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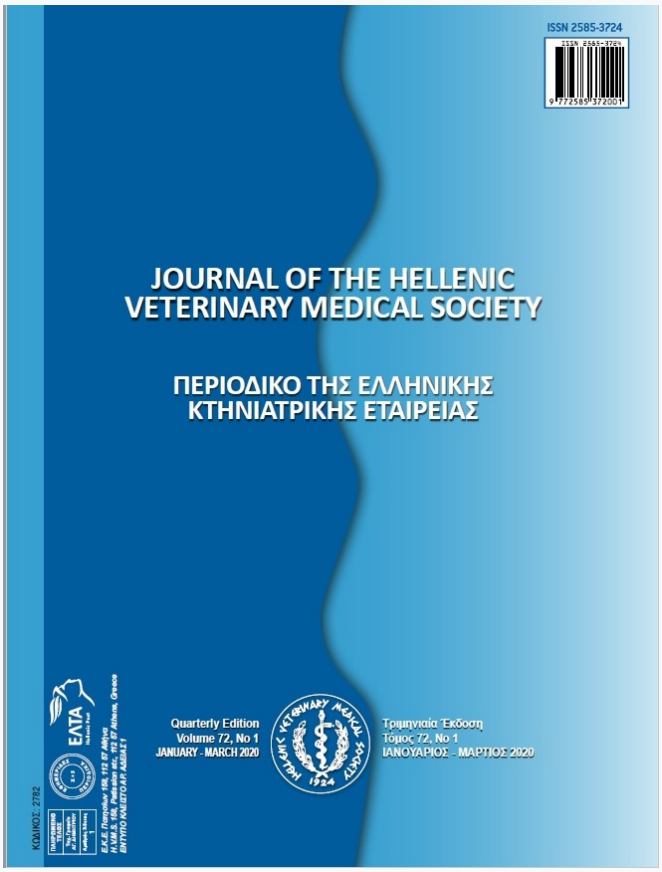

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Survival of Shiga Toxin-Producing *Escherichia coli* (STEC) Serogroups During Production and Storage of Yogurt

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ABSTRACT: In this study, the survival of *Escherichia coli* O157:H7 and non-O157 STEC serogroups of O26, O111, O103, and O145 were investigated during production and storage of yogurt. For this purpose, pathogens were individually inoculated into milk after pasteurization along with the starter culture (approximately 7.00±1.00 $\log_{10}$ cfu/g). After incubation at 44°C (about 180 min), yogurt samples were capped and stored at 4°C for 20 days. Pathogens were enumerated at 0, 5, 10, 15, and 20th days of storage. Lactic acid content (%) and pH of the samples were also screened. Moreover, mesophilic *Lactococcus* spp. and mesophilic *Lactobacillus* spp. were enumerated during production of yogurt.After incubation, the number of *E. coli* O157, O26, O103, O145, O111were 6.76±0.45, 6.64±0.53, 7.12±0.43, 6.00±1.39, 5.89±1.37 $\log_{10}$ cfu/g, respectively. A significant decrease was determined in all groups during the storage of yogurt samples at 4°C ($p<0.05$). It was detected on the 20th day of storage that the number of *E. coli* O157:H7 and non-O157 STEC serogroups of O103 and O145 were under the detection limit. However, STEC O26 and O111 were viable around 1.51±0.98 and 1.18±0.62 $\log_{10}$ cfu/g respectively. Results of the study showed that *Escherichia coli* O157:H7 and non-O157 STEC serogroups might pose a potential health risk during production and storage of yogurt.

Keywords: Yogurt, *E. coli*, VTEC, Acid Adaptation, Dairy Products

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INTRODUCTION

Escherichia coli (*E. coli*) is a natural member of the intestinal flora of all warm-blooded animals, including humans. Additionally, some *E. coli* strains can cause gastrointestinal illnesses in humans (ECDC and EFSA, 2011). Shiga-like toxin-producing *E. coli* serogroups are pathogenic bacteria which are known as Shiga Toxin-Producing *E. coli* (STEC) or Vero-Toxin-Producing *E. coli* (VTEC). Some STEC strains cause serious diseases including hemorrhagic colitis, hemolytic uremic syndrome (HUS), and kidney failure (CDC, 2015).

