 5.84ab |
| 6 | ND | 7.78±6.98a | ND | 7.57 ± 6.84b |
| 9 | ND | 8.39±7.83a | ND | 7.77 ± 6.89ab |
| 12 | ND | 9.11±8.90a | ND | 8.07 ± 7.76b |
| 15 | S | 5.61±4.82a | ND | 5.48 ± 4.95b |
| 18 |  | S | S | 5.19 ± 4.45ab |
| 21 |  |  |  | 4.34 ± 4.08b |
| 24 |  |  |  | S |

(G1): served as (control); (G3): 9log 10CFU/ ml of O111; (G4): 2% SC served as( control); (G6): 2% SC + 9log 10CFU/ ml of O111.

\***S**: Spoiled samples**.**

**\*ND:** not detected

\*values indicated are the mean ± S.E (n=3).

\*Values in the same column denoted by different letters (abc) differ significantly (p < 0.05) from each other. **)**

**4. DISCUSSION**

Although milk pasteurization removes almost all pathogenic *E. coli*, inactivation of *E. coli* shiga toxins has not been proven. However, following consumption of pasteurized milk, an outbreak in North Cumbria, England, during 1999 with HUS cases have been reported and no living bacteria were found in the milk samples **(Goh *et al*., 2002).**

Epidemiological studies suggest that non-O157 Shiga toxin-producing *E.coli* (STEC) is a major player associated with foodborne disease outbreaks **(Johnson *et al*., 2006).**

The ten most clinically relevant STECs belong to serogroups O26, O103, O111, O145,O157, O91, O113, O128, O45, and O121 have been reported in milk and dairy products causing severe illness in humans including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome and may result in death **(Farrokh *et al*.,2003)**.

Kareish cheese serve as an ideal medium for STEC bacterial growth due to synergistic effect of different factors exist in cheese as (low pH, water activity, NaCl and starter culture) **(Galvez et al., 2007)** .

It is evident from table (1&2) that STEC strains (O26,O111) were absent from all examined Kareish cheese in both( G1): (control) &( G4): 2%SC (control) throughout the entire period. This is may be due to effective pasteurization of milk to produce high quality cheese to protect consumers health **(Abdalla *et al*., 2006).** Our results agreed to those established by EOSQ (2005) for cold stored soft cheese, that it must be free from *Enteropathogenic E. coli*.

The mean values of (O26) counts (log10 CFU/g ± S.E)at Zero time were 6.67±5.32 and 6.46±5.66 for G2( Inoculated with 1ml of O26) & G5(2%SC + 1 ml of O26 inoculum) respectively. On storage, STEC counts sharply increased in their numbers and reached their maximum at the 12th day of refrigerated storage 9.08±8.65, 8.01±7.69 Cfu/g. Finally, the STEC counts decreased gradually until the end of shelf life time of examined kareish cheese samples it decreased to reach 5.53±4.85, 4.63±4.55 (at15 th, 21 th day) for G2 & G5 respectively(Table 1).

The obtained results agreed to those recorded by **(Schlesser *et al*., 2006; Montet *et al*., 2009; Bellio *et al*., 2018)** whom stated that STEC counts increased by (0.5-2; 1-2.2 and 2.0 log10 CFU/g) for Cheddar, Camembert- type cheese& Fontina Protected Designation of Origin cheese respectively.

An increase in the levels of STEC was observed in G2( Inoculated with 1ml of O26) & G5(2%SC + 1 ml of O26 inoculum) either due to bacterial growth of STEC, or to concentration of bacteria during curd formation, which generally increases the STEC concentration by 5-10 fold in soft cheese such as kareish, feta and telemes **(Maher *et al*.,2001; Govaris *et al*., 2002)**. Also, the rate of STEC growth is higher in G2 than G5 depending on the cheese-making scheme (addition of 2% SC) **(Farrokh *et al*., 2013).**

The extended shelf- life of low salt (3%) soft cheese with 2% starter culture up to 18th , 24 th &24 th day of refrigerated storage in G4(served as control) ,G5(1 ml of O26 inoculum) & G6 (1 ml of O111 inoculum) respectively Table (1&2) . This result may be attributed to the suppressive effect of several antimicrobial metabolites such as; organic acids, hydrogen peroxide, diacetyl, antifungal compounds and phenyllactic acid, produce by the added starter LAB which protect the products from spoilage-causing microorganisms **(Anjum *et al*., 2014)**. Also, presence of starter LAB during manufacturing of Kareish cheese lead to decrease in the pH of cheese by action of acids that act antagonistically against STEC strains **( Dineen *et al*., 1998 ; Mahaut *et al*.,2000).**

Survival of STEC strains can be enhanced by cross-protection when subjected to combination of many factors such as; acid, salt concentration, heat and presence or absence of SC **(Rowe and Kirk, 1999 ; Abu-Ghazaleh.2012)**.