STEC are generally non-pathogenic to ruminants including cattle, goat, sheep, deer, and elk; on the contrary, these organisms are pathogenic for humans. The major vector of STEC is cattle. Other animals including pigs and birds can spread STEC to the environment. Shiga toxigenic *E. coli* O157 is responsible for most of the STEC outbreaks. On the otherhand, non-O157 STEC outbreaks are recently increasing. Non-O157 serogroups of O26, O103, O111, O121, O145, and O45 were listed as ''Big Six'' by the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) due to the increasing incidence of food-borne diseases caused by these serogroups (USDA FSIS, 2010). Each year 265.000 STEC infections occur in the USA, 36% of which are the infections caused by STEC O157 and the remaining part is caused by non-O157 STEC. Some food products pose a high risk of *E. coli* O157:H7 infection (CDC, 2014), such as raw milk and soft cheeses made from raw milk. *E. coli* O157:H7 and O26:H11 are the most common serogroups of STEC outbreaks from dairy products (Baylis, 2009; Farrokh et al., 2013).

There are studies which show that *E. coli* O157:H7 can survive in yogurt, Colby, Romano and Feta cheese, Kashar cheese, sour cream, buttermilk, white cheese, and goat cheese fora couple of days to weeks (Arocha et al., 1992; Ioanna et al., 2017; Bellio et al., 2018; Oztekin 2019). According to Arıcan and Andıç (2011) *E. coli* O157:H7 could survive the storage of set yogurt in pH values of 4.0 and 4.6. In several studies, it was stated that since the pathogen can survive for several weeks in different dairy products, *E. coli* O157:H7 poses a food safety risk even in low levels (Arocha et al., 1992; Tosun et al., 2007).

Yogurt is consumed widely in Turkey and annually approximately 2.293.431 tons are produced (AERI, 2005). Likewise, it is one of the most consumed dairy products in the world (Sahar and Rahman, 2019). The report of a yogurt-borne *E. coli* O157:H7 outbreak (Morgan et al., 1993) shows the possibility of contamination (Massa et al., 1997). The decrease in pH may provide an advantage for *E. coli* serogroups during the production of yogurt. The pH ranges of yogurts that are produced in the world are approximately between 3.5 and 4.6 (Glass et al., 1992). There is no available data in the literature about the survival of non-O157 serogroups during the production and storage of yogurt. Thus, the aim of the present study is to investigate the survival of *E.coli* O157:H7 and non-O157 serogroups of O26, O111, O103, and O145 during the production and storage of yogurt.

MATERIALS AND METHODS

The raw cow milk was purchased from a local market that sells products from nearby villages and was shortly brought to the laboratory in a thermobox at 4°C (15-30 minutes). Approximately 5.5 liters of milk were used for each repetition and a total of approximately 16.5 liters of milk were used for the study. Pre-made yogurt was used as the starter culture and 2% (w/v) of it was added to the pasteurized cow milk. This pre-made yogurt was provided from a local plant in Tunceli (Simge Süt Ürünleri LTD. ŞTİ.).

Preparing of STEC strains for inoculum

All STEC strains were obtained from a proficiency test held by Istituto Superiori di Sanita (Italy) and stored at -20°C as reference material. Additionally, for daily use STEC serogroups were preserved on agar slants at 4°C. Strains from these slants were inoculated to Tryptic Soy Broth (10 ml) (LABM, UK) and incubated at 37°C for 24 hours (repeated twice). After that the cultures were centrifuged for 5 min at 3500 rpm. The cells were washed with sterile 0.9% NaCl solution and centrifuged once more. Those cultures were diluted with 0.1% sterile peptone water to obtain a cell density of 10^7-10^8 cfu/ml.