After 12 days of refrigerated storage, the behavior of non O157 STEC strains can be affected by the microbial hurdles found in cheese (Milk micro flora as well as Lactic acid bacteria) .Generally there is a decline in the numbers of STEC in G2 ( Inoculated with 9 log 10CFU/ ml of O26), G3( Inoculated with 9 log 10CFU/ ml of O111), G5( 2%SC + 9 log 10CFU/ ml of O26 inoculum) &G6 ( 2%SC + 9 log 10CFU/ ml O111 inoculum) till signs of spoilage at 18 th, 18 th, 24 th&24 th day of refrigerated storage in G2, G3, G5& G6 respectively, Table (1&2). Our results agreed to those established by **Govaris *et al*., 2002 and Hudson *et al*., 1997** whom mentioned that; in Feta cheese, *E.coli* O157: H7 was not detectable after 44 days of refrigerated storage , also they observed a 3-log cycle reduction in viable counts of *E.coli* O157: H7 after 27, 30, and 27 days for Colby, Romano, and Feta cheeses respectively.

Although, **Ramsaran *et al*. (1998)** observed that, with in an inoculation level of 4log10 CFU/ml , the pathogen population in cheese was more than 6 log10 CFU/g after 75 days of refrigerated storage.

**Montet *et al*., 2009** found that non- O157 STEC strains survived in Camembert-type cheese during storage for up to 20 days.

The results recorded in Table (2) revealed that the initial mean values of (O111) counts (log10 CFU/g ± S.E)at Zero time were 6.50±5.98, 6.06 ± 5.75,On storage, the counts were sharply increased in their numbers and reached 9.11±8.90, 8.07 ± 7.76 at 12 days of storage, finally it decreased till appearance of spoilage signs to reach 5.61±4.82, 4.34 ± 4.08 at 15, 21days for G3 (Inoculated with 9 log 10CFU/ ml of O111)& G6(2%SC + 9 log 10CFU/ ml of O111) respectively. The obtained results were nearly similar to those recorded by **(Mohammadi *et al*.,** [**2009**](https://onlinelibrary.wiley.com/doi/full/10.1111/1750-3841.12547#jfds12547-bib-0023)**)** whom found that the counts of *E. coli* O157:H7 were reduced by 3.1 to 3.8 log10 CFU/g more in Iranian white cheese made with starter LAB during refrigerated storage than in those prepared without starter cultures.

 Certainly, during refrigerated storage of cheeses and other fermented dairy products at <10 °C, the numbers of viable STEC decrease during normal storage. This was shown in Feta cheeses aged for 30 d **(Hudson et al., 1997).**

As well as, Careful inspection of table(1&2) we can concluded that there was a significant increase in numbers of STEC from the initial count in G2 & G3( Kareish cheese manufactured without SC) (by 2.4- 2.6 log10 CFU/g), Moreover G5, G6( Kareish cheese manufactured with 2%SC) were increased by ( 1.55- 2.01 log10 CFU/g). our results were similar to those recorded by **Osaili *et al.* (2014)** whom found that presence of starter (LAB) during manufacture of white brined cheese significantly enhanced reduction of *E.coli* O157: H7 counts overall its storage time.

Growth of STEC during manufacture and storage of soft cheese is inhibited by addition of NaCl at concentrations ≥ 8.5%. But, it could withstand salt concentrations till 5.59% **(Glass *et al*.,1992)**.

In conclusion , low salt soft cheese (3% NaCl) with added starter lactic acid bacteria (SC) at concentration of 2% had prolonged shelf-life than those without (SC). Also, viable counts of STEC in starter culture treated groups were lower than those in un treated ones. This means presence of starter culture affect survival of non O157:H7, significantly. Consistently, non O157 STEC was able to survive low salt concentration (3%) beyond end of refrigerated storage.

**5. CONCLUSION**

It is apparent that presence of starter culture (LAB), the low water activity, low PH and increasing salt concentration reduces STEC counts. These results indicate that STEC can survive law salt concentration in experimentally produced kareish cheese so, control measures including good manufacturing practices and hazard analysis critical control point programs during its processing are necessary to reduce the risk of STEC contamination.

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**7. CONFLICT OF INTERESTS**

authors declare that they have no conflict of interest.

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