The production of contaminated yogurt

5.5 liters of raw cow milk were transferred to a large steel pot and pasteurized at 95°C for 5 minutes. After the pasteurization process, the milk was cooled to 48°Cby using a water-bath. At this stage, 100 ml of milk were sampled for determination of the acidity. Pre-made yogurt was used as the starter and milk was inoculated with the pre-made yogurt at a ratio of

2% (w/v) in the steel pot. Microbiological sampling of 2x25 ml samples for Lactic acid bacteria quantification. After this stage, milk was aseptically divided into five sterile beakers (1500 ml). STEC strains [O157:H7 (ATCC 43894), O26, O111, O103, and O145] were inoculated individually to each beaker and stirred about 5 minutes by sterile spoons. In order to determine the inoculation level (Fermentation Pre) 2×25 ml of this mixture was sampled from each beaker and transferred to the sterile stomacher bags. The contaminated milk samples in beakers were transferred to each of the prepared 250 mL plastic containers for approximately 200 mL each, and allowed to incubate at 44°C. When the pH of the samples reached to pH 4.7, the containers were removed from the incubator and stored at 4°C. In the meantime, 2×25 g samples were taken from different plastic containers (Fermentation Post) and pathogens were enumerated after incubation. The rest of the microbiological and chemical analyses were conducted at 5, 10, 15, and 20 days of the storage.

Microbiological analysis

Each yogurt sample was aseptically stirred by using sterile glass stirrers before sampling. For each strain, 25 g of sample was weighted into sterile stomacher bags and 225 ml of sterile 0.1% peptone water (LABM, Lancashire, UK) was added and then mixed for 2 min by a bag mixer. Analyses were made by spread plate technique.

Sorbitol MacConkey agar (LABM, Lancashire, UK) was used for enumeration of *E. coli* O157:H7 and STEC non-O157 strains. Plates were incubated at 35°C for 24 hours (Dikici et al., 2015).

De Man, Rogosa, Sharpe agar (MRS agar, LABM, Lancashire, UK) and M17 agar (LABM, Lancashire, UK) were used for enumeration of mesophilic *Lactobacillus* spp., *Lactococcus* spp. respectively. Surface plated plates were incubated at 37°C for 48 hours for the enumeration of *Lactococcus* spp. (Rogga et al., 2005). *Lactobacillus* spp. was enumerated by the double layered petri dishes technique after incubation at 30°C for 72 hours (ISO 15214, 2015).

Chemical analysis

For each experimental group, the pH values were measured by a pH-meter (Termo Scientific, Orion-3Star, Singapore) at 5, 10, 15, and 20 days of storage.

The lactic acid content (%) was determined according to AOAC920.124 methods at each experiment day (AOAC, 1990). The analyses were performed separately for each experimental group and repeated twice.

Statistical analysis

Bacterial counts were converted to $\log_{10}$ cfu/g. The data for each pathogen were subjected to the ANOVA test in accordance with repetition x number of samplesxtime to determine fixed effects and interactions between variables. The mean values were separated using Fisher's least squares method according to the General Linear Models (GLM) procedures and the statistical significance level was accepted as 5%. Analysis of the data was made by using the Statistical Analysis System (SAS) (SAS, 1999).

RESULTS

In this study, the survival of STEC serogroups *E. coli* O157:H7, O26, O111, O103, and O145 were investigated during the production of yogurt. The pH value of pasteurized milk samples was 6.81± 0.11.The pH value of pasteurized milk after adding starter culture was 6.36±0.11 (Table 1). The pH of the milk, in which pre fermentation yogurt was used as starter culture, was 4.77±0.01 after incubation(Table 1). Significant decreases in pH values were observed in all groups between 0 and 5 days of the storage($p<0.05$). After 5 days of storage, although the pH value of all samples decreased, there was no significant difference among pH values ($p>0.05$). The pH value of yogurt samples ranged between 4.16-4.03 at the end of the storage (Table 1).

Table 1. The changes in pH values during the production and storage of contaminated yogurt samples ($\log_{10}$ cfu/g) (n:6)

Serogroups	The pH values during production and storage					
	Fermentation Pre	Fermentation Post	5	10	15	20
O157	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.18 ± 0.05^{C}	4.17 ± 0.06^{C}	4.16 ± 0.06^{C}	4.11 ± 0.06^{C}
O26	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.20 ± 0.05^{C}	4.13 ± 0.04^{C}	4.12 ± 0.05^{C}	4.05 ± 0.09^{C}
O103	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.26 ± 0.07^{C}	4.23 ± 0.03^{C}	4.17 ± 0.04^{C}	4.16 ± 0.04^{C}
O145	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.17 ± 0.03^{C}	4.13 ± 0.03^{C}	4.12 ± 0.05^{C}	4.06 ± 0.02^{C}
O111	6.36 ± 0.11^{A}	4.77 ± 0.01^{B}	4.19 ± 0.08^{C}	4.14 ± 0.05^{C}	4.11 ± 0.06^{C}	4.03 ± 0.11^{C}

A-C: Means in the same line with different superscripts are statistically different ($P<0.05$)

Although statistically significant decline ($p<0.05$) was observed before and after incubation, no significant change occurred in the lactic acid content of the samples on the other days of storage ($p>0.05$) (Table 2).The lactic acid content of all samples from experimental groups was similar. Therefore the obtained values from experimental groups were averaged and given in Table 2.

The high levels of spiking with STEC serogroups can be explained as to monitor the dramatic changes of numbers of pathogens during incubation and storage. The number of STEC serogroups increased during incubation of yogurt (Table 3); however, this increase was not statistically significant ($p>0.05$). It was determined that the number of STEC O157, O103, and O145 decreased about 6.00+1.00 $\log_{10}$ cfu/g during storage and were below the detectable level at the end of the storage period. However, the number of STEC O26 and O111 were determined as 1.51±0.98 and 1.18±0.62 $\log_{10}$ cfu/g, respectively at the end of the 20 days of storage (Table 3).

Table 2. The lactic acid % values of during the production and storage of contaminated yogurt samples (n:6)

Serogroups	Fermentation		Storage Day			
	Pre	Post	5	10	15	20
O157	0.15±0.02^{A}	0.84±0.01^{B}	0.81±0.12^{B}	0.91±0.01^{B}	0.90±0.10	0.87±0.12^{B}
O26	0.18±0.02^{A}	0.71±0.01^{B}	0.85±0.08^{B}	0.93±0.01^{B}	0.87±0.18	0.82±0.10^{B}
O103	0.16±0.10^{A}	0.73±0.01^{B}	0.79±0.16^{B}	0.95±0.01^{B}	0.85±0.15^{B}	0.88±0.14^{B}
O145	0.17±0.02^{A}	0.72±0.01^{B}	0.80±0.14^{B}	0.90±0.01^{B}	0.82±0.17^{B}	0.83±0.07^{B}
O111	0.19±0.04^{A}	0.70±0.01^{B}	0.80±0.10^{B}	0.96±0.01^{B}	0.81±0.15^{B}	0.85±0.11^{B}

AB: Means in the same line with different superscripts are statistically different ($P<0.05$).

Table 3. The microbiological changes during the production and storage of contaminated yogurt samples ($\log_{10}$ cfu/g) (n:6).

Serogroups	Fermentation		Storage Day			
	Pre	Post	5	10	15	20
O157	6.79±0.45^{A}	7.01±0.31^{A}	4.59±1.59^{B}	3.14±0.47^{B}	3.55±1.24^{B}	< 1.0
O26	6.64±0.53^{A}	7.32±0.37^{A}	5.07±0.98^{B}	3.42±0.73^{C}	2.27±0.72^{CD}	1.51±0.98^{D}
O103	7.12±0.43^{A}	7.29±0.43^{A}	5.47±1.18^{B}	4.28±1.08^{BC}	2.52±1.32^{C}	<1.0
O145	6.00±1.39^{A}	7.41±0.35^{A}	4.66±1.03^{A}	1.95±1.15^{B}	1.83±1.47^{B}	<1.0
O111	5.89±1.37^{A}	7.35±0.45^{A}	4.95±1.03^{A}	3.20±1.87^{B}	2.36±1.04^{B}	1.18±0.62^{B}

A-D: Means in the same line with different superscripts are statistically different ($P<0.05$)

.Pre-Fermentation *Lactobacillu*s spp. and *Lactococcus* spp counts were5.02+0.22, 4.93+0.31 $\log_{10}$ cfu/g respectively. The number of mesophilic *Lactobacillus* spp. and *Lactococcus* spp. of experimentally contaminated yogurt samples were 7.04±0.51, 7.42±0.21 $\log_{10}$ cfu/g respectively after incubation. The numbers of these bacteria were around the targeted number of starter bacteria in yogurt (7 $\log_{10}$ cfu/g).There were no significant changes in the populations of mesophilic *Lactobacillus* spp. and *Lactococcus* spp. The number of mesophilic *Lactobacillus*spp. in the samples of experimental groups O157, O26, O103, O145, O111 were 7.82±0.46, 7.88+0.31, 7.65±0.53, 7.62±0.40 and 7.80±0.38 $\log_{10}$ cfu/g, respectively. The number of *Lactococcus* spp. in the same groups were7.91±0.22, 7.90±0.19, 7.88±0.27, 7.94±0.51 and 7.93±0.42 $\log_{10}$ cfu/g, respectively.

DISCUSSION

Although yogurt is a heat-treated dairy product, a possible post-process contamination might alter the safety of the product and cause serious health hazards for consumers. According to the study of Dehkordi et al. (2014), 50 out of the 600 dairy products were contaminated with *E. coli* and yogurt was the most contaminated one. The most prevalent *E. coli* serogroups were O157 and O26. Since several studies reported that STEC serogroups are acid resistant pathogens that can survive in various dairy products for several weeks to several months (Govaris et al., 2002; Solomakos et al., 2009; Miszcyzcha et al., 2012; Ioanna et al., 2017; Bellio et al., 2018), the reliability of acidic foods such as yogurt is questionable.

In this study, it was determined that *E. coli* O157:H7 and non-O157 STEC serogroups can survive in yogurt environment (Table 3). *E. coli* O157:H7, non-O157 STEC O103, and O145 had the lowest survival rate and viable counts dropped below the detection lim-

it at day 20 of storage (Table 3). It was reported that the inhibitory effect of starter cultures on *E. coli* O157 and non-O157 was dependent on strain and species. Similar results were reported in goat milk fermented with yogurt starter cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus*) that inhibited non-O157 *E.coli* strains (Fayemi and Buys, 2017). In another study it was shown that yogurt starters had no adverse effect on *E.coli* O157:H7 individually but showed an antimicrobial effect when used together (Dineen et al., 1998). The reason for that is reported to be the high antimicrobial substances produced from the beginning (Ogueke, 2008). In the studies conducted on fermented milk and dairy products, the reasons for the decrease in the number of pathogenic microorganisms during fermentation and storage were reported as the number and type of lactic acid bacteria as well as the substances which are produced during acidic fermentation such as organic acid, hydrogen peroxide, bacteriocin, diacetyl, ethanol (Dineen et al., 1998; Leistner, 2000; Rogga et al., 2005; Dikici, 2008; Ogueke, 2008; Callon et al., 2016; Fayemi and Buys, 2017; Bellio et al., 2018).

STEC O26 and O111 maintained their viability during storage of 20 days. STEC O26 and O111 counts in the inoculated milk were 6.64±0.53 $\log_{10}$ cfu/mL and 5.69±1.37 $\log_{10}$ cfu/mL respectively. Significant decreases of viable counts were determined during storage ($p<0.05$) and STEC O26 and O111 counts at the end of the storage were determined as 1.51±0.98 $\log_{10}$ cfu/g and 1.18±0.62 $\log_{10}$ cfu/g respectively (Table 3). Therefore, it can be concluded from these results that during the production and storage of yogurt, STEC O26 and O111 were more resistant than the other serogroups in this study. Since STEC O26 is the second most prevalent STEC serogroup (Lajhar et al., 2017), it is expected that these bacteria are more resistant to stressors.

There is no report on the survivability of non-O157 STEC serogroups in yogurt in the literature and such reports regarding dairy products are limited. The studies carried out by Miszczycha et al. (2012) and Bellio et al. (2018) show that the number of STEC increased during the first step of cheese production. In these studies researchers determined that the increase of LAB and the decrease of pH were not effective on the inhibition of *E. coli* O157:H7. In both studies, the number of STEC increased and then decreased as a result of ripening at the first step of cheese making.

In the study of Arıcan and Andıç (2011), the number of *E. coli* O157:H7 was found to be similar to that of our study during the fermentation and storage of yogurt (Table 3). Likewise, in the study of Tosun et al. (2007), the viability of acid-adapted and non-adapted *E. coli* O157:H7 were investigated in fermented dairy products. In this study, non-adapted *E. coli* O157:H7 maintained its viability during storage of symbiotic yogurt for 26 days at 4°C. Massa et al. (1997) investigated the viability of O157:H7 in traditional and bifido yogurt. They reported that O157:H7 was still detectable at the end of 7 daysof storage. As a consequence, it can be concluded from the study of Akdemir and Evrendilek (2007) that *E. coli* O157:H7 can survive in acidic foods. This pathogen can survive in several acidic foods such as sweet pickles (pH: 2.8) (Tsai and Ingham, 1997), yogurt (pH: 4.5) (Massa et al., 1997), and mayonnaise (pH: 3.65) (Weagant et al, 1994). Although yogurt is considered as a rather safe product due to its natural acidic environment, this pathogen can survive in acidic foods such as yogurt through its resistance to the low pH (Bracket et al., 1994; Conner and Kotrola, 1995; Dineen et al., 1998; Bellio et al., 2018; Yousef and Courtney, 2003).

The number of studies on the behavior of non-O157 STEC serogroups in foods is quite low. Especially, the studies on dairy products are very rare. The possible contamination to any product after the heat treatment may pose a potential risk to the safety of food and public health. Therefore, in this study the safety of yogurt was investigated to demonstrate the consequences of a post-heat treatment contamination with STEC.

CONCLUSIONS

According to the results obtained from the present study, *E.coli* O157:H7 and non-O157 strains of O26, O111, O103, and O145 could survive yogurt environment. Even though *E.coli* O26 and O111 were still detectable on the 20th day whereas O157, O103 and O145 were not; the differences in the survivor numbers were not very significant. Therefore, it cannot be concluded that these strains were more resistant to yogurt production and storage steps than other strains used in this study, without further investigation.

The incubation step did not decrease the number of pathogens; on the contrary, the number of all pathogens increased at this step. The possible risks resulting from post-process contamination of yogurt can be seen clearly from these results that STEC O157:H7 and non-O157 STEC serogroups could survive the production and storage of yogurt. Considering the fact that the infective dose of STEC pathogens

can be very low, consumption of contaminated yogurt could be threatening. Various studies have shown that pathogens, which are capable of adapting to acidic environments, are more likely to cause diseases.

As a zero tolerance bacteria to be listed by the authorities, STEC, the public health hazard should be taken into account and additional countermeasures should be taken. foods should be monitored in terms of STEC contamination throughout production and storage period. Risk assessment of non-O157 STEC serogroups should be made by each country and the STECshould be included in the legislation; the isolation and identification procedures must be improved, rapid and reliable screening systems need to be developed.

CONFLICT OF INTEREST

None declared by the authors.

